Volume 34

PROGRESS IN MEDICINAL CHEMISTRY

Edited by

G.P. ELLIS and D.K. LUSCOMBE

ELSEVIER

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1997

ELSEVIER
AMSTERDAM • LAUSANNE • NEW YORK • OXFORD • SHANNON • TOKYO

Elsevier Science BV
P.O. Box 211
1000 AE Amsterdam
The Netherlands

Library of Congress Cataloging in Publication Data:

Please refer to card number 62-2712 for this series.

ISBN 0-444-82632-7
ISBN Series 0-7204-7400-0

This book is printed on acid-free paper

Printed in The Netherlands

Preface

In this volume, there are five reviews of topical subjects. The availability of drugs for the successful treatment of many diseases results in an increase in the number of people who live to their eighties and nineties at which time Alzheimer's disease is most prevalent. The steady progress which is being made in our understanding of this complex disease and in the development of drugs for its treatment are reviewed in Chapter 1. Chemotherapy of another disease which often resists treatment may have another weapon in its armoury if recent work on modifying a naturally occurring anticancer compound (camptothecin) succeeds in reducing its side-effects; recent efforts in this field (described in Chapter 2) may yield a drug of value in treating cancer of the colon and stomach.

Transporting a drug molecule to a specific site of action continues to present a challenge. The use of phosphates and phosphonates to protect the drug from hydrolysis or other form of damage *en route* is reviewed in Chapter 3. The destruction of pathogenic microbes is of vital importance both inside and outside the human body; this is particularly true of hospitals where there has recently been an increase in the diagnostic and surgical use of endoscopy. Sterilization using aldehydes (especially glutaraldehyde) is surveyed in Chapter 4.

The difference in biological potency between enantiomers of compounds such as adrenaline, ephedrine and morphine is well-known. Manufacturers of more complex synthetic asymmetric drug molecules are now turning their attention to the merits of offering the patient only the biologically active enantiomer. Chapter 5 is an account of the progress that has been made in this expanding area of medicinal chemistry.

We thank the authors for condensing the ever-expanding literature of these topics. We are grateful to publishers who own the copyright of material reproduced with their permission and to our own publishers for their encouragement.

July 1996

G.P. Ellis
D.K. Luscombe

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1 Pharmacology of Alzheimer's Disease

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INTRODUCTION

Effective drug therapy for Alzheimer's disease is one of the major goals of 'the decade of the brain', proclaimed first in the United States and shortly thereafter in Europe. Two countries have already granted marketing authorisations for one compound, tacrine (see p. 46) [1], indicated for Alzheimer's disease. For those who are not specialists in this area, it is important to note that these authorizations recognize the achievements reached during the preliminary stages of research and nothing more. Tacrine provides symptomatic relief in the early stages of the disease by reducing memory impairment but does not have any known effect relating to the theoretical cholinergic basis of the disease. This drug is a temporary, partial and modest preliminary step in our progress towards finding a truly effective agent which is dramatically lacking in our current pharmaceutical armamentarium.

The history of tacrine does, however, provide an excellent illustration of the problems encountered in the development of drugs for use in the wider field of dementia and thus merits careful consideration. Both modesty and patience are essential when approaching the problem of dementia. How can it be otherwise since research to date has been relatively unsuccessful and drug development to final clinical proof of efficacy is such a long process? Advances will depend on stimulating exchanges amongst researchers in molecular biology, genetics, clinical medicine and pharmacology as well as co-ordinating the efforts of regulatory agencies. The general international consensus on the importance of adopting common co-ordinated strategies in the fight against Alzheimer's disease emphasizes the exceptional nature of this field of research.

In addition to discussing possible therapeutic agents, a review of the pharmacology and chemistry of drugs with anti-Alzheimer's disease potential would be incomplete without raising a number of major problems facing research in dementia. We shall therefore examine the clinical symptoms and

diagnosis in man, experimental models for screening new compounds, and strategies for testing the effect of promising compounds in man. We shall also present a critical assessment of several phase III trials currently being conducted in several countries. It is hoped that this will help to stimulate fundamental research and its transcription into the final stages of drug development, and the ultimate goal of marketing a truly safe and effective drug responding to the needs of our patients and their families.*

DESCRIPTION OF ALZHEIMER'S DISEASE

Alzheimer's disease is characterized by a progressive deterioration of the cognitive functions (dementia) associated with specific histological lesions of the cerebral cortex. The disease is age-related. An early form begins between the age of 45 and 60 years and occurs in about 0.1% of the population at risk. A late form, which has increased in frequency in developed countries due to longer life expectancy, is seen in subjects aged over 60 years. Alzheimer's disease occurs in about 5–6% of the population over 65 years of age. Prevalence is multiplied by a factor of two for every 5 years increase of age and consequently in the population over 80 years of age, it reaches 20%.

The disease always begins insidiously, the three types of symptoms of memory impairment, behavioural disorders and failure of specific mental functions developing progressively.

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*The following abbreviations are used in this review: AAMI, age-associated memory impairment; Ach, acetylcholine; ADAS, Alzheimer's disease assessment scale; ADRDA, Alzheimer's Disease And Related Disorders Association; ALAT, alanine amino transferase; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolyl propionate; APP, β-amyloid precursor protein; ASLA, aspartate amino transferase; Aβ, β-amyloid; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; BMAA, β-methylamino-L-alanine; BOAA, β-*N*-oxalylamino-L-alanine; BPRS, brief psychiatric rating scale; CGI, clinical global impression; CIBIC, clinician's interview-based impression of change; DFT, dementia of frontal type; DSM IV, Diagnostic and Statistical Manual, 4th edn; DSP-4, *N*-2-chloroethyl-*N*-ethyl-2-bromobenzylamine; EEGQ, quantified electroencephalogram; FR, free radicals; GLU, glutamate; M_1, muscarinic-type 1 receptor; MAO-B-I, monoamineoxidase type B inhibitors; MAOI, monoamine oxidase inhibitor; MMS(E), Mini-mental state (examination); MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; N, nicotine; NBM, *nucleus basalis magnocellularis;* NFT, neurofibrillary tangle; NGF, nerve growth factor; NINCDS, National Institute of Neurological & Communicative Disorders & Stroke; NMDA, *N*-methyl-D-aspartate; NMRI, nuclear magnetic resonance imaging; NSAID, non-steroidal anti-inflammatory drugs; NT, neurotransmitter; PET, positron emission tomography; PK/PD, pharmacokinetic/pharmacodynamic relationship; PPA, progressive primary aphasia; PRAD, probable Alzheimer's disease; SAM, senescence-accelerated mouse; SPECT, single photon emission computed tomography; THA, tetrahydroaminoacridine.

(i) Memory impairment which is limited to recent events is the first symptom occurring in 75% of cases and directly affects the patient's everyday life and his or her relationships with family and friends. On examination, the disease can be distinguished from age-associated memory impairment. Temporal, followed by spatial disorientation occurs early in the disease. The patient is unable to recall three words after an interfering event such as counting backwards, and cannot use compensatory association techniques to improve memory performance. At onset, these symptoms are highly suggestive of Alzheimer's disease but they also occur in a number of other degenerative dementia diseases including Lewy body disease and degenerative diseases of the frontal lobe.

(ii) In 40% of cases, behavioural disorders is the first manifestation of the disease, with or without associated memory impairment. Usually, the patient becomes self-centred, more or less oblivious to his or her surroundings, and appears to be sad or anxious. A diagnosis of depression is often considered, but several symptoms are markedly different. In Alzheimer's disease, there is no feeling of guilt or self-accusation. Sensorial pleasures such as eating out, music, and affection, for example, with children and grandchildren, are not disturbed. On examination, the memory loss is too great to be explained by the effects of depression in which temporal and spatial orientation is at least partially maintained and processes of associative assistance can improve memory performance. Signs of depression may also be observed in the early stages of certain frontal lobe dementias. Uninhibited social behaviour is exceptional in the early stages of Alzheimer's disease and is more often a sign of frontal lobe dementia.

(iii) In about 5% of cases, the first sign of disease is an isolated dysfunction of specific mental functions. The most common manifestation is impaired language performance with missing words, often attributed to poor memory and having little impact on everyday life. In fact, such progressive manifestations of degenerative aphasia comprise a syndrome usually resulting from frontal lobe degeneration or focal lobar atrophy. Apraxia is exceptionally the inaugural sign but can also be observed in corticobasal degeneration and focal lobar atrophy.

The disease invariably worsens with time but with wide interindividual variability. Generally, cognitive impairment as measured on the Mini-Mental State Examination (MMSE) declines by 3 to 4 points per year. In slow decliners, however, loss may be less than 1 point per year and, in fast decliners loss may be as much as 7 points or more per year. In addition, mental decline usually follows a linear pattern or shows temporary plateaux between periods of more or less rapid decline. Performance also varies from one day to the next and occasionally within a given day. Nevertheless, major long-

term variations in mental performance are unusual in Alzheimer's disease and are more suggestive of Lewy body disease.

Severity is evaluated by assessing the degree of cognitive impairment (for example, a MMSE score above 18 defines minor dementia, 10 to 18 moderate dementia and below 10 severe dementia) or by measuring the loss of independence in everyday activities (in minor dementia a certain degree of independence is retained, in moderate dementia assistance is required and in severe dementia the patient is totally dependent). Specific evaluation scales such as the Clinical Dementia Rating [2] or the Global Deterioration Scale [3] integrate both types of information.

Unless histological proof is obtained, diagnosis is a question of probability. The NINCDS-ADRDA [4] criteria are usually used for diagnosis with good sensitivity (above 90%) but with low specificity, particularly in early stages (about 70%).

There is no biological marker for Alzheimer's disease. However, three complementary explorations are useful for diagnosis. A computed tomography or nuclear magnetic resonance imaging (NMRI) of the brain can eliminate vascular dementia or focal brain lesions. Ventricular dilatation or widened arachnoid spaces localized in the posterior regions of the hemispheres and atrophy of the hippocampal regions are suggestive of Alzheimer's disease. The electroencephalogram and especially quantified EEG usually show a slow basal rhythm. Single Photon Emission Computed Tomography (SPECT) is probably the most useful technique but the images acquired are not specific. The most characteristic feature is deficient brain perfusion, usually in the temporoparietal regions, although different regional distributions are seen. The perfusion defect may be asymmetrical or even lacking in the early stages of the disease.

Neuropathological examination of brain tissue reveals three types of abnormality: senile plaques, neurofibrillar degeneration and neuronal loss. Senile plaques are the most characteristic feature. The centre of the plaques contain a deposit of Aβ-amyloid protein surrounded by neuronal debris. Plaques occur diffusely in layers I and III of the cerebral cortex. Neurofibrillar degeneration is an intracellular phenomenon caused by abnormal phosphorylation of the tau proteins in the cytoskeleton. It occurs in layer V, especially in the hippocampal regions and in the associative cortex. Neurone loss follows synaptic loss and predominates in the same regions. Debate continues on two elements: the relationship between these histological abnormalities and clinical signs, and the relationships among clinical signs. There is a clear relationship between the degree of dementia and the number of senile plaques and neurofibrillar degenerations but the relationships or links between histological signs are far from sufficient to explain the clinical signs.

One frequently suggested theory is based on the fact that the Aβ protein deposit is the earlier phenomenon which would be followed, in certain subjects and in certain specific regions, by neurofibrillar degeneration. These lesions would perturb axonal flow, disturb neurotransmission and synaptic activity, then finally lead to neurone loss. However, for other authors, senile plaques and neurofibrillar degeneration are only markers of a certain type of degeneration and not the cause of the clinical signs which would be related to synaptic loss and neurone death.

Therapeutic goals have been designed on the basis of these concepts. Two types of action have been proposed: symptomatic amelioration and attempts to retard the clinical course of the disease (pathophysiological action). The symptomatic approach is directed specifically at correcting neurotransmission abnormalities, in particular, cholinergic dysfunction. Non-specific drugs (antidepressants, anxiolytics or neuroleptics) are also used to improve behavioural manifestations. This symptomatic action is essential when dementia is moderate or severe. Attempts to retard disease progression, on the other hand, are essential in the early stages of the disease. They can be oriented towards either specific disease mechanisms (drugs aimed at inhibiting amyloid deposition or development of neurofibrillar degeneration) or non-specific mechanisms of cell death. Only drugs in this latter category are currently in the developmental stage in man. An awareness of the problems caused by impaired cognition on the patients' personal and interrelational life is essential as has been demonstrated by the placebo effect in therapeutic trials.

Well-conducted therapeutic trials are highly dependent on adequate patient selection among the wide number of types of dementia. The question of differential diagnosis is thus raised. Alzheimer's disease is responsible for approximately 50% of all dementias [5].

Since no reliable biological marker of the disease is currently available, and since certain diagnosis requires specific histological evidence, patient selection must rely solely on clinical manifestations and a few complementary examinations aimed at eliminating secondary dementia. The development of widely accepted diagnostic criteria such as the DSM IV (*Table 1.1*) or the NINCDS-ADRDA [4] has been a major step forward, but these criteria are actually more adapted to the diagnosis of primary dementia than for Alzheimer's disease since they are largely based on eliminating secondary dementia. Their specificity is excellent in cases with a typical clinical picture, but falls to about 70% in less typical presentations [6], a percentage which corresponds approximately to the frequency of Alzheimer's disease among presenile degenerative dementia [7].

When a clinician examines a patient with primary dementia, he finds him-

Table 1.1. DSM IV CRITERIA FOR ALZHEIMER'S DISEASE

A. The development of multiple cognitive deficiencies manifested by both
   1. memory impairment (impaired ability to learn new information or to recall previously learnt information)
   2. one (or more) of the following cognitive disturbances:
      (a) aphasia (language disturbance)
      (b) apraxia (impaired ability to carry out motor activities despite intact motor function)
      (c) agnosia (failure to recognize or identify objects despite intact sensory function)
      (d) disturbance in executive functioning (i.e. planning, organizing, sequencing, abstracting)

B. The cognitive deficiencies in Criteria A1 and A2 each cause significant impairment in social or occupational functioning and represent a significant decline from a previous level of functioning.

C. The course is characterized by gradual onset and continuing cognitive decline.

D. The cognitive deficiencies in Criteria A1 and A2 are not due to any of the following:
   1. Other central nervous system conditions that cause progressive deficiencies in memory and cognition (e.g., cerebrovascular disease, Parkinson's disease, Huntington's disease, subdural hematoma, normal-pressure hydrocephalus, brain tumour)
   2. Systemic conditions that are known to cause dementia (e.g., hypothyroidism, vitamin B_{12} or folic acid deficiency, niacin deficiency, hypercalcaemia, neurosyphilis, HIV infection)
   3. Substance-induced conditions.

E. The deficiencies do not occur exclusively during the course of a delirium.

F. The disturbance is not better accounted for by another Axis I disorder (e.g., major depressive disorder, schizophrenia).

self in an uncomfortable position, hesitating between an 'overly' sensitive attitude where all cases of primary dementia would be considered to be an authentic case of Alzheimer's disease with the risk of misdiagnosis in about 25% of the patients or an 'overly' specific attitude where all atypical cases would be considered non-Alzheimer until formal proof is obtained. Indeed several clinical presentations can mimic Alzheimer's disease. We shall not discuss here differential diagnosis which can be correctly recognized after complementary explorations (in particular, brain scan). Other situations raise no particular problem when the neurological examination reveals evidence incompatible with probable Alzheimer's disease (such as prion disease).

AGE-ASSOCIATED MEMORY IMPAIRMENT

In 1986, Crook *et al.* [8] introduced the concept of age-associated memory impairment (AAMI), assuming that memory performance declines with age and that it is related to age-dependent biochemical and structural changes. The criteria proposed were: age over 50 years, memory complaints involving everyday life, results on standardized memory tests below those obtained by young subjects, lack of global intellectual deficiency and lack of an individual cause which could explain the memory complaint. It rapidly became clear that the concept as defined by Crook *et al.* does not correspond to a homogeneous clinical entity but rather identifies a large spectrum of physiological and pathological situations both in certain normal but anxious subjects and in certain patients in the early stages of dementia [9].

Based on the DMS III-R criteria, the differentiation between age-associated physiological memory complaints from an authentic early stage dementia is theoretically clear since, by definition, age-associated memory impairment remains isolated and has no effect on the patient's social or professional life. These criteria, however, are highly subjective as it is sometimes quite difficult to identify the isolated nature of the impairment, particularly in patients with a low level of general culture. It is generally recommended that a patient is seen again a few months later in order to evaluate changes and identify any possible aggravation.

PSEUDODEMENTIA DEPRESSION

The DSM III-R criteria stipulate that the diagnosis of dementia cannot be retained unless no non-organic factor can be identified as sufficient to explain the impaired cognitive performance. This is, however, often difficult to achieve since signs of depression, frequent in the early stages, may either be an indication of the causal element or simply concomitant manifestations. Clinical criteria for pseudodementia [10] have been proposed but have not been validated. It is recommended to start antidepressant treatment as a test. The situation may be clarified if the signs of depression disappear, but states of depression severe enough to mimic an authentic dementia are usually drug resistant and would require electroconvulsive therapy.

FRONTAL LOBE DEMENTIA

Until recently, degenerative dementia with predominant frontal syndrome has been considered to be a rare manifestation of Pick's disease, progressive subcortical gliosis, or even certain forms of Alzheimer's disease. Teams in

Sweden [11, 12] and England [13] have demonstrated, on the basis of clinical and pathological data, that the frequency of frontal dementia is higher than expected, reaching about 10% of the presenile dementias. These authors proposed the term dementia of frontal type (DFT). Neary *et al.*[14] would accept that the underlying substratum is a heterogeneous combination of nosologies, with certain DFT actually being Pick's disease, but the histological lesions are usually non-specific, with associated neurone loss, laminar spongiosis and focalized gliosis. Other terms such as presenile dementia with motor neurone disease described by Mitsuyama in 1984 [15] and dementia lacking distinctive histology reported by Knopman *et al.* in 1990 [16] could be similar to the DFT concept.

The clinical picture here is different from classical Alzheimer's disease. Behavioural disturbances, diminished social interrelations and impaired language of the expression type are the main signs while the instrumental cognitive functions remain relatively untouched. Nevertheless, in our opinion, the DSM III-R criteria and the NINCDS-ADRDA criteria can be applied for these patients: they are often described as forgetful and their psychic inertia and attentional disorders can make it difficult to identify the normality of instrumental functions. The predominant behavioural disorders, the absence of temporo-spatial disorientation, the absence of a clinically patent amnesic syndrome, and the spectacular improvement of memory performance by indexing are usually sufficient to differentiate this clinical condition from Alzheimer's disease. The fronto-temporal atrophy and hypometabolism of the frontal lobes are also significantly different from the posterior signs of Alzheimer's disease.

PRIMARY PROGRESSIVE APHASIA AND OTHER FOCAL ATROPHIES

The syndrome of progressive primary aphasia (PPA) described in 1982 by Mesulam [17], is defined as an isolated progressive deterioration in language performance for at least 2 years. Attention, memory, visuo-spatial capacity and behaviour must be preserved during these first two years [18]. This presentation does not correspond to a homogeneous entity but is a clinical syndrome. The histological lesions of Alzheimer's disease are only observed in 30% of the cases. This percentage may be nearly zero in cases with an expressive type of aphasia. For Mesulam, the most distinctive sign in PPA and PRAD (probable Alzheimer's disease) is the remarkable conservation of everyday activities.

Following Mesulam's initial work, other signs revealing focal atrophy have been described including progressive apraxia [19], posterior cortical atrophy [20] and progressive prosopagnosia [21]. A common element in

these different clinical entities is their occurrence in the proscenium in non-demented patients, at least at onset. The underlying nosological entities do not comprise a homogeneous group, but usually do not correspond to Alzheimer's disease. Using the NINCDS-ADRDA criteria, these cases cannot be considered to be probable Alzheimer's disease, but are termed possible Alzheimer's disease.

The lack of an amnesic syndrome is part of the PPA definition, but care must be taken to avoid accepting the patient's complaint as memory loss when actually suffering from an incapacity to find words. The limited nature of the neuropsychological picture and the lack of early dementia can sometimes be difficult to identify even for a well-trained and experienced clinician.

SEMANTIC DEMENTIA

This is a new entity, sometimes difficult to differentiate from progressive primary aphasia or from probable Alzheimer-type dementia. It was individualised by Snowden *et al.* in 1989 [22]. Semantic dementia is a loss of performances which Tulving grouped under the term semantic memory [23]. The main clinical feature is the loss of the sense of words, things and persons.

Here again, the criteria of probable Alzheimer's disease are undoubtedly applicable. Patients fail classical memory, language and recognition tests. There are however certain differences between semantic dementia and probable Alzheimer's disease [24]. Clinically, these patients are not forgetful, nor are they disoriented, or forget what they have done or what they have to do. There is no manifestation of visuo-spatial impairment or anosognosia, nor loss of self-sufficiency even after a disease course of more than 10 years. These patients are capable of using compensatory strategies to overcome their deficiency and maintain their areas of interest. The atrophy or hypometabolism is strictly localized in the temporal or fronto-temporal regions. Based on reports in the literature, it would appear that the epidemiological data are similar to those for focal atrophy with a clear predominance of presenile cases and a slight over-representation in males. Finally, among the complete observations reported with histological results, two cases were found to be Pick's disease and in two others only non-specific anomalies could be identified.

LEWY BODY DISEASE

Lewy's bodies have been demonstrated in certain patients with Parkinson's syndrome who developed dementia early, not only in the substantia nigra but also in the cortex. There is frequent association of the histological fea-

tures of Alzheimer's disease suggesting a possible relationship between Lewy's body dementia and Alzheimer type dementia.

Clinically, the diagnosis is based on early signs of Parkinsonism, episodes of confusion and fluctuating performance and frequent psychiatric symptoms (delirium, hallucinations). If the dementia is relatively isolated, then clinically Lewy body disease cannot easily be differentiated from Alzheimer's disease.

In conclusion, there are a large number of diagnostic pitfalls to avoid when using the NINCDS-ADRDA or the DSM III-R criteria. These scales are unable to identify the underlying nosology with certainty for every clinical situation presenting progressive deterioration of cognitive function. Until reliable biological markers are available, we must rely on the contribution of functional imaging, which itself must be validated, to increase the specificity of clinical criteria. More importantly, Alzheimer's disease cannot simply be considered to be the summation of various symptoms (amnesia + aphasia + apraxia). It must be considered as one of the clinical entities existing in a continuous clinical spectrum analysed in neuropsychology and so well illustrated in the recent book published (in French) by Sabouraud [25].

ANIMAL MODELS

The search for valid animal models capable of predicting human pathophysiology, and applicable to *in vivo, ex vivo* and *in vitro* research, is fundamental in pharmacology. Despite a certain amount of controversy over the validity of extrapolating data from animal models to human physiology, this is an essential step before carrying out clinical trials in humans. Some of the models developed to date for Alzheimer's disease have proved to be quite useful, but unfortunately none can be accepted as truly valid. These models have nevertheless enabled us to clarify the cause of the pathogenic process and outline potential therapeutic strategies.

No single animal model of Alzheimer's disease which perfectly mimicks the complex disease in man is known. Most are simple attempts at reproducing certain features of the disease (behavioural deficits, histological stigmata, metabolic disturbances). Since memory loss and neurotransmitter deficiency are characteristic features of Alzheimer's disease, non-transgenic models have predominantly focused on lesioning specific pathways in experimental animals and relating this to deficits in learning and memory. Models have been developed with the aim of mimicking the main risk factors in man, for example, ageing, trisomy 21, mutations on the gene encoding for the β-amyloid precursor protein APP, and presence of the allele on chro-

mosome 19 encoding for type 4 apolipoprotein E. These models thus use old animals or animals with artificially induced anomalies.

AGED ANIMALS

Aged non-human primates

In non-human primates, behavioural disturbances and brain anomalies similar to those seen in aged humans and to a greater extent in individuals with Alzheimer's disease, are known to be age-related. The most appropriate model would appear to be the rhesus monkey (*Macaca mulatta*) which has a life span of approximately 35 years. Several Alzheimer-like phenomena including neuron degeneration, abnormal axons, neuritis and amyloid deposits in senile plaques and around blood vessels are all seen in the brains of old monkeys. Moreover, the levels of specific neurotransmitter circuit markers decrease in some older animals [26, 27]. Despite the potential of this model, the number of animals available for study and questions of cost limit its use for routine research.

Other aged animals

While non-human primates may be the most relevant animals, practical and economic factors have made rodents the most widely used model. The small size of the mouse can be a handicap, but there are several well-defined strains which offer a wide range of features. For example, NMRI strain-aged mice provide evidence for cognitive impairment (Morris water maze, passive avoidance). But in this model, neurochemical changes do not correlate with performance in learning and memory tasks [28]. In one specific strain, the senescence- accelerated mouse (SAM), age-related changes in learning and memory have been reported [29].

There has been much work done on the aged rat. For this model, there is a complete battery of behavioural tests demonstrating deficient cognition and the potentially corrective effects of pharmacological agents. Spatial or avoidance learning tasks are typically used to assess Alzheimer-like changes in rodents (Morris water maze, 8-arm radial arm maze for example). Higgins *et al.* used *in situ* hybridization to discriminate between different forms of the β-amyloid precursor protein transcripts in young adult, non-impaired aged, and behaviourally impaired aged rats. They were able to demonstrate a rise in the level of Kunitz protease inhibitor (KPI) containing APP mRNAs, as compared with APP-695 mRNA, in forebrain neurone, a rise

which occurs selectively in the subpopulation of aged rats exhibiting spatial memory deficits [30].

The comparison between groups of young and aged animals must take into account the effect of age-related physical incapacitation or behavioural performance (tumours, spontaneous sensorimotor changes, pain sensitivity, visual impairment). The fact that aged subjects are only one subgroup of the minority of the surviving animals also introduces a selection bias.

In certain breeds of dog, β-amyloid also accumulates in aged animals. The prevalence of diffuse plaques and the absence of dystrophic neuritis has also been described, findings similar to the plaques seen in the human cerebellum. There is no significant correlation between the extent of β-amyloid deposition and neurone loss. The aged canine brain appears to represent a model of early plaque development [31]. Neuritis plaques have also been observed in aged bears (a rather inconvenient model) but not in rats and mice. The aged animal has the advantage of being an integrated model, but unfortunately is rather a model of normal than of pathological ageing.

NEURODEGENERATION MODELS

Basal forebrain lesions

The reasons for developing a choline-deficient animal model include the following arguments [32]:

(a) A cholinergic effect is most consistently implicated in Alzheimer's disease associated with a loss of cells in Meynert's *nucleus basalis*. This region contains large cholinergic neurones that project to the amygdala, hippocampus and the entire neocortex.

(b) Cognitive deficits have been observed in normal subjects after pharmacological disruption of cholinergic transmission.

(c) Long-term memory function has been shown to be facilitated after treatment with cholinomimetics.

In the rat, the *nucleus basalis magnocellularis* (NBM) is considered to be analogous to the human *nucleus basalis* of Meynert. When lesions of this structure are induced either by injection of neurotoxins (kainic or ibotenic acid) or by electrolytic manipulation or surgery, the expression of cholinergic markers in the brain is decreased in association with profound impairment in recent memory. But cell destruction is not limited to cholinergic neurones. Unlike Alzheimer's disease, this memory impairment frequently recovers and drugs that are effective in these animal models are not as effective in humans with Alzheimer's disease. Another glutamate analogue, quisqualic acid, destroys 80–90% of all cholinergic neurones within the NBM

without memory impairment, which would raise doubts concerning the cholinergic neurone theory of memory in this structure [33].

This type of lesion has also been studied in primates. The conclusions are similar: performance usually recovers to the control level, the compounds which are effective in the model do not predict a beneficial action in man, and memory deficits do not correlate with the degree of cholinergic cell loss. Thus, monkeys with basal forebrain lesions may be more useful as models of the attentional deficits associated with Alzheimer's disease than as models of learning and memory impairment [34].

Another approach is to study trisomy 16 mice (Ts16). Chromosome 16 in mice is homologous to chromosome 21 in humans, including the gene coding for APP so the Ts16 is an animal model of Down's syndrome. As Ts16 mice do not survive birth, researchers [35] have transplanted foetal Ts16 basal forebrain tissue into the hippocampus of young adult mice. Ts16 transplants demonstrated age-related cholinergic neuronal atrophy but Aβ amyloid containing plaques were not observed.

Cholinotoxin

Ethylcholine aziridinium (AF64 A), a compound with close structural similarity to choline, acts directly at the cholinergic nerve ending; it is also more specific for the cholinergic system. Intraventricular administration of this compound induces a long-term choline-deficiency in animals as indicated by a significant reduction in all cholinergic biological markers and cognitive disturbances. Tests such as the radial arm maze and the T-maze task (test of working memory losses due to hippocampal choline-deficiency) performed on AF64 A-treated rats should serve as a suitable model for the earlier stages of Alzheimer's disease [32].

Glutamatergic denervation

The hypothesis that a glutamatergic deficiency could exist in Alzheimer's disease is a more recent suggestion with less supporting evidence than the cholinergic hypothesis. The glutamatergic system may be particularly interesting because of its implication in excitotoxicity and memorization. Also as an alternative, glutamatergic denervation is proposed. In the rat, transection in the white matter between the temporal cortex and lateral entorhinal cortex resulted in a severe mnemonic dysfunction which is effectively ameliorated by pharmacological methods [36].

Administration of aluminium

The older hypothesis in which aluminium was said to be involved in the progress of Alzheimer's disease has generated much controversy [37]. It nevertheless inspired the development of early models for Alzheimer's disease. Intracerebral administration of aluminium in rabbits brings about neurofibrillary degeneration in several brain regions and a decrease of choline acetyl transferase in the striatum, but not in the cerebral cortex and hippocampus where the decrease mostly occurs in Alzheimer's disease [38], no amyloid plaques are formed and few changes in other neurotransmitter systems and behaviour have been reported [39].

TRANSGENIC ANIMALS

The generation of diseases in animal models by means of genetic engineering will revolutionize experimental pathology. The search for overexpression of genes expected to be implicated in the genesis of Alzheimer's disease is a promising approach which has not yet become operational. At the present time, none of the transgenic animals reproduce the features of Alzheimer's disease-type pathology. All the studies to date have used mice, the idea being to transfer human genes to these animals. Gene transfer has focused on the genes coding for the different types of APP as shown in the trials discussed below (for reviews, see [26,40,41]).

In transgenic mice expressing a human APP-751 cDNA under the control of a neurone-specific enolase promotor, it has been possible to detect expression of mRNA from the transgenes. Furthermore, certain brain regions of the transgenic mice displayed extracellular β-amyloid immunoreactive deposits [42]. As other authors have been unable to demonstrate such expression, these results remain to be confirmed.

Another line of animals express the C-100 terminal of human APP driven by the brain dystrophin promotor. The 4–6 month-old transgenic mice studied showed an accumulation of Aβ immunoreactive material and mRNA from the transgene was detected [43].

In another experiment where overexpression of the C-100 fragment was driven by the JC viral early region promotor (a human papovirus with brain-specific tissue tropism), brain specific expression of mRNA from the transgene was also detected and increased Aβ immunoreactivity occurred although no Aβ deposits were observed [44]. Expression of the isoform APP-695 under control of the metallothionein promotor led to impaired spatial memory without neuropathological abnormalities [45].

When the C-99 terminal with APP signal sequence was placed under the

control of a cytomegalovirus enhancer and a chick β-actin promotor, high levels of mRNA expression from the transgene were described but immunohistochemical and histopathological studies did not reveal differences between transgenic and control mice and no neuronal degeneration was found in the 13-month-old transgenic animal [41].

Most recently, Games *et al.* [46] published an unusual construct which included the full-length human APP complementary DNA with the APP-717 val→phe mutation under the control of the platelet-derived growth factor promotor. This construct directs much higher levels of APP expression. These mice have neuritic plaques and neuronal and synaptic loss with reactive gliosis with a neuroanatomical distribution as in Alzheimer's disease. There was no evidence of the occurrence of neurofibrillary tangles. This model appears to be by far the most promising but as yet an insufficient amount of data is available on its usefulness. Two previously published mouse models have been retracted, one because it turned out to be an artefact, the other because a misconducted investigation was suspected [47].

There has been some debate as to the reasons why these models of Alzheimer's disease have been relatively unsuccessful [48], especially since similar transgenic models have been quite useful in other disorders including prion diseases, cystic fibrosis and the Lesch-Nyhan syndrome.

Mice, or rodents in general, might simply be a poor model since these animals never develop amyloid plaques spontaneously when ageing. Or perhaps the native murine sequence protects against β-amyloid deposition. All the transgenic animals have had APP-derived sequences with artificial promotors, so the levels of expression of the transgene under these conditions is relatively low and the constructs are expressed with an inappropriate tissue distribution. It is likely that the actual nature of the APP transcript is important in pathogenesis. Perhaps the sequences used to date are not the best sources of β-amyloid deposition. Finally, it is possible that the role of Aβ deposition in the aetiology of Alzheimer's disease has been overestimated.

In any case, intensive research has been conducted in this field and many other possibilites are under study. One method is to introduce into the mouse genome genes cloned in yeast artificial chromosomes (YACs) which have the capacity to handle full-length genes so the entire APP gene can be used. Another possibility is to focus less on the APP gene and try to introduce into the mouse the human gene coding for ApoE4, or to render the animals deficient for the *E4* allele. Homozygous ApoE-knockout mice display significant loss of synapses and marked disruption of the dendritic cytoskeleton. They also demonstrate poor hippocampal compensatory synaptogenesis following removal of entorhinal-cortex projections [49].

OTHER APPROACHES

Other models have been described which reproduce learning impairment and memory loss. These models are based on lesioning or pharmacological blockade (scopolamine) of specific neurotransmitter pathways, or as in the Brattleboro strain of rats where vasopressin production is perturbed due to an hereditary disorder. These models will undoubtedly make a contribution, but they are aimed at studying memory and do not necessarily elucidate the characteristic features of Alzheimer's disease.

Different aproaches have been proposed. Several biochemical markers of mitochondrial dysfunction have been characterized in Alzheimer's disease, especially a decrease in activity of the mitochondrial enzyme cytochrome oxidase in blood platelets. In rats given a chronic infusion of sodium azide, an inhibitor of cytochrome oxidase, both spatial and non-spatial learning impairment have been demonstrated [50].

Another hypothesis is that the β-amyloid protein would be toxic for neurones. Using this theory, models would not attempt to induce β-amyloid synthesis in the animal but rather be based on injecting amyloid bodies, isolated from the brains of individuals with Alzheimer's disease, into the rat hippocampus and cortex. Other *in vitro* investigations focus on neurone cell cultures derived from different zones of the brain. The most convincing results have been reported using Aβ peptide injected into the brains of aged non-human primates, but have not been reproducible. It is not clear whether differences in methodologies for synthesizing or purifying Aβ peptides account for differences in toxicity [51].

NEURONAL DEATH

Why do neurones die? For both acute neuronal diseases and neurodegenerative pathology, the answer to this fundamental question will provide the key to modifying the process which, after a more or less long latency period, turns stimulus into cell death. Calcium plays a fundamental and universal role. Loss of calcium homeostasis is seen either as the direct cause of neurone death or as a secondary event resulting from other phenomena (excitotoxicity, β-amyloid protein, trophicity, free radicals) suspected to be implicated in neurone death. Calcium homeostasis is thus the central theme of several proposals focusing on the interactive nature of the problem [52, 53].

CALCIUM HOMEOSTASIS [54, 55]

In the central nervous system, calcium is implicated in the regulation of membrane excitability, neurotransmitter release, axonal transport, dendrite morphology and regulation of numerous enzymatic reactions. Intracellular concentration of free calcium must be precisely regulated to maintain a strong Ca^{++} gradient between the cytoplasm (10^{-7}M) and the extracellular space (10^{-3}M).

In the central nervous system, calcium can enter the cell from the extracellular space via four types of well-defined calcium ion channels [56]. The L channels (long-lasting) have a high activation threshold and are slow to inactivate. They are widely distributed in neurones and are a specific target of calcium antagonists. The function of the T channels (transient) are less well understood. They are implicated in rhythmic activities. Only synaptic nerve endings have N channels (neuronal). These channels are directly implicated in Ca^{++} dependent neurotransmitter release. The P channels (Purkinje) were discovered more recently and are the most abundantly found type related to synaptic transmission in the mammalian brain. P channels apparently play an important role in neuronal integration as well as in neuronal death. During the ageing process, the ratio of P-channels to calbindin is altered, an excess of P-channels being found in cells undergoing degeneration [57]. Other types and subtypes of receptors (Q- and R-type for example) are currently being described in the central nervous system.

Under conditions of the resting cell, all these voltage-dependent channels are closed. Activated by depolarization, they allow huge amounts of calcium to enter the cell. These channels are controlled by complex regulation systems involving different neurotransmitters.

The *N*-methyl-D-aspartate (NMDA) receptor is an example of a typical agonist-dependent calcium channel in the central nervous system. These receptors are quiescent in basal conditions and are blocked physiologically by magnesium. Their activation requires a strong depolarization to overcome the magnesium blockade and the fixation of two ligands, an excitatory amino acid, usually glutamate, and a co-activator, glycine. When stimulated, NMDA receptors modify sodium, potassium and calcium ion conductance. They form a highly complex and precisely controlled receptor system, but when the regulation mechanisms fail, the intracellular concentration of calcium rises dangerously and causes a cascade of events leading to neuronal death.

Ca^{++}/Na^{+} counter-transportation is a reversible mechanism where three Na^{+} ions are exchanged for each Ca^{++} ion. In this reversible electrogenic system, Ca^{++} enters the cell at depolarization. The calcium pump functions

using either a high Ca^{++} affinity ATPase Ca^{++}-dependent mechanism with a small transport capacity or a Ca^{++}/Na^{+} exchange system which allows Na^{+} to enter into the cell.

Neurones also maintain a large stock of intracellular calcium in the endoplasmic reticulum and mitochondria. Two classes of distinct receptors regulate the mobilization of the intracellular calcium pool contained in the organelles. One type is activated by inositol 1,4,5-triphosphate (IP3) and is inhibited by cytosolic concentrations of calcium above 0.3 mM and by heparin. The other receptors are ryanodine/caffeine-dependent and control Ca^{++}-induced Ca^{++} release [58]. Although the mitochondria can store a large quantity of calcium, their Ca^{++} affinity is low. The mitochondrial pool is only involved in overload situations. Re-storage is regulated by the calcium pump on cell and organelle membranes. Within the cell, Ca^{++} is linked to specific calcium-binding proteins which have different structures depending on the neuronal populations considered (calmodulin, calbindin, parvalbumin, calcineurin, etc). These proteins participate in neutralizing free calcium and thus act as a buffer system.

CALCIUM, AGEING AND ALZHEIMER'S DISEASE

It has been suggested that perturbed calcium regulation plays a role in age-related neurodegenerative diseases. According to this hypothesis, based on very little and controversial evidence, disrupted calcium homeostasis is involved in both normal and pathological ageing.

Several convergent age-dependent mechanisms could lead to both a rise in intraneuronal Ca^{++} concentration and to a decreased capacity of the neurones to eliminate the excess of Ca^{++}. This minimal variation in intracellular Ca^{++} could, after a long latency period, finally lead to effects similar to those observed after short-term, but important, perturbations of calcium concentrations.

Electrophysiological studies have evidenced ageing-dependent alterations of Ca^{++}-mediated potentials and currents in hippocampus neurones of aged rats. It appears that ageing is associated with a greater voltage-activated influx of Ca^{++} through L-type channels [59]. Findings relating to Ca^{++}-binding proteins in Alzheimer's disease have been contradictory. For example, some authors have found no changes in the number of parvalbumin-immunoreactive neurones in certain parts of the brain whilst others have found a decrease in the number and cell size of these neurones. Inversely, a decrease in the levels of calbindin-D28K has been found in the temporal, parietal and frontal cortex of brains of patients with Alzheimer's dis-

ease while other authors have reported unchanged calbindin-D28K in RNA and protein levels in cortical areas [60].

In Alzheimer's disease, a relationship between β-amyloid production and loss of calcium homeostasis has been postulated, but not demonstrated [61]. This rationale is nevertheless the basis of clinical trials using calcium antagonists, particularly with nimodipine.

EXCITOTOXICITY

The concept of excitotoxicity was proposed as early as 1957 by Lucas and Newhouse [62] who demonstrated that glutamate is toxic for retinal cells. In the 70s, the work published by Olney and Ho [63] proved that glutamate and similar compounds administered to rodents during the neonatal period lead to acute neurodegeneration in retinal neurones and periventricular structures. Excitotoxicity is related to an excessive flow of calcium into the neurone resulting from an overstimulation due to different categories of excitatory amino acid receptors [64]. An excess of neurotransmitters produces several effects:

1. Overstimulation of ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazol propionate (AMPA) receptors causes sodium influx which has three sodium-related consequences: (a) change in osmolarity causing either cell swelling or even lysis, or simply micro-leakage via the membrane allowing calcium to enter the cell according to its electrochemical gradient; (b) excess intracellular Na^+ forces the $3Na^+/Ca^{++}$ exchange to favour sodium extrusion and thus calcium entry; and (c) the depolarizing entering sodium current activates the voltage-dependent calcium channels and consequently further favours calcium entry.

AMPA receptors were long thought to be impermeable to Ca^{++}. Today it is known that only one of the four sub-units of the receptor, Glu-R-B, is impermeable to calcium. The lack of Glu-R-B expression in certain structures or its transitory disappearance in particular pathological conditions, indirectly implicates a phenomenon of calcium overload.

2. Direct stimulation of the NMDA receptors leads to depolarization which inhibits magnesium-related blockade and represents also a major pathway for Ca^{++} entry.

3. Finally, the activation of metabotropic receptors coupled with phospholipase C, by inducing a higher level of IP_3, mobilizes calcium from the intracellular stocks and also contributes to disrupted calcium homeostasis.

The increased intracellular concentration of free calcium triggers a series of hyperactive enzyme reactions with deleterious effects [65]. There are several examples:

(a) phospholipase A_2 causes production of arachidonic acid or platelet-activating factor. Platelet-activating factor stimulates glutamate release whilst arachidonic acid inhibits recapture by the glial cells and neurones. The result is an excessive neurotransmitter concentration. In addition, the catabolism of arachidonic acid generates free radicals which cause lipid peroxidation.

(b) Nitric oxide (NO) synthases provide another example. The chemistry of this gas is complex. It can exist in several states which have different, even opposing effects. The nitrosonium ion NO^+ links to the redox modulator site of the NMDA receptor inhibiting its activity, but the reaction between NO and the superoxide anion O_2^- forms peroxynitrite $ONOO^-$ which decomposes into highly toxic free radicals. The result is a complex system and, depending on its site of production and the environment, NO may or may not lead to neurone death.

(c) when activated, endonucleases fragment DNA and initiate apoptosis.

(d) phospholipase C generates IP_3 which induces calcium release from intracellular stocks.

(e) calpains I and II lead to degradation of the cytoskeleton.

(f) different families of kinases can cause abnormal phosphorylation of the tau protein or other cytoskeleton proteins that, in turn, lead to the formation of neurofibrillary tangles. Overstimulation of APP phosphorylation also modifies proteolytic production of the different, but more or less amyloidogenic, fragments.

(g) the proteases which in the xanthine dehydrogenase/xanthine oxidase equilibrium favour the oxidase form, metabolize hypoxanthine and generate free radicals.

What is the series of events leading to the abnormal glutamate accumulation that triggers excitotoxicity [66]? Glutamate is essentially stored within cells at an intracellular concentration of about 10 mmol/l. The normal extracellular concentration is about 0.6 μmol/l while the excitotoxicity concentration is estimated to be 2–5 μmol/l. Glutamate is recaptured by the astrocytes and neurones via a sodium-dependent high-affinity uptake system. Any defect that impairs the ability of cells to maintain the sodium gradient might cause the glutamate-uptake system to fail. Glutamate may also be released into the extracellular fluid as a consequence of swelling of the astrocytes. For example, energy failure dampens Na^+/K^+-ATPase activity and therefore increases the intracellular sodium concentration.

Another possible source of excess glutamate is abnormal release from its storage sites in neuronal vesicles. This, for example, is what happens during hypoxia, ischaemia or hypoglycaemia. The simplest cause of excess extracellular glutamate is injury to cells. One of the reasons is excitotoxicity itself, which explains why the phenomenon is self-sustaining. This also happens

during trauma. In addition to the effect on Na^+/K^+-ATPase, energy failure and depletion of intracellular ATP impedes the conversion of glutamate to glutamine in astrocytes leading to intracellular accumulation of glutamate. At some concentration of intracellular glutamate, the capacity of the inward glutamate transporter would be overrun causing the concentration of extracellular glutamate to rise.

EXCITOTOXICITY AND ALZHEIMER'S DISEASE

A wide variety of acute and chronic neurological diseases may be mediated, at least in part, by a final common pathway of neuronal injury involving excessive stimulation of glutamate receptors. Excitotoxicity is known to have an important effect on neurone degeneration in certain acute disease states such as ischaemia, trauma and epilepsy. For neurodegenerative diseases, it is assumed that the same type of effect would occur. There are several arguments in favour of this hypothesis.

Dietary consumption of excitotoxins

Dietary intake of vegetable compounds with a chemical structure similar to that of excitatory amino acids has been incriminated in many neurological pathologies: *β-N*-oxalylamino-L-alanine (BOAA) a toxic component of the chick pea, triggers neurolathyrism (spastic paraplegia) [67]. The Guam island syndrome associating amyotrophic lateral sclerosis, Parkinson's disease and dementia have all been associated with the consumption of *β*-methylamino-L-alanine (BMAA) contained in the flour prepared from cycade nuts (*Cycas circinalis*) [68]. Blue mussel poisoning which occurred in Eastern Canada in October 1987 was linked to a marine diatom, *Nitschia pungens*, that serves as a foodstuff for blue mussels. The implicated excitotoxin was domoate (an agonist of the kainate receptor). Anterograde amnesia was a common persistent finding in the victims.

Glutamate and neurone plasticity

Long-term potentiation is considered to be an experimental model of memory at the cellular level. In the CA_1 region of the hippocampus, a low frequency stimulation frees glutamate which, by activating the AMPA receptors leads to sodium influx and production of a post-synaptic potential of a certain amplitude. Under these conditions, the NMDA receptor is inactivated, blocked by Mg^{++} ions.

Stimulation by a high frequency tetanizing pulse causes a major depolar-

izing influx of sodium, inhibiting NMDA receptor blockade and allowing calcium to enter the cell. Comparatively, the amplitude of the post-synaptic potentials obtained before and after tetanizing stimulation shows that after tetanization, the response rises for a period which can last several days. This long-term potentiation can be related either to an increased sensitivity of the AMPA receptors to glutamate or to increased glutamate release. If NMDA receptor antagonists are administered during the high frequency stimulation, long-term potentiation does not occur. Induction of long-term potentiation is dependent of a rise in intracytosolic calcium concentration. A retrograde messenger (arachidonic acid and/or NO) transfers the information from the post-synaptic to the pre-synaptic element. Excitatory neurotransmission is also involved in developmental plasticity - glutamate serves a trophic role on developing neurones - the NMDA receptor may participate in neurone elongation, branching and the refinement of synaptic position during early development [69].

Due to the crucial role of aminoexcitatory neurotransmission in learning and memory and in neurone plasticity, this may have an important role in diseases where memory impairment is a major symptom.

Pre- and post-excitatory amino acid markers in Alzheimer's disease [70]

Post-mortem studies on human brains have established that many neurones of the cortex and hippocampus involved in Alzheimer's disease are glutamatergic neurones. Many of the neurones that are damaged in Alzheimer's disease utilize excitatory amino acids as neurotransmitters and also receive widespread inputs from other excitatory amino acidergic neurones. In addition, the concentration of glutamate in regions of the brain damaged most in Alzheimer's disease are lowered and there is a correlation between the glutamate level in the cerebrospinal fluid in Alzheimer's disease patients and the degree of dementia.

Post-synaptic receptors are also altered in the brain of patients with Alzheimer's disease. NMDA and metabotropic receptors are decreased with less marked effects on AMPA receptors. These changes occur in areas of the brain most pathologically affected by the disease. Several pieces of evidence are inconsistent with a primary role for excitotoxic mechanisms in the aetiology of Alzheimer's disease, but it is nevertheless probable that these mechanisms participate in the pathogenesis of Alzheimer's disease which explains the development of many compounds affecting all levels of aminoexcitatory transmission.

β-Amyloid neurotoxicity

Amyloid deposition is a central event in the aetiology of Alzheimer's disease. The β-amyloid protein comes from a precusor protein containing 695 amino acids with a long extracellular segment, a transmembrane segment, and a short intracytoplasmic domain. The Aβ portion has 28 amino acids situated in the extracellular segment and a 12 to 14 amino acid transmembrane sequence.

APP 695 belongs to a family of proteins including the APP 751 and APP 770 isoforms which contain the Kunitz-like protease inhibitor domain, APP 714, and also a shorter form with only 563 amino acids. The soluble forms of APP which contain the KPI domains have also been identified as heparin-activated inhibitors of coagulation factor Xia and as protease nexin II. APP is post-translationally processed by numerous proteolytic pathways which lead to secretion of various fragments of APP or intracellular fragmentation and degradation. Proteolytic cleavage by γ-secretase (between Lys 16 and Leu 17 using APP 659 numbering) produces the Aβ amyloidogenic protein. The γ-secretase cleavage site is integrated into the cell membrane. It generates an extracellular aminoterminal fragment of APP containing a complete β-amyloid peptide sequence, potentially amyloidogenic. The α-amino-secretase cleavage (between Met 596 and Asp 597) generates a soluble fragment which corresponds to the 17–42 residues of the Aβ sequence and is not amyloidogenic. Shifts from one pathway to another determine the likelihood of Aβ and presumably senile plaque formation [71, 72].

Aβ amyloid deposits occur in two morphological classes: the non-fibrillar diffuse plaques, non-congophilic, or pre-amyloid which are not associated with significant neuronal pathology, and the mature fibrous senile or neuritic plaques (end-stages of the distinct α-amino-and β secretase pathways?).

Is amyloid deposition a causal agent or merely a secondary event resulting from neuronal damage? To date, there is no answer to this question, nor to its corollary: is β-amyloid neurotoxic? The initial evidence of Aβ's potential neurotoxic activity was reported by Yankner *et al.* [73] on transfected PC 12 cells with the C-terminal sequence of APP including Aβ. *In vivo*, the results of Aβ injections are contradictory. Kowal *et al.* applied the β1-40 fragment directly to rat brain parenchyma and showed that the neurodegenerative effect was antagonized by substance P [74]. But microinjection of synthetic amyloid β protein in monkey cerebral cortex failed to produce neurotoxicity [75]. It has also been proposed that Aβ generally causes little direct neurotoxicity by itself but can enhance the vulnerability of neurones to a variety of other insults such as excitotoxicity or peroxidative damage. For other workers, Aβ would act as a trophic factor [76, 77]. The same questions were

raised during earlier attempts to create Alzheimer's disease in animal models using Aβ. There appears to be at least three variables involved [78]. Firstly, although the peptides cannot be differentiated on the basis of their physical or chemical properties, the aggregation of Aβ varies according to its source of preparation, the aggregation state of the peptide being crucial for the manifestation of degeneration. Secondly, the density of the exposed cells also affects cytotoxicity, high-density cultures being more resistant. Thirdly, all investigators have reported that the injection of vehicle alone causes some degree of tissue damage and that this effect is not easily distinguishable from the degenerative effect induced by an Aβ solution.

Mattson *et al.* developed a promising though speculative theory relating APP mis-metabolism to loss of calcium homeostasis [79–81]. Normal APP metabolism is regulated by the action of secretases producing soluble APP fragments which would play a physiological role in Ca^{++} regulation via specific receptors. The transduction mechanism would implicate GMPc. When activated, these receptors would cause a drop in $[Ca^{++}]_i$ and thus be implicated in neuroprotection and synaptic plasticity. *A contrario*, in Alzheimer's disease, mutations, overexpression or age-related changes may favour β-amyloid peptide formation and generate Aβ aggregates which themselves destabilise $[Ca^{++}]_i$, alter neurite outgrowth, and render neurones vulnerable to excitotoxicity.

According to Pollard *et al.*[82], the Aβ forms cation-selective channels which are capable of transporting calcium and some other monovalent cations when incorporated in artificial phospholipid bilayer membranes. Whether such an effect occurs in intact cells requires further study. These theories assume that Aβ is a substance not normally produced by cells but only in brain tissue of certain aged mammals and in patients with Aβ-type amyloidosis. This assumption would be in contradiction to recent reports which show that in cell cultures, under normal metabolic conditions, soluble Aβ is produced. Establishing a functional role for Aβ remains a challenge.

Probably, Aβ deposition is a necessary, but not sufficient factor for the pathogenesis of Alzheimer's disease. Perhaps there is a chronic imbalance between Aβ production and clearance leading to the gradual accumulation over decades of Aβ deposition far in excess of those produced during ageing. When Aβ reaches a critical local concentration, an aggregation of monomers followed by oligomers may occur, leading to fibril formation (see [61] for review).

We have discussed briefly a very complex and constantly changing subject. Other studies will be needed to clarify the situation. Nevertheless, it can be stated that the formation of amyloid plaques is a pathogenetically critical feature of the disease. A pharmacological strategy which could interfere

with one or more steps in this complex cascade remains to be developed. Molecules that inhibit the proteinases that produce the β-amyloid protein from APP, block assembly of soluble monomeric Aβ to insoluble aggregated Aβ, or interfere with the toxic response of neurones and other cells that contribute to the chronic inflammatory process around the neuritic plaques offer a potential therapeutic target for Alzheimer's disease [83, 84].

NEUROTROPHIC FACTORS

Neurotrophic factors are chemical substances secreted by target post-synaptic cells into synaptic clefts. They are taken up by the pre-synaptic terminal and transported retrogradely along the axon to the cell-body where they modify neuronal metabolism, often by affecting protein synthesis. Experimental blockade of neurotrophic factors results in nerve cell damage or death.

Neurotrophins play an important role in the normal development of the nervous system, but their function in the mature central nervous system is poorly understood. Nerve growth factor (NGF) is necessary for the survival and maintenance of the basal forebrain cholinergic system.

NGF is secreted by neurones in the hippocampus and is transported via the fimbria fornix to basal forebrain cholinergic neurones. In Alzheimer's disease, a disruption in NGF production could explain the degenerations of the *nucleus basalis* of Meynert. In Alzheimer's disease, neurones are only lost from the rostral part of the locus ceruleus where neurones are responsive to cortical neurotrophic factors [85]. Actually, it appears that NGF production in the brain of patients with Alzheimer's disease is normal. Inversely, decreased expression of brain-derived neurotrophic factor (BDNF) has been detected in post-mortem Alzheimer patients' brains. Furthermore, NGF receptor density has been shown to be significantly reduced. Loss of neurotrophin receptors may result in neuronal degeneration, as available factors are unable to convey trophic support to dependent neurones. In addition, possible interactions between trophic factors and amyloid protein deposits have been investigated. Administered *in vitro*, NGF would potentiate β-amyloid neurotoxicity. The gamma subunit of the 75 NGF complex appears to possess peptidase activity and could contribute to APP mis-metabolism [86].

Another completely different approach has been seen with epidemiological studies [85] emphasising the role of trophic factors. In the region around Shanghai, in China, two subgroups of population have been formed: one subpopulation of illiterate persons and another subpopulation of educated persons. In the first group, the prevalence of Alzheimer's disease is higher

whilst in the second group, onset comes earlier. Experimental studies suggest that learning is associated with increased numbers of cortical neuronal synapses and elevated levels of neurotrophic substances. Thus, a longer time is required for the Alzheimer's disease process to interfere sufficiently with the enhanced levels of neurotrophic factors to result in neuronal death.

An ideal neuroprotective agent would act to prevent death by halting the progression of degenerative changes and, in addition, initiate trophic events that repair and reverse the inflicted damage [87]. Several studies have shown the protector effect of NGF in a model of cerebral ischaemia [88] or hypoglycaemia [89] where NGF would antagonize neurone death in the hippocampus.

The use of trophic factors as a therapeutic approach has been envisaged but the number of obstacles is substantial. NGF does not cross the blood-brain barrier and possible undesirable effects are still poorly investigated. The high cost of recombinant NGF is also of significant importance.

NEUROFIBRILLARY TANGLES

Neurofibrillary tangles (NFTs) together with the protein β-amyloid, are the major pathological features of Alzheimer's disease. NFTs consist mainly of paired helical filaments which result from the assembly of abnormal protein tau. Normal protein tau links to microtubules, thus contributing to the stability of the cytoskeleton which is essential to neurone function. The tau protein's ability to bind to microtubule segments is partly determined by the number of phosphate groups attached to it. NFTs carry many more phosphate groups than cover the normal protein, including many at sites not ordinarily phosphorylated. Extra phosphate might derail the normal process. The masses of NFTs may further obstruct cellular transport and damage the neuron. The cause(s) of these abnormal phosphorylations is(are) poorly understood [90].

Reduction in activation of phosphate enzymes removes phosphate groups from tau. In fact it seems that phosphatases are suppressed in the neurones of Alzheimer's victims. A role for aluminium has also been suspected. Aluminium salts could link to phosphate groups modifying the molecular conformation of the protein, rendering it less accessible to enzymes.

A relationship with transmission of the Apo$E$4 allele has also been established. The Apo$E$3 binding to tau may regulate the function of tau. Apo$E$3 may act as a beneficial cofactor or a sequestration agent to prevent phosphorylation for a portion of the tau pool. This regulation would not be present in individuals who express only E4 protein [91].

EARLY PHASES OF CLINICAL DEVELOPMENT OF DRUGS FOR ALZHEIMER'S DISEASE

The term 'early phase' in the clinical development of drugs includes work in the classical Phases I and II, the first stages of administration to man and the detection of the first pharmacological effects as a function of dose and drug kinetics. The question raised here is whether, when developing drugs with potential effects in Alzheimer's disease, these early phases have any specificities. Whatever the answer to this question, it is obvious that the objectives in these early phases are similar to those for other classes of drugs. They are predominantely aimed at evaluating acceptability, determining the precise pharmacokinetic characteristics of the studied compounds in healthy volunteers (young and elderly) and identifying the dose-effect and pharmacokinetic/pharmacodynamic relationships (PK/PD) after repeated administration. We shall focus on the problems which *a priori* appear to be specific for drugs with potential effects in Alzheimer's disease, excluding compounds which would act on the associated non-cognitive signs such as depression, agitation and dyssomnia. Such an approach may appear rather theoretical for at least two reasons. First, a multitude of drugs of doubtful efficacy and for which early studies appear to be lacking have been proposed (at least in France) for cognitive impairment in senility ('nootropic' drugs, for example). Second, most of the compounds for which *a priori* there appears to be a promising therapeutic possibility have been administered to patients (as treatment) without any of the prerequisites regularly expected for other classes of drugs. The example of tacrine for which the question of dose was raised almost at the same time as the marketing authorization was delivered is quite instructive! Today it is urgent to emphasize the mandatory nature of early phase trials, the necessity of standardization, and that early phase trials are time-saving (for patients) and money-saving (for society) and should be conducted within the framework of a rational development scheme.

REQUIREMENTS FOR INITIAL CLINICAL TRIALS

The decision to undertake trials

This highly complex decision is generally based on the preclinical assessment of product toxicity, the biological basis of the mechanism of action and on animal test results. This apparently obvious first- step often creates a good many problems. Indeed there are large numbers of sometimes contradictory potential pharmacological targets for such drugs [92]. For example,

transmission of memory messages depends mainly on glutamate which is simultaneously a source of excitotoxicity. Current trends in research also influence decision-making since it is quite tempting to work on the most recent advances in biology. Inversely, there are few animal tests capable of providing a positive effect on cognition and the tests which do exist often tend naturally to give overly optimistic results. It is no exaggeration to state that the decision to initiate a phase I trial is basically a question of industrial management with a goal of choosing compounds with the best probability of reaching the marketing phase.

Theoretical basis of mechanism of action

Phamacologists recognize that drugs do not have a unique mechanism of action and that, in the case under consideration, the mechanism of action chosen to justify a potential interest in a product is either the one most evident in *in vitro* or animal tests or the one which is currently the most fashionable. There are many examples. Drugs stimulating neurone metabolism, whilst totally ineffective in Alzheimer's disease, correspond to the regional hypometabolism suspected a few years ago and widely confirmed by new techniques of neuro-imaging. For tacrine, a cholinesterase inhibitor, the pro-cholinergic effect is emphasized although the drug also affects histamine and potassium channels [1]. It is also important to mention that transduction mechanisms are sometimes modified in Alzheimer's disease, as is the case for cholinergic receptors [93].

But opposite situations are also seen. For example, S12024, a drug acting on vasopressin and noradrenaline (norepinephrine) passed through the early phases rapidly and has already reached comparative phase III trials [94]. The traditional calcium antagonists are also in phase III, even if it is clearly demonstrated (recently) that they cannot affect the specific neuronal calcium channels implicated in the massive calcium influx incriminated in neuronal death [95]. A final example concerns free radicals. This old and latent theory is still quite active today. It led to the development of a powerful scavenger molecule, exifone [96], which was quickly withdrawn due to its hepatotoxicity and especially because this mechanism of action is no longer in the main stream of thinking in international neurology. Here again we see that the mechanism of action is not necessarily a sound basis for discussion and that the relationship between mechanistic theory and production of proof of clinical effectiveness is not always easy to perceive (*Table 1.2*).

Table 1.2. MECHANISTIC APPROACH IN THE DEVELOPMENT OF DRUGS FOR ALZHEIMER'S DISEASE

Mechanism	*Pharmacology*
1. Apolipoprotein (Apo)	
Lack of Apo E3	
Alteration of protein tau	Supplementation in Apo E3
Contribution to NFT	
Microtubules instability	
Presence of Apo E4	
Amyloid insolubility	Blockade of Apo E4
Deposit in the plaques	
2. Loss of neurotransmitters (NT)	
Acetylcholine	THA, M_1 agonists
GABA	Anti$GABA_A$ receptors
Nicotinic receptors	Agonists
Glutamate (Glu)	Glu antagonists (NMDA)
Other NT	MAO inhibitors, S12024
3. Cell death	
Calcium overload	Calcium antagonists
Trophicity	Neurotrophic factors
4. Oxidative stress	
	MAO-B inhibitors
	Free radical scavengers
5. Other mechanisms	
Amyloid; protein tau	Effect on kinases or proteases
Hormones	Oestrogens
Inflammation	NSAIDs; glucocorticoids
Circadian rhythm	Melatonin agonists

Symptomatic action versus aetiopathology

It is often stated that early phase studies are impossible in Alzheimer's disease because the corrective action of a truly effective drug on the inevitably long progression of the physiological process would be too slow. On this basis, levodopa, which is so important for Parkinson patients, would still be on the laboratory shelf. For the same reason, deprenyl (MAOI-B) - initially developed for its purely symptomatic effect - is now being studied for its possible cytoprotector action [97, 98]. In Alzheimer's disease, there has been discussion (and some efforts at verification) as to whether drugs initially con-

sidered to give symptomatic relief might also have a cytoprotector effect. The question raised is to determine whether early-phase studies can provide short-term evidence for the detection of a 'pharmacodynamic' effect, which in turn would predict a protector effect slowing the progressive nature of the disease. This is the typical question in clinical pharmacology, whatever the disease under consideration (notion of intermediary criteria, surrogate endpoints). This type of discussion is somewhat disorientating and not very convincing for pure clinicians. Nevertheless, the answer is yes, surrogate endpoints are useful, even if no reference drug is available to provide ultimate proof. Stated clearly, this means that intermediary criteria (discussed below) are the expression of a product's pharmacodynamic activity which will finally (as verified in phase III trials) be shown to be therapeutically useful. Schematically, the debate between symptomatic action (supposedly immediately verifiable) and aetiopathogenic action (which can only be verified after long-term trials) appears, in pharmacology, to be an artificial one. This can easily be demonstrated in other classes of pharmaceutical agents dealing with less sensitive diseases.

Methodology

According to Drachman and Sweater [99] in their book on 'Guidelines for drug trials in memory disorders', it is obvious that methodology problems have contributed to discredit the pharmacological class of cognitive enhancers. We agree that these early-phase studies must comply with the strict rules required for all drugs by the regulatory authorities (double-blind, placebo, several doses, single and repeated doses), and that all instruments (EEG, psychometry, biology) are validated beforehand. In these same guidelines [100], attention is drawn to the risk of using methods of questionable value such as the administration of scopolamine (an anticholinergic agent assumed to have an effect on memory) or hypoxia (successful tests with piracetam derivatives). Finally, the biomathematic approach to early trials in Alzheimer's disease does not raise any further questions if the conditions stated above are clearly taken into account, if the assessment tools are validated beforehand and if the effect has been shown to be quantifiable within the range of desired variability. Consequently, the nature of early trials in Alzheimer's disease would not appear to be specific and would not limit their application since the goal is to quantify pharmacodynamic effects compared with placebo and to verify that the product is well tolerated.

AN EXAMPLE: THE HIPPOCAMPUS IN ALZHEIMER'S DISEASE

There are a number of arguments which imply that a dysfunction in the hippocampus could be the inaugural event in Alzheimer's disease. Unlike Parkinson's disease, the clinical signs of Alzheimer's disease are apparent when the cholinergic loss is still moderate (-35%). Pathology findings (senile plaques, neurofibrillary tangles) [101], neurochemistry and even anatomy (thickness of the medial temporal lobe) are inaugural clues pointing to the fact that memory is one of the first functions to be affected in the early stages of Alzheimer's disease.

The mechanistic aim of pharmacology

Here, the objective is to halt the pathophysiological process occurring in the hippocampus and to re-establish the normal function of this anatomical and functional element of the central nervous system. More precisely, the anatomical target could be the gyrus dentatus, the focal point between the cortical and subcortical afferents and the hippocampus. Increased cholinergic activity and stimulated cell response in this structure should be the principal objective. One well documented fact should be recalled here, namely, that cholinergic transmission is a phasic rhythmic phenomenon suggesting that tonic stimulation (the usual effect of an agonist for example) could not mimic this type of neurotransmission. This would be the explanation for the indirect pharmacological approach to these cholinergic neurones via functional interactions between neurotransmitters: opiates, dopamine, glutamate, GABA. Experimentally, blockade of $GABA_A$ receptors (with flumazenil for example) amplifies cholinergic rhythmic activity, favouring memory and spatial orientation as well as theta activity on the EEG. Basing research on drugs with this mechanism means that the compounds to be assessed will have an impact on memory and orientation and that an effect at this level will be observable using 'hippocampus-specific' criteria.

'Hippocampus-specific' endpoints

(a) Psychometry. This raises the delicate question of 'hippocampus-specific' tests. Psychometric tests evaluate a capacity more than a specific anatomical structure; for example, the Wisconsin card sorting test explores the capacity to develop a strategy and adaptation much more than the frontal lobe *stricto sensu*. According to such reasoning, all tests capable of evaluating functions which primarily involve the hippocampus could provide surrogate endpoint criteria. Essentially, the tests available explore different components of

memory, components which are chronologically impaired in the initial stages of Alzheimer's disease. Batteries of reliable standardized tests (Cantab, SM9, BEM144, Wechsler-memory...) which can be repeated without a learning phenomenon should be favoured over global tests in order to assess the effect of drugs over time.

The search for the pharmacological activity of a (new or old) product thus requires the use of a battery of tests in volunteers and patients in the early stages of the disease. These tests evaluate different types of memory (*Table 1.3*) and are always reinforced by careful follow-up of attention and vigilance. These tests are sensitive to the deleterious action of drugs (mainly psychotropic agents) but have been shown to be sensitive to pro-memory agents. Using such a battery of tests, our team was able to demonstrate that a nootropic drug, EGb761 [102], a D2 dopaminergic agonist (piribedil) and a reversible MAOI-A, (moclobemide) [103] all have a positive impact on delayed recall of words and/or images. In our opinion, the fact that positive effects can be obtained in man, associated with recognized effects in experimental animal models, justifies continuing clinical development and relates the dose range to a recognized effect.

Table 1.3. MAIN PSYCHOMETRIC TESTS USED IN EARLY PHASE EVALUATIONS

1. Memory (declarative/episodic/procedural)	Digit span Benton's visual retention test Buschke's task Associated pair learning test Word list learning test Posner's memory test Dual coding task SM9 battery SM5 battery Toronto/Hanoï tower test
2. Attention, vigilance	Continuous performances task (CPT) Phasal alert task Divided attention task Tracking task Critical flicker fusion point CRT DSST SKT
3. Anxiety, inhibition, stress	DRL task Gonogo's task Stroop's task

(b) Electrophysiology. Quantified electrophysiology (EEGQ) appears to be an excellent intermediate criterion reflecting activity both quantitatively and qualitatively. For the hippocampus, it would be useful to verify the impact focusing on this structure (theta activity). Spectral analysis of modifications in different frequency bands (compared with placebo) provides information of the product pattern requiring prudent interpretation [for example, increased slow activity (4–8 MHz) and rapid activity (20–30 MHz) reflects drug facilitating cognitive tests, particularly memory]. In addition to EEGQ, other more precise but less applicable techniques, including evoked cognitive potentials [9] and magneto-encephalography [104], have been developed to provide objective evidence of pro- cognitive activities. This type of intermediary criteria can also provide quantification of the duration of cerebral activity for the drugs studied. In practice, these electrophysiological criteria are highly dependent on changes in neuro-imaging techniques (SPECT, PET-scan) and are not easily applicable to clinical research where repeated measurements are required on a large number of subjects.

(c) Biology. Besides the mandatory biological tests to detect possible toxicity, several other tests could, exceptionally, be carried out in early phase studies. For example, plasma assays of certain substances may provide indirect proof of the mechanism of action of a drug, for example, prolactin and dopamine, or erythrocyte esterases and anticholinesterases.

Choice of representative subjects

We have emphasized the importance of recruiting both young and elderly [105] healthy volunteers in early clinical trials. It is also important to evaluate, again with the same criteria as presented above, the pro-memory action in groups of subjects with the precise deficit of episodic memory impairment. A real problem is to select the correct type of patients. Age-associated memory-impairment (AAMI) as defined by the criteria described by Crook *et al.* [8] could define a target population for phase II trials despite the fact that recent debate now questions the validity of such a clinical entity [9]. In our experience, besides healthy elderly volunteers (70–85 years), hospitalized patients with disturbed mental function should be enrolled with the aim of detecting drug activity rather than a therapeutic effect (which in any case would be evidenced by large sample sizes). This particularly fragile population would provide valuable information on product tolerance not only in the short-term, but also more realistically, after 1 or 2 months treatment (again versus placebo). In selecting the test population, elimination of different pathologies such as depression and vascular dementia is essential with

long-term evaluation being based on specific or global scales (MMS, Hachinski, SCAG, SKT...).

Conclusion

It thus appears that the hypothesis of setting a target structure, such as the hippocampus, beforehand is only an approximate approach, at least for now, to a specific strategy for drug development. The choice of intermediary criteria alone responds to the question raised (memory tests, EEGQ). The mechanisms of action of hippocampus-targeted drugs would not appear to be particularly pertinent, at least for the time being, although the advent of genetic therapy and the possibility of *in situ* gene vectors could considerably change the situation.

PERSPECTIVES

The lack of early phase studies of compounds for the treatment of Alzheimer's disease appears as a historical gap in drug development. This concept can largely be explained by the speculative nature of the pharmacology of Alzheimer's disease, dominated for too long by results obtained in animal studies and by considerations about mechanism of action. The modern approach in some centres undertaking Alzheimer's disease research is aimed at developing real strategies for conducting phase III trials of drugs in man. The importance of supporting such research into reliable intermediary criteria for Alzheimer's disease is obvious and illustrated by recent progress in cognitive psychology of memory [106, 107]. The development of a drug with a certain, even if minor, efficacy is urgently needed in our search for new drugs to treat Alzheimer's disease since it would provide a reference which is so lacking at present. Considering the populations which could be included in this type of clinical trial, it is important that the assessment criteria be compatible with computer processing and remain accessible for subjects with a certain degree of degradation. Later phase III trials for Alzheimer's disease are both a subject of discussion and of active research and explains why some international consensus is beginning to be developed or at least being encouraged in directives from the official authorities [108]. A new chemical entity could receive marketing authorization if two phase III clinical trials with patient follow-up of at least 6 months have shown positive results compared with placebo. In addition, a significant clinical improvement should be recorded using a validated scale (for example, the ADAS-Cog), an assessment of daily quality of life and a global assessment by relatives and the caregivers. A consensus meeting has recently been held in

Canada where the research options have been established in this field. Parts of this report have been published [108a]. The authors comment on the magnitude of the problems faced by Canadian society as a result of an aging population. The most important issues are, on the one hand, an increasing incidence of dementia and Alzheimer's disease, and on the other, the present limited therapeutic options available. Guidelines are required for optimizing the development of new compounds. The different steps (from Phase I to Phase IV) are described as well as the methods of measuring drug efficacy. It is recommended that an indication for symptomatic treatment of Alzheimer's disease must be supported by a demonstration of efficacy within at least one of the individual domains affected (cognition, behaviour, function). For an indication of disease stabilization, a global staging of efficacy would be mandatory. Phase IV studies should complement pre-marketing studies by the collection of long-term clinical and pharmaco-economic data.

It is probable that the main assessment criteria to be included in phase III clinical trials will evolve rapidly. They would take into account the socio-economic benefit obtained or the improvement in the most characteristic signs of Alzheimer's disease, these symptoms not necessarily or uniquely being cognitive signs.

DRUGS AND ALZHEIMER'S DISEASE

GENERAL PRINCIPLES

To attempt to review the drugs indicated or potentially useful in Alzheimer's disease is a risky process for a number of reasons. The list is long: (1) in 1995, only one compound, tacrine, had been initially authorized for marketing in two countries, France and the United States; (2) few compounds are currently in the development stage and they belong to quite different chemical and pharmacological classes; (3) the final therapeutic objective (symptom relief or halting the pathophysiological process) is not always clear; (4) neurochemical targets of the main drugs currently being assessed in clinical trials do not correspond to the most characteristic features of the disease (β-amyloid deposition, neurofibrillar degeneration); (5) few potential drugs have been assessed in phase III trials using acceptable methodologies, most being screened in so-called 'pilot' studies with an insufficient population sample; (6) the imbalance between disease stage and sensitivity to measuring instruments is a classical problem despite the fact that slow progression of the disease at onset (and thus the difficulty in measuring small variations) and the acceleration of the clinical signs in the moderate stages (MMS be-

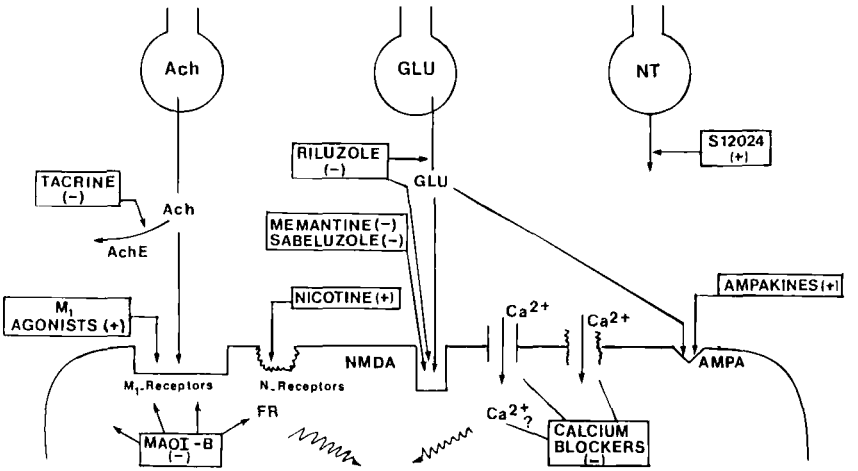


Figure 1.1. Schematic mechanisms of action of the main anti-Alzheimer drugs (see page 3 for key to abbreviations). (-), antagonism; (+), agonism.

tween 12 and 18) is well known; (7) the nearly total absence of clinical pharmacology studies (dose range, assessment of undesirable effects, detection of psychometric actions other than memory effects, interactions such as food/drug interactions).

The slow degradation at the beginning of the disease together with the discrete nature of the clinical signs means that a symptomatic effect, even if non-specific, could be demonstrated (improved vigilance, memorisation, etc) without the compound being a true anti-Alzheimer agent. This type of reasoning should be adapted every time a symptom-targeted product is assessed in the early stages of the disease.

Schematically, certain areas of research are considered to be highly promising. These include MAO-B inhibitors, enhancers of cholinergic transmission, and calcium antagonists. Other areas such as free radical scavengers, glutamate blockers or modulators, and anti-inflammatory agents remain hypothetical. Finally, other areas appear to have recently been abandoned, for example, nootropic drugs, stimulation of cell metabolism or products with membrane properties (*Figure 1.1*). Entirely new areas (for example, gene therapy) are in the animal experimentation stage. While there is complete agreement between theoretical and neurobiological data, solutions to certain questions of ethics and technical difficulties need to be resolved before development of such therapeutic procedures in this area can proceed.

In the following section, we have attempted to avoid the pitfall of over-

emphasizing 'potentially' promising agents which are more a question of fashion than of effectiveness. We shall focus on molecules which have already reached a stage of development such that they can be looked upon as treatments for the 'immediate future'.

SELEGILINE

Medicinal chemistry

Selegiline [(*R*)(-)-*N*,α-dimethyl-*N*-(2-propynyl)benzeneethanamine, Eldepryl, Deprenyl, 1] is a phenethylamine derivative: which is pharmacologically active in the levo form. It is indicated as adjuvant treatment with levodopa in Parkinson's disease, and is supplied as a 5 mg tablet of selegiline hydrochloride. It belongs to the class of type B inhibitors of monoamine oxidase (MAOI-B). At high doses (>40 mg/day in man), selegiline loses its MAOI-B specificity and also inhibits MAO-A.

$$PhCH_2CHMeNMeCH_2C{\equiv}CH$$
(1)

Justification for use in Alzheimer's disease

The main property of this drug is to inhibit catabolism of certain amino acids (phenylethylamine, dopamine). The compound is assumed to improve amine-dependent neurotransmission systems and is thus appropriate for certain symptoms of Parkinson's disease. More importantly, MAO-B inhibition opposes an age-related increase in enzyme activity which could rise by as much as 20 to 30% per decade, particularly in certain structures of the central nervous system (thalamus, nucleus caudatus, hippocampus, amygdala, cingulum). This effect could be the reason why rats treated with selegiline live longer than control animals [109]. MAO-B inhibition in the mitochondria antagonizes oxidation due to both endogenous and exogenous agents (neurotoxins such as MPTP) and the production of free radicals. This argument is applicable to Alzheimer's disease since MAOIs can be considered to be potential enhancers of cognition and neurocytoprotectors [110]. This mechanism has been fruitful in another degenerative disease, Parkinson's disease, where the two therapeutic effects, symptomatic relief [111] and protection against motor decline [112], have been proven.

Pharmacological properties

The symptomatic action of MAO-B inhibitors is mediated by blockade of the MAO-B enzyme involved in dopamine degradation, which results in increased dopamine availability at the synapse. This is not, however, the only mechanism of action. The symptomatic effect is also mediated by inhibition of amine uptake and a major increase in phenylethylamine concentrations in the striatum. Phenylethylamine is a trace amine which can amplify dopaminergic transmission. In addition, it has been proposed that blocking MAO-B metabolism of N-acetylated polyamine derivatives could modulate the activity of inhibitor glutamatergic efferents at the subthalamic level. The exact role of selegiline in amphetamine metabolism is not clearly understood.

The neuroprotector role of selegiline is of particular importance as it not only protects against the toxic action of MPTP, but also leads to less neuronal damage induced by other exogenous agents (6-hydroxydopamine, dihydroxytryptophan). Why should we not therefore imagine that this drug could have the same effect on potential environmental toxins analogous to MPTP? Several pesticides and insecticides which occasionally drain into well-water have been incriminated not in Alzheimer's disease, but in Parkinson's disease, although epidemiological studies have not been able to confirm this. It should also be noted that while the MPTP intoxication model reproduces all the clinical features of Parkinson's disease, it does not reproduce the neuropathology so that it would be erroneous to imagine that the disease is caused by simple exposure to exogenous toxins.

The more interesting hypothesis is that endogenous oxidative stress is particularly damaging to dopaminergic neurones and is related to hydrogen peroxide production during enzymatic degradation of dopamine in the MAO-B catalyzed reaction. Beyond this action on dopamine metabolism, selegiline could increase dismutase superoxide and catalase activity, and reduce the concentration of oxidized glutathione in the long term [113]. In addition, an increase in N-acetylated polyamines by MAO-B inhibition could decrease calcium-related cytotoxicity generated by glutamatergic receptors. All these suggestions, although hypothetical, reinforce the idea that MAO-B inhibition has a multi-faceted effect. Furthermore, selegiline not only prevents toxicity due to several exogenous and endogenous substances, but also protects against DSP-4 toxicity, apparently by a mechanism independent of its MAO-B inhibitory effect. Lastly, it has been suggested that selegiline has a neurotrophic effect by increasing survival of neurones which have already been submitted to a potentially lethal effect. It should be noted that

selegiline appears to significantly increase Trk C (neurotrophin T3 receptors) in the frontal and parietal cortex.

Pharmacokinetics

When administered orally, selegiline is completely and rapidly absorbed, maximal concentration being reached in 0.5 to 2 hours. Amphetamine metabolites are produced on its first passage through the liver. Selegiline is strongly linked to proteins (albumin/globulins) with more than 90% being in a bound form. The volume of distribution is 300 litres. Rapid tissue penetration is facilitated by its basic and lipophilic characteristics [114].

Elimination occurs mainly via renal filtration in an unmetabolized form, but also as amphetamine derivatives (desmethylselegiline, *l*-amphetamine, and *l*-metamphetamine). The elimination half-life is short (0.5 hours) and the half-life of its derivatives is relatively long: 2–5 hours for desmethylselegiline, 20 hours for *l*-methamphetamine and *l*-amphetamine. The role of the amphetamine derivatives is still subject of debate. It is known that racemic transformation does not occur and that the *l*-amphetamine derivatives are much less active than the *d*-amphetamine derivatives, but it is possible these derivatives participate in the clinical action of selegiline. Current work based on selective MAO-B inhibitors with no amphetamine metabolites (Ro 19–6327) should provide answers on this point.

Cerebral uptake of radiolabelled ^{11}C selegiline is rapid. Peak uptake occurs after 2 to 3 minutes and is followed by progressive elimination if the dextro form is administered or if the subject had received the compound beforehand. Conversely, in subjects having no prior exposure to the compound, the levo form produces a retention plateau. Action then occurs on cerebral MAO-B. A 10 mg dose of seligiline totally inactivated cerebral MAO-B and, since selegiline is an irreversible enzyme inhibitor, inhibition lasts for the enzyme turn-over period which varies with the brain structure implicated but which can be as long as 40 days. After a single dose of selegiline, detectable MAO-B activity reoccurs in platelets after 2 days at the earliest and 4 to 5 days at the latest [115].

Clinical trials in Alzheimer's disease

One must appreciate that before conducting clinical trials for any drug intended to have a symptomatic effect or a protective effect in Alzheimer's disease, methodological difficulties have to be resolved. Indeed an apparent 'stimulating' effect on disease symptomatology may be mistaken for a cytoprotector effect (the difference can only be determined by reference to the

placebo effect at the end of the study). Several clinical trials with acceptable methodologies have recently been reviewed by Wiseman and McTavish [116]. At 10 mg/day, patients treated for 2 to 6 months showed significant improvement in cognitive functions. In the study reported by Mangoni *et al.* [117], 112 patients given selegiline for 3 months showed significant improvement in Blessed's dementia scale, scores of activities of daily living and on items of orientation, memory and concentration. However, these encouraging results could not be reproduced by Burke *et al.* who reported upon two studies [118, 119]. These latter authors used less sensitive global scales (clinical dementia scale, MMS, BPRS, geriatric evaluation by relatives) in 39 patients over a 15 months study period. A later trial provided interesting results which demonstrated that combining daily doses of selegiline (10 mg) with tacrine (80 mg) improves cognitive function as assessed by the Alzheimer's disease assessment scale [120]. The findings reported in these trials suggest that selegiline at a dose of 10 mg/day can be beneficial for patients. Hopefully, current trials will confirm the clinical effectiveness of selegiline in the treatment of Alzheimer's disease.

Selegiline given to Alzheimer's disease patients does not induce any more undesirable effects than it does when administered to Parkinson's patients. No behavioural disorders other than a few cases of hallucinations, confusion or insomnia, are observed.

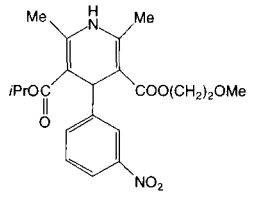
Perspectives of MAO-B inhibitors

Selegiline has certainly opened a new area of therapeutic research in Alzheimer's disease. The aim of using MAO-B inhibitors is to prevent progressive decline. The intense research activity in this area is demonstrated by the list of drugs which have been examined, although few have reached phase III trials in Alzheimer's disease. At present, it would be premature to differentiate between the different products (for example, mofegiline or lazabemide) either in terms of clinical effectiveness or acceptability. The role of monoamine oxidases on nervous system physiology is highly complex and must be related to other systems suspected of playing a pathogenic role in Alzheimer's disease, such as amyloid substance [121]. The following are some of the many MAO-B inhibitors which are being developed; milacemide, Ro 16–6491, and MD 220–661 (reversible for MAO-B); J 508/AGN 1133 and AGN 1135 (irreversible for MAO-B), eutonyl (partly selective MAO-B), almoxatone and MDL 72 638 (exhibit enantiomeric selectivity), MDL 72 145 (potentiates levodopa) and Ro 19–6327 (short acting).

NIMODIPINE

Medicinal chemistry

Nimodipine (Nimotop, 2) is a dihydropyridine: the isopropyl 2-methoxyethyl diester of 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-pyridine-3,5-dicarboxylic acid. This molecule is marketed in a 30 mg oral formulation (as Nimotop) and is indicated for use in vascular diseases, particularly cerebrovascular diseases due to its high lipid affinity and the fact that it easily crosses the blood-brain barrier.



(2)

Nimodipine is a calcium antagonist. In this pharmacological class, dihydropyridines are blockers of voltage-dependent L-type calcium channels. These ion channels facilitate Ca^{++} ion movement through cell membranes. The channels are found throughout the organism, occur in several types, can interact [122] and are involved in cell death [123], neurocytotoxicity [124] and in the consequences of cell phenomena due to hypoxia/ischaemia, particularly in the brain [125–127].

This pharmacological class is one of the most complex since its members act on a variety of newly discovered calcium channels within the central nervous system with either synaptic or somatic localization and numerous functions. Little is known about the impact of calcium antagonists on chemical neurotransmission, neuronal functions and intracellular calcium homeostasis, particularly in neurones and glial cells. This wide area of research and the action of these drugs on cerebral vascularization have been the subject of a number of recent reviews [95, 128–130]. For dihydropyridines, it is also important to note the peripheral (cardiovascular) effects which in turn affect the central nervous system.

Justification for use in Alzheimer's disease

Based on a mechanistic approach, the initial question is to determine whether the possibility of re-establishing calcium homeostasis, apparently perturbed in Alzheimer's disease, using direct access to the brain with dihydropyridine, has a beneficial effect. While neuronal calcium accumulation can be demonstrated in the physiological ageing process [131], the situation in Alzheimer's disease is far from clear [132]. According to Heizmann and Braun [133], Ca^{++}-binding proteins would be the site of characteristic anomalies in neurodegenerative diseases. However, there is little evidence to suggest that dihydropyridines such as nimodipine act at this level. One recent theory assumes the opposite effect that moderate increases in free Ca^{++} protects against apoptosis [134] and that voltage-gated Ca^{++} channel blockers induce apoptosis in cortical neurones [135]. Likewise, the effect of calcium channel blockers on synapse function as reported by Dunlap *et al.* [130] opens up a new unexplored area of research, such as the role of nickel in blocking E-channels [136].

Pharmacological properties

Nimodipine exerts its major pharmacological properties on blood vessels, particularly in the brain [137]. These spasmolytic and vasodilator effects which are predominantly responsible for the anti-hypoxic effect of dihydropyridines, have not been specifically studied in Alzheimer's disease. Likewise, the fact that calcium antagonists can only express pharmacological properties in pathological situations or specifically act either on the endoplasmic reticulum or on calciproteins, has not been clearly explored in dementia [138, 139]. The typical behavioural and cognitive pharmacological effects have, on the other hand, been clearly demonstrated in animal models. In the early 1980s [140], the specific effects on memory, recall and training were demonstrated in the rabbit and rat [141–145]. These pharmacological properties are those classically required in preclinical studies when selecting drugs for further development in Alzheimer's disease.

Pharmacokinetics

The pharmacokinetic constants of nimodipine have been carefully determined: strong protein affinity (98%), hepatic elimination (clearance 850 ml/min), half-life elimination (60 min), hepatic metabolism, and plasma concentration at equilibrium (27 ± 2 ng/ml). Compared with other dihydropyridines, it is important to recall its high lipid affinity and slow renal elimina-

tion in elderly subjects. These data explain why clinical trials with nimodipine given orally require 3 daily doses of 30 to 60 mg.

Clinical trials in Alzheimer's disease

Several preliminary trials have shown that nimodipine has cognition stimulating effects in man, particularly in the elderly subject with cognitive impairment of various origins (vascular and degenerative). For example, Parnetti *et al.* [146] reported on 352 patients given 30 mg three times a day of nimodipine for 3 months and 403 patients for 6 months. Likewise, Ban *et al.* [147] showed the greater effect of 90 mg nimodipine compared with placebo in 178 elderly subjects treated for 3 months, as also did Tollefson [148] on 227 Alzheimer patients given 30 mg nimodipine three times a day for 3 months versus placebo. These results are in agreement with animal data, and preliminary results obtained with pharmaco-EEG data [149]. These results are ample justification for conducting new multicentre clinical trials and appear promising since the objective criteria needed today were positively affected by the treatments (CGI, MMS, scales of cognitive degradation, scales of daily activity). The results of two large multicentre trials being carried out in Europe using standardized methodology are awaited shortly and will hopefully justify the use of nimodipine in Alzheimer's disease at oral doses of about 180 mg/day. These trials have already verified the safety of the product when given to a particularly fragile elderly population. The side-effect most frequently reported have been dizziness (28%), hypotension (19%) [150], pollakiuria, and headache with flush.

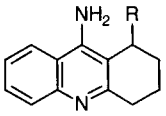
Perspectives for calcium antagonists

The large number of molecules in this class is not matched by an equivalent number of known trials in Alzheimer's disease. Undoubtedly, most pharmaceutical firms are awaiting the shortly-to-be-published results of the two clinical trials mentioned above. It is clear however that calcium antagonists have one common property, that of providing improvement in symptoms for certain cognitive functions in elderly subjects. The precise mechanism of action is still a matter of uncertainty although the hypothesis that these drugs result in the pharmacological correction of cerebral vascular anomalies (with marked regional specificity well-described in Alzheimer's disease) is often emphasized [151].

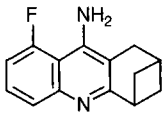
TACRINE

Medicinal chemistry

Tacrine (THA, Cognex, 3), velnacrine (4) and SM 10888 (5) are members of the 9-amino-1,2,3,4-tetrahydroacridine family [1]. They have one common pharmacological property, that of selective inhibition of acetylcholinesterase in the central nervous system. Compared with classical anticholinesterases used in the treatment of myasthenia gravis, tacrine easily crosses the blood-brain barrier.



(3) R = H
(4) R = OH



(5)

Tacrine causes reversible allosteric inhibition of acetylcholinesterase and butyrylcholinesterase *in vitro* by binding to a hydrophobic region near the anionic a or g sites on the surface of the enzymes [152]. A mixture of competitive and non-competitive inhibition has been recorded [153]. In therapeutic use, these enzyme inhibitors can be generally termed anti-myasthenic agents [154]. The following recall of the properties of these agents is useful to explain the potential peripheral effects of tacrine and the characteristic problems raised by this class of pharmacological agent.

Justification of use in Alzheimer's disease

The initial rationale for the use of tacrine in Alzheimer's disease is based on solid arguments favouring a depopulation of specific cholinergic neurones in Alzheimer's disease. This neurone loss, particularly in the *nucleus basalis* of Meynert, has been correlated with the degree of cognitive deficit in man, the basis of the cholinergic theory relating to Alzheimer's disease [155, 156]. This theory has been widely corroborated by the results of numerous pharmacoclinical studies which have shown the deleterious effects of anticholinergic substances such as scopolamine on memory and orientation in young and elderly healthy volunteers [157–159]. Episodes of memory impairment, artificially induced by anticholinergic agents, are the initial signs of Alzheimer's disease [160]. Furthermore, acetylcholinesterase hyperactiv-

ity and butyrylcholinesterase hyperactivity in senile plaques and neurontangles occur in cortical, limbic and subcortical structures in patients with Alzheimer's disease [161]. Finally, inhibition of acetylcholinesterase in the nervous system leads to an increase in synaptic acetylcholine which is assumed to induce an agonist effect on all central cholinergic receptors, not only muscarinic but also nicotinic receptors. These central nicotinic receptors are strongly suspected of playing a role in the genesis of the pathophysiological process of Alzheimer's disease and in the function of numerous cognitive activities such as memory, attention, vigilance and visual discrimination [162–164]. An ultimate argument in favour of the cholinergic theory of Alzheimer's disease results from a better understanding of factors contributing to cholinergic neuron survival, particularly factors such as NGF [165].

Pharmacological properties

The pro-cholinergic effect by inhibition of acetylcholinesterase has been proven, but is not the only action of these compounds (*Table 1.4*). In their review of the subject, Wagstaff and McTavish [1] discussed the therapeutic potential of the different properties of tacrine and recalled that it is impossible to attribute the clinically observed phenomena exclusively to one or other of this drug's properties. Certain features would suggest there is a possible cytoprotector action (anti-glutamate effect [166, 167], an MAO inhibitor effect and a cytoskeleton effect) associated with the symptomatic effect currently in the limelight resulting from the action initially described for neurotransmitters.

Table 1.4. PHARMACOLOGICAL EFFECTS OF TACRINE

Inhibits acetylcholinesterase and butyrylcholinesterase activity
Inhibits muscarinic and nicotinic receptor binding
May restore density of nicotinic receptors in patients with Alzheimer's disease to control values
Increases synthesis and release of acetylcholine under certain conditions
Inhibits cyclic AMP phosphodiesterase
Inhibits potassium-evoked release of excitatory amino acids
Inhibits monoamine oxidase activity
Affects noradrenaline (norepinephrine), dopamine and serotonin (5-hydroxytryptamine) uptake and release
Inhibits histamine-*N*-methyltransferase, increasing histamine levels
Blocks potassium, sodium and calcium ion channels
Stimulates insulin secretion
Increases glucose metabolism
Alters the physical properties of membranes
Inhibits glutamate/NMDA toxicity

In animal models of behavioural disorders, tacrine has been shown to be effective for reversing memory deficiency, improving learning and orientation performance at effective doses from 0.3 to 10 mg/kg. These dose-dependent effects sometimes show an inverted bell-shaped curve, characteristic of many agents with effects on cognition.

Pharmacokinetics

As for any drug causing enzyme inhibition, interpretation of concentration/effect or pharmacokinetic/pharmacodynamic relationships must be considered with caution. This is particularly true for tacrine since enzyme inhibition is not its only property. Several important features should be emphasized: (1) there are wide interindividual variations in the main pharmacokinetic parameters, (2) correlations have been established between bioavailability and improvement in clinical scores and even with pure memory tests, (3) there appears to be a correlation between plasma levels and increase in liver enzymes, (4) a typical gender effect is present, and finally (5) a possible explanation for the presence of non-responders is the fact that these patients exhibit low bioavailability. The pharmacokinetic properties of tacrine can be summarized as: bioavailability 17 to 24%, peak plasma concentration at 1.3 to 2.0 hours, distribution volume ranging from 3.7 to 6.8 l/kg, protein binding approximately 75%, predominant hepatic metabolism by cytochrome P450 IA2 and secondarily by cytochrome P450 IID6 resulting in five main metabolites including 1-hydroxytacrine (velnacrine) with a peak half-life of 0.5 to 1.5 hours after oral administration. The plasma elimination half-life is 1.4 to 2.8 hours after single oral dose and 2.9 to 3.6 hours after repeated oral doses. Plasma clearance occurs at a rate of 144 to 168 l/h. Urinary elimination predominates.

There are few data available on the kinetic effect except those obtained with pharmaco-EEG (increase in alpha, decreased theta and delta correlated with efficacy) or PET scan [168]. These data were reviewed by Freeman and Dawson [152] and by Schneider [169].

Clinical trials in Alzheimer's disease

Since the inaugural publication by Summers *et al.* in 1986 [170], there have been a large number of clinical trials carried out throughout the world which have given rise to much discussion [171–174]. It would be unreasonable to reconsider all this information here. Nevertheless, it is important to underscore the rather disorganized nature of this body of knowledge which actually contains little phase I and phase II data and has varied greatly in the

choice of the optimal dose, frequency of administration or treatment duration. Only the more recent trials, including the study reported by Knapp *et al.* [175] deserve more careful analysis. These authors enrolled 663 subjects and obtained evaluable data in 263 at the end of the 30 week trial. At 18 weeks treatment in 338 patients, tacrine given at a daily dose of 120 mg (n = 171) was effective compared with placebo (n = 136) using the CIBIC and ADAS cognitive scales. Likewise, patients who completed the study with 160 mg had a mean improvement in the ADAS-Cog of 4.1 points and 2.6 points on the MMS scale compared with placebo. Schematically, when the official authorities accepted tacrine for marketing authorization, it was considered to be effective when compared with placebo, with an active daily dose of 80–160 mg, and a 12 month period in which could be observed such pertinent improvements as attention, memory, word recognition and language [176].

Drug safety

In common with other anticholinesterase agents, tacrine induces a number of peripheral parasympathetic effects including nausea, vomiting, diarrhoea, abdominal cramps (9–40%), pollakiuria (6–25%), diaphoresis (3–17%) and ptyalism (3–10%), whilst agitation (5%), insomnia (6%) and confusion (3%) are rarely observed.

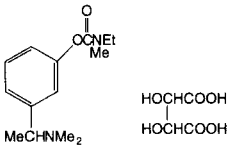
The rise in ASAT and ALAT induced by tacrine is a frequent feature (50% or less) and reflects hepatotoxicity which limits its use in Alzheimer's disease. These dose-dependent rises in liver enzymes generally regress in one month and are related to the effect of tacrine on hepatic cytochrome P450 [177]. Several cases of symptomatic acute hepatitis have been reported. The precise mechanism of liver toxicity is currently under study in order to explain, among other things, why the rechallenge doses of tacrine are higher than those given initially which cause a transaminase rise. Trials which attempt to minimize this hepatotoxicity through dose optimization have begun in France (co-therapy with sylimarin). Confirmation of tacrine's toxicity will come from an intensive follow-up of the first 5,000 patients treated with the drug currently being carried out by the French Pharmacovigilance Group (the PACO study).

PERSPECTIVES FOR THE CHOLINERGIC APPROACH

Anti-cholinesterases

There are several anti-cholinesterase agents (e.g., Donpezil, Metrifonate,

Eptastigmine, alanthamine, and MDL 73, 745) currently in phase III clinical trials based on the tacrine experience. It would appear reasonable to predict that a product with a minimal efficacy but without the undesirable peripheral effects and especially without the risk of hepatotoxicity would be an interesting alternative to tacrine [178].



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SDZ ENA 713 (ENA 713, (+)(*S*)-*N*-ethyl-3-[(1-dimethylamino)ethyl]-*N*-methylphenyl carbamate hydrogen tartrate, 6) is an acetylcholine esterase inhibitor of the carbamate type. This carbamate inhibitor mimics acetylcholine as a substrate for acetylcholine esterase but produces a carbamoylated enzyme, unlike tacrine which inhibits both competitively and non-competitively. The slow hydrolysis of carbamoylated acetylcholine contributes to the long duration of action of SDZ ENA 713. This compound is more potent in inhibiting acetylcholine esterase in rat cortex and hippocampus than in other brain regions such as the striatum, pons and medulla. SDZ ENA 713 is highly brain selective. When the brain enzyme is inhibited by 80%, the acetylcholine esterase activity in peripheral organs such as liver, lung and heart is only marginally affected (less than 20%). In the alert non-mobile-behavioural model in animals, SDZ ENA 713 is 16 times more potent than tacrine given orally. Based on hippocampal EEG data, the drug has been shown to have a long duration of action (>6 hours). The drug has been administered to over 700 patients with Alzheimer's disease in a daily dose range of 1–12 mg being well tolerated by the majority of patients. Significant liver enzyme elevations (three times the upper limit of normal) are extremely rare (1.8%) compared with tacrine.

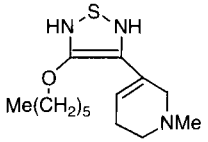
Furthermore, there have been no drug withdrawals due to enzyme elevations in patients treated with SDZ ENA 713 while approximately 25% of patients treated with tacrine discontinue due to liver enzyme elevation. There appear to be no associated cardiac, renal or central nervous system adverse effects. The most commonly reported adverse effects are nausea, vomiting and diarrhoea of mild intensity.

Efficacy of SDZ ENA 713 has been established in an analysis of patients

considered valid in two independent multicentre studies. In the first multicentre study conducted in Europe, in 402 patients with Alzheimer's disease, fixed doses of 3 mg twice daily were shown to be significantly superior to placebo for the clinical global impression test and a variety of other psychometric measures after 12 weeks of treatment. A subsequent multicentre trial was conducted in 114 patients with Alzheimer's disease in Europe and Canada. Significantly more patients given fixed daily doses of 6, 9 or 12 mg twice daily for 8 weeks were judged to be 'responders' based on ratings of the CIBIC-plus compared with patients treated with placebo.

M_1 muscarinic agonists

It would appear logical to select a drug for Alzheimer's disease which acts directly on post-synaptic muscarinic receptors (M_1 type receptors) rather than using enzyme inhibitors. Indeed, many such products are currently in phase III in the United States. Xanomeline (7) followed modern schemes of product development as explained above. Two French products, miramelin and SR 46559A (8) are in phase II. The affinity of SR 46559A for M_1 receptors is 6 times greater than for M_2 receptors and apparently does not carry a risk of a 'cholinergic syndrome'. In preclinical pharmacology, several M_1 agonists are under study. All have been effective in animal models of cognition impairment. One such compound is AF102B, a quinuclidine derivative [179]. SB 202026 is also a powerful partial agonist of M_1 receptors [40 times more powerful than RS86 (9)] and can be used in humans at daily doses between 100 and 150 μg.



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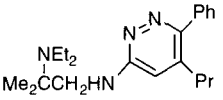

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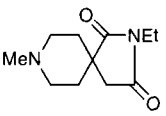

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Nicotinic agents

Despite a very negative connotation, nicotine is recognized as having positive effects on memory, attention, anxiety and even depression [180]. In clinical pharmacology, improvement in several cognitive performances has been demonstrated [181, 182]. The lack of a tolerance phenomenon has also been proven. These results have been reproduced in patients with Alzhei-

mer's disease with improved word recall, attention and information processing [183, 184].

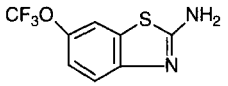
Nicotine stimulates central nicotine receptors and also affects other neurotransmission systems, increasing tyrosine hydroxylase activity and elevating noradrenaline (norepinephrine) release from the hippocampus [185]. These effects have surprising kinetics since induced modifications persist long after treatment withdrawal. The administration of nicotine to patients raises certain problems, but this line of research has resulted in the development of compounds without the undesirable effects of nicotine itself and are referred to as cholinergic channel activators (for review see [186]).

Nicotine agonists form a very active new field of pharmacological research in the central nervous system, especially since the neuroprotector properties have been clearly demonstrated [187]. These facts should be examined in the light of epidemiological data which suggest a dose-related protector effect of smoking in Alzheimer's disease [188]. Recent discussion has focused on the risk of Alzheimer's disease, smoking and certain genotypes. Apparently the allele at Apo *E* is not associated with risk of Alzheimer's disease [189].

GLUTAMATERGIC APPROACH

Medicinal chemistry

A large heterogeneous series of chemical compounds has been recognized as having an effect on excitatory amino acid transmission. For the time being, none of these compounds has reached phase III clinical trials in Alzheimer's disease. We shall approach this question by speculating on the possibilities offered by three of them: riluzol (2-amino-6-trifluoromethoxybenzothiazole, 10) (a phase III study in amyiotrophic lateral sclerosis), sabeluzol (11) and memantine (3,5-dimethyl-1-adamantamine) [190].



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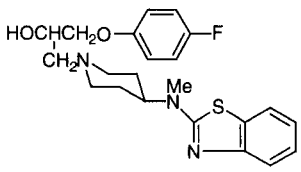


(11)

These drugs limit or block glutamatergic neurotransmission by various mechanisms (decreased release, receptor blockade, effect on synthesis). They do not belong to a single pharmacological class, and the action on glutamate is rarely unique (ion channels almost always being affected). This explains their wide spectrum of pharmacological effects and thus the variety of their potential uses.

Justification of use in Alzheimer's disease

Theories on the processes leading to neurodegenerative diseases often implicate glutamate, but there are many questions to be answered. One line of thought in amyotrophic lateral sclerosis [191] has contributed to the preferential development of riluzol with very encouraging phase III results. Inversely, for Alzheimer's disease, the glutamatergic approach is still highly speculative [192] or at least can only be based on very fragile arguments: loss of NMDA receptors in hippocampus of patients [193], development of NFT in glutamatergic neurones [194], stimulation of glutamate neurotoxicity by amyloid substance [195], raised glutamate levels in cerebrospinal fluid in patients [196]. Excessive glutamatergic transmission as an acute phenomenon appears to be accepted but can hardly explain a chronic process such as Alzheimer's disease [197]. Furthermore, glutamate is required to consolidate the trace of memory, and since memory impairment is one of the first manifestations of Alzheimer's disease, blocking the system would appear to be antagonistic [198]. This observation has led certain authors to propose that drugs capable of modulating glutamate release rather than focusing directly on receptors [199, 200] would be most useful in treating Alzheimer's disease.

Pharmacological properties

Riluzol antagonizes glutamatergic transmission via different mechanisms: indirect blockade of NMDA receptors (modification of receptor phosphorylation status), inhibition of excitatory amino acid release by an agonist effect on an unknown receptor bound to protein G or interaction with voltage-dependent sodium channels (depressor effect on neurones with a high tonic activity spontaneously). The pharmacological properties of the compound can be explained by these mechanisms: anti-seizure, anti-ischaemia, antagonism of MPTP cytotoxicity [201].

Sabeluzol is a substance which stimulates learning processes and recall, has an anti-hypoxic and anti-seizure effect. The two enantiomers contribute to the pharmacological activity. This compound increases long-term potentiation at 2.5 mg/kg in the rat and counteracts chronic glutamate toxicity in

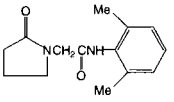
neurone cultures (10^{-8}M). Binding studies indicate interactions with several classical neurotransmission systems as well as with sigma receptors and calcium-dihydropyridine sites. Three double-blind trials have been conducted in Alzheimer's disease at doses between 10 and 20 mg twice daily [202].

Memantine has been known since 1963 and was used in several human diseases such as Parkinson's disease and stroke. This compound affects several transmitters [203] and has an anti-hypoxia and anti-ischaemia effect. It is a non-competitive antagonist of NMDA receptors and blocks Ca^{++} entry through serotonin operated channels and binds to the phencyclidine recognition site in the NMDA-operated Ca^{++} channel [204, 205].

Perspectives for the glutamatergic approach

It is difficult to judge the desirability of the glutamatergic approach since no pure glutamatergic compound has been found to be effective in Alzheimer's disease. The data currently available suggest that these compounds would be more effective in stroke. Finally, the toxicity of certain ligands has been studied *in vitro* and has dampened the enthusiasm of many clinicians.

Conversely, a new line of inquiry examined glutamatergic activity, not as a blocker of neurotransmission but as an agonist of AMPA type receptors. A new class of compounds, called ampakines, act as long-term boosters of the learning process. Aniracetam [206] is considered as the major product in this series. It improves performance in all classical behavioural tests in animal models. The question which has to be answered is whether these compounds simply have symptomatic effects on memory processes or whether they have complementary properties such as inhibition of cell death [207]. Furthermore, certain compounds currently classed as ampakines such as oxiracetam and piracetam have not been shown to be effective in Alzheimer's disease. Others, such as nefiracetam (12) are in the animal experimentation stage and appear to be promising because of their impact on the neuronal cell adhesion molecules (NCAM) [208] and their effect on synapse plasticity.



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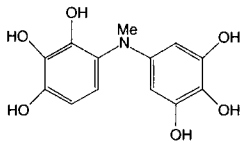
FREE RADICAL SCAVENGERS

There is an abundant body of literature on the role played by free radicals in cell-death and their interaction with other deleterious compounds such as glutamate, iron and calcium [209–211]. However, in Alzheimer's disease, the arguments favouring the free radical theory are not particularly solid and are all based on indirect actions [212]. Lack of evidence contrasts with the large number of chemical compounds counteracting over-production of free radicals: the series of aminosteroids (lazaroids including U-74006F), alpha-tocopherol (vitamin E) and a whole series of compounds which, in Europe, are classed as cognitive enhancers. In our experience, only exifone (13), a powerful scavenger, has been shown to be effective in a study of 38 patients tested for 6 months. Effectiveness was judged on the basis of MMS scores versus placebo (unpublished results). This drug, which has since been withdrawn (from the French pharmacopoeia 6 months after authorization) because to liver toxicity, was found to be superior to placebo for cognitive impairment in Parkinson's disease [213]. The contrast in results, inspired by this same free radical theory, has led certain groups to assess the effect of tocopherol or desferoxamine (chelator of aluminium and iron) in Alzheimer's disease [214]. These theories, more in fashion in Parkinson's disease [215] have not been abandoned by those convinced that free radical metabolites have a potential neurotoxic role in Alzheimer's disease. More recently, the melatoninergic theory, based on the capacity to oppose overproduction of free radicals, has been debated and agonists of melatonin synthesis are currently under study [216].



(13)

COGNITION ENHANCERS

The pharmacology of substances capable of non-specific stimulation of cerebral function and of improving cognitive performances have dominated neuropsychopharmacology since the beginning of the decade [217]. There is an impressive list of compounds known to be active in animals via various

mechanisms [218]. Certain drugs such as hydergine, piracetam, pentoxifyllin, ganglioside and vincamine have dominated the market for the 'aged brain' [219]. In fact, the use of all these drugs raises two questions: firstly, there is a gap between the biological and neurochemical effectiveness and the almost imperceptible effects observed in man. Secondly, the indications for which these cognition enhancers should be used remains unclear [220]. For the moment, correctly conducted trials in Alzheimer's disease have been unable to demonstrate any objective proof of efficacy for piracetam (and is derivatives), for ginkgo biloba, or for hydergine [221]. These failures need to be discussed and analyzed, especially since the principal mechanism of action in dementia (that is, to simulate neurone metabolism) is in agreement with the theoretical data most recently obtained with neuroimaging techniques. *In vivo* NMR spectroscopy and PET scan have confirmed that focalized metabolic perturbations occur in the brain of patients with Alzheimer's disease. These anomalies are precisely those which would be corrected by cognition enhancers [222].

To further complicate the picture, it is important to recall that several of these compounds were found to be active in phase II clinical trials either in healthy young or elderly volunteers or in dementia patients. In our experience, these beneficial results in the early stages of development can only be interpreted as proof of cognitive action, but not as sufficient evidence for proposing their use in Alzheimer's disease.

OTHER STRATEGIES

Certain therapeutic strategies currently under assessment should be mentioned because they offer innovative options (Table 1.5). S12024 is now in phase III in an international multicentre trial. This compound which affects adrenergic and vasopressinergic neurotransmission (a pathophysiological mechanism rarely put forward in Alzheimer's disease) has been shown to have a pro-memory effect in animal tests and to be non-toxic. It was thus satisfactorily studied in early clinical trials [223, 224]. A recent study including 404 patients (phase IIb, treatment for 3 months) has just demonstrated that at a dose of 100 mg, S12024 is superior to placebo for 3 officially recognized criteria. The development of such a compound is highly instructive since it illustrates that pharmacological innovation outside established theories particularly fashionable at a given moment can be useful.

Non-steroid anti-inflammatory drugs (NSAIDs) are also being studied in Alzheimer's disease. This approach is novel since it was based initially on epidemiological data which showed an inverse association between NSAID consumption and the occurrence of Alzheimer's disease [225, 226]. It is evi-

Table 1.5. MAIN THERAPEUTIC AVENUES IN ALZHEIMER'S DISEASE

Drug	*Main pharmacological effect*	*Dosage per day in humans*	*Positive effects in phase III studies*	*Longest duration of blinded follow-up*	*Problems*
SELEGILINE	inhibition of MAO-B	10 mg	Yes	24 weeks	Symptomatic effect or cyto-protection Right dosage?
NIMODIPINE	L-Type calcium channel blocker	90–180 mg	Yes	24 weeks	Vascular or neuronal action?
TACRINE	Inhibition of central acetylcholinesterase	80–160 mg	Yes	30 weeks	Peripheral cholinergic side effects Hepatotoxicity
S 12024	Increase of neurotrans-mission (vasopressine; alpha adrenergy)	100–200 mg	No (phase II: yes)	12 weeks	Dosage choice
MIRAMELINE SR 46559 A XANOMELINE AF102B SB202026	M_1 agonists, increase of muscarinic activity		No		Extra CNS toxicity
RILUZOLE	Glutamatergic transmission		No		Developed in ALS
SALBELUZOLE			No		Many other properties
MEMANTINE	antagonists		No		

dent that NSAIDs slow down the Alzheimer's disease process. MMS, the Boston naming test and delayed condition of the Benton visual retention test have shown in 28 patients that objective assessment measures are affected by NSAIDs [227].

The beneficial effect of oestrogenotherapy in Alzheimer's disease is based on several factors. Oestrogen receptors in the brain have a distribution which is similar to the histological lesions of the disease (basal telencephalon, cerebral cortex and hippocampus). Oestrogens reinforce the synthesis of NGF receptors [228]. 17-Beta oestradiol favours non-amyloidogenic processing of APP [229]. Certain retrospective studies [230] have shown that menopausal women who have taken substitution oestrogen therapy have a lower risk for Alzheimer's disease and that among the patients with the disease, the degree of cognitive impairment is less in those who had taken hormone substitution therapy. To our knowledge, there has been no prospective study aimed at confirming the potential of oestrogen therapy on the course of Alzheimer's disease once the disease has begun.

CONCLUSION

To those interested in gene therapy, the approach to discovering and developing drugs for use in Alzheimer's disease which is presented here may appear old-fashioned compared with recent advances in cell biology, cell trophicity and cell-death [231]. We nevertheless have focused our discussion on the considerable delays (with the corollary of cost for society) which result when developing a drug for this condition. In one sense, Alzheimer's disease is a concrete example of the pharmaco-economic debate [232]. The ideal drug for Alzheimer's disease should limit the social and economic devastation caused by this disease. However, this aspect of the problem has been timidly expressed by the health authorities who have recommended quality of life measurement and drug benefit scales for daily life activities to be carried out in phase III clinical trials. This debate was recently emphasized in an article in the French literature where the author noted the continuous deceleration in current research despite the exponentially growing number of researchers and funds devoted to research [233]. The acceleration in basic, fundamental and consequently 'pre-competitive' research without a specific therapeutic application, appears to be typical in Alzheimer's disease. Piecemeal knowledge of this disease and of neurodegenerative diseases in general is certainly a major obstacle to much needed pharmacological progress. What is clear today is that the lesions causing dementia are not diffuse lesions but involve, at least at onset, a limited number of susceptible sites

[25]. Thus, Alzheimer's disease is only one of the neurodegenerative diseases affecting the most noble functions of the individual. The analysis of these diseases (and thus their assessment) must call upon analysis-dependent plans interacting with logical technical, social and ethical considerations [234]. Finally the pathophysiological mechanisms, whether dependent or independent of an individual's genetic make-up, have been identified (neurofibrillary degeneration, metabolism of the β-amyloid protein, abnormal phosphorylations) and should thus become designated targets for drugs. Modern pharmacology has yet to resolve the difficult problems raised by a multifocal approach, in other words, to re-establish harmonious function between the brain structures (frontal lobe/associative cortex) typically damaged in Alzheimer's disease. The present response, which will most certainly be judged to have been poorly adapted, is an attempt to affect the mechanisms assumed to be initiating,or which are common to degenerative diseases (free radicals, calcium, excitotoxicity, trophic factors). The impact on the major neurotransmission systems (if it is assumed that the receptors function normally) appears to be a logical approach. The rest of the story will be of a more general nature and concerns the means of correctly and rapidly assessing the effects of new potentially useful drugs. This is the justification for the emphasis in this review on early phase clinical trials in Alzheimer's disease and the reference to pilot studies using small samples but rigorous objective assessment measures. It is impossible to finish without mentioning the role played by the regulatory bodies (FDA, European Medicines Evaluation Agency) in drug innovation in dementia. Many industrial firms in Europe have initiated major phase II trials using the efficacy criteria dictated by the authorities. This means that in 10 years from now these drugs will appear on the market with perhaps a rather outmoded mode of action. The idea would thus be to incite more innovative methodologies from these bodies (for example, more formal requirements for phases I and II, earlier medico-economic analysis, revisions of assessment criteria for phase III, directives in terms of biotechnological products or corresponding to gene therapy). A coordinated approach by the pharmaceutical industry and public research organisations is, in our opinion, the only guarantee for future progress in the treatment of Alzheimer's disease.

REFERENCES

1 Wagstaff, A.J. and McTavish, D. (1994) Drugs Aging 4, 510–540.
2 Hughes, C.P., Berg, L., Danziger, W.L., Coben, L.A. and Martin, R.L. (1982) Br. J. Psychiatry 140, 566–572.

3 Reisberg, B., Ferris, S.H., de Leon, M.J. and Crook, J. (1982) Am. J. Psychiatry 139, 1136–1139.
4 McKhann, G., Drachman, D., Folstein, M., Katzman, R., Price, D. and Stadlan, E.M. (1984) Neurology 34, 939–944.
5 Habib, M., Poncet, M., Dans Habib, M., Joanette, Y. and Puel, M., eds. (1991) Démences et Syndromes Démentiels, Masson, Paris.
6 Blacker, D., Albert, M.S., Bassett, S.S., Go, R.C.P., Harrell, L.E. and Folstein, M.F. (1994) Arch. Neurol. 51, 1198–1204.
7 Neary, D., Snowden, J.S., Bowen, D.M., Sims, N.R., Mann, D.M.A., Benton, J.S., Northern, B., Yates, P.O. and Davison, A.N. (1986) J. Neurol. Neurosurg. Psychiatry 49, 163–174.
8 Crook T., Bartus, R.T., Ferris S.H., Whitehouse, P. and Gershon, S. (1986) Development. Neuropsychol. 2, 261–276.
9 Derouesné, C., Kalafat, M., Guez, D., Malbezin, M. and Poitrenaud, J. (1994) Int. J. Geriatr. Psychiatry 9, 577–587.
10 Wells, C.E. (1979) Am. J. Psychiatry 136, 895–900.
11 Brun, A. (1987) Arch. Gerontol. Geriatr. 6, 193–208.
12 Gustafson, L. (1987) Arch. Gerontol. Geriatr. 6, 209–223.
13 Neary, D., Snowden, J.S., Northen, B. and Goulding, P. (1988) J. Neurol. Neurosurg. Psychiatry, 51, 353–361.
14 Neary, D., Snowden, J.S. and Mann, D.M.A. (1993) Dementia, 4, 154–159.
15 Mitsuyama, Y. (1994) J. Neurol. Neurosurg. Psychiatry 47, 953–959.
16 Knopman. D.S., Mastri, A.R., Frey, W.H., Sung, J.H. and Rustan, T. (1990) Neurology 40, 251–256.
17 Mesulam, M.M. (1982) Ann. Neurol. 11, 592–598.
18 Mesulam, M.M. and Weintraub, S. (1992) in Heterogeneity in Alzheimer's Disease (Boller, F., Forette, F., Khachaturian, Z., Poncet, M. and Christen, Y., eds.) pp. 43–66, Springer Verlag, Berlin.
19 De Renzi, E. (1986) Cortex 22, 171–180.
20 Benson, D.F., Davis, R.J. and Snyder, B.D. (1988) Arch. Neurol. 45, 789–793.
21 Tyrrell, P.J., Warrington, E.K., Frackowiak, R.S.J. and Rossor, M.N. (1990) J. Neurol. Neurosurg. Psychiatry, 53, 1046–1050.
22 Snowden, J.S., Goulding, J. and Neary D. (1989) Behav. Neurol. 2, 167–182.
23 Tulving, E. (1972) in The Organisation of Memory (Tulving, E. and Donaldson, W., eds.), Academic Press, New York.
24 Belliard, S. (1994) La Demence Sémantique, Thèse de Médecine, Université de Rennes.
25 Sabouraud, O. (1995) Le Langage Et Ses Maux, Odile Jacob Ed. Paris (France).
26 Price, D.L. and Sisodia, S.S. (1994) Annu. Rev. Med. 45, 435–446.
27 Price, D.L., Martin L.J., Sisodia, S.S., Wagster, M.V., Koo, E.H., Walker, L.C., Koliatsos, V.E. and Cork, C.C. (1991) Brain Pathol. 1, 287–296.
28 Gower, A.J. and Lamberty, Y. (1993) Behav. Brain Res. 57, 163–173.
29 Miyamoto, M., Kiyota, Y., Yamazaki, N., Nagaoka, A., Matsuo, T., Nagawa, Y. and Takeda, T. (1986) Physiol. Behav. 38, 399–406.
30 Higgins, G.A., Oyler, G.A., Neve, R.L., Chen, K.S. and Gage, F.H. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3032–3036.
31 Cummings, B.J., Su, J.H., Cotman, C.W., White, R. and Russell, M.J. (1993) Neurobiol. Aging 14, 947–960.
32 Hanin, I. (1992) Ann. N.Y. Acad. Sci. 648, 63–70.
33 Wenk, G.L. (1990) Acta Neurobiol. Exp. 50, 219–223.
34 Wenk, G.L. (1993) Behav. Brain Res. 57, 117–122.

35 Holtzman, D.M., Li, Y., Dearmond, S.J., McKinley, M.P., Gage, F.H., Epstein, C.J. and Mobley, W.C. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 1383–1387.
36 Myhrer, T. (1993) Neurosci. Biobehav. Rev. 17, 195–202.
37 Eichhorn, G.L. (1993) Exp. Gerontol. 28, 493–498.
38 Hofstetter, J.R., Vincent, I., Bugiano, O., Ghetti, B. and Richter J.A. (1987). Neurochem. Pathol. 6, 177–193.
39 Pepeu, G., Pepeu, I.M. and Casamenti, F. (1990) Drug Design Delivery 7, 1–10.
40 Aguzzi, A., Brandner, S., Sure, U., Rüedi, D. and Isenmann, S. (1994) Brain Pathol. 4, 3–20.
41 Fukuchi, K.I., Ogburn, C.E., Smith, A.C., Kemkel, D.D., Furlong, C.E., Deeb, S.S., Nochlin, D., Sumi, S.M. and Martin, G.M. (1993) Ann. N.Y. Acad. Sci. 695, 217–223.
42 Quon, D., Wang, Y., Catalano, R., Scardina, J.M., Murakami, K. and Cordell, B. (1991) Nature (London) 352, 239–241.
43 Kammesheidt, A., Boyce, F.M., Spanyoannis, A.F., Cummings, B.J., Ortegon, M., Cotman, C., Vaught, J.L. and Neve, R.L. (1992) Proc. Natl. Acad. Sci. 89, 10857–10861.
44 Sandhu, F.A., Salim, M. and Zain, S.B. (1991) J. Biol. Chem. 266, 21331–21334.
45 Yamaguchi, F., Richards, S.J., Beyraither, K., Salbaum, M., Carlson, G.A. and Dunnet, S.B.(1991) Neuro Report 2, 781–784.
46 Games, D., Adams, D., Alessandrini, R. and 31 other authors (1995) Nature (London) 373, 523–527.
47 Marx, J. (1992) Science (Washington, D.C.) 255, 1200–1202.
48 Lannfelt, L., Folkesson, R., Mohammed, A.H., Winblad, B., Hellgren, D., Fuff, K. and Hardy, J. (1993) Behav. Brain Res. 57, 207–213.
49 Poirier, J. (1994) Trends Pharmacol. Sci. 17, 525–530.
50 Bennett, M.C., Diamond, D.M., Stryker, S.L., Parks, J.K. and Parker, W.D. (1992) J. Geriatr. Psychiatry Neurol. 5, 93–101.
51 Price, D.L., Borchelt, D.R., Walker, L.C. and Sisodia, S.S. (1992) Neurobiol. Aging 13, 623–625.
52 Nicotera, P. and Orrenius, S. (1992) Ann. N.Y. Acad. Sci. 648, 17–27.
53 Young, G.W. (1992) J. Neurotrauma 53, S9-S25.
54 Carafoli, E. (1987) Annu. Rev. Biochem. 56, 395–433.
55 Miller, R.J. (1991) Prog. Neurobiol. 37, 255–285.
56 Bertolino, M. and Llinas, R. (1992) Annu. Rev. Pharmacol. Toxicol. 32, 399–421.
57 Llinas, R., Sugimori, M., Hillman, D.E. and Cherksey, B. (1992) Trends Neurosci. 15, 351–356.
58 Ehrlich, B.E., Kaftan, E., Bezprozvannaya, S. and Bezprozvanny, I. (1994) Trends Pharmacol. Sci. 15, 145–149.
59 Landfield, P.W., Thibault, O., Mazzanti, M.L., Porter, N.M. and Kerr, D.S. (1992) J. Neurobiol. 23, 1247–1260.
60 Heizmann, C.W. and Braun, K. (1992) Trends Neurosci. 15, 259–264.
61 Selkoe, D.J. (1994) Annu. Rev. Neurosci. 17, 489–517.
62 Lucas, D.R. and Newhouse, J.P. (1957) Arch. Ophthalmol. 58, 193–201.
63 Olney J.W. and Ho, O.L. (1970) Nature (London) 227, 609–611.
64 Choi, D.W. (1988) Trends Neurosci. 11, 465–469.
65 Meldrum, B. and Garthwaite, J. (1990) Trends Pharmacol. Sci. 11, 379–387.
66 Lipton, S.A. and Rosenberg, P.A. (1994) N. Engl. J. Med. 330, 613–622.
67 Meldru, B. (1993) Brain Res. Rev. 18, 293–314.
68 Allain, H. and Bentué-Ferrer, D. (1994) La Lettre du Pharmacologue 8, 196–198.
69 Cotman, C.W., Geddes, J.W., Ulas, J. and Klein, M. (1990) in Progress in Brain Research :

Plasticity of Excitatory Amino Acid Receptors: Implications for Aging and Alzheimer's Disease (Coleman, P., Higgins, G. and Phelps, C., eds.) 86, pp 55–61, Elsevier, Amsterdam.
70 Young, A.B. (1993) in Molecular and Cellular Approaches to the Treatment of Neurological Disease: Role of Excitotoxins in Heredo-degenerative Neurologic Diseases (Waseman S.G., ed.) pp 175–189, Raven Press, New York.
71 Fraser, P.E., Lévesque, L. and McLachlan, D.R. (1993) Clin. Biochem. 26, 339–349.
72 Ashall, F. and Goate, A.M. (1994) Trends Biol. Sci. 19, 42–46.
73 Yankner, B.A., Dawes, L.T.R., Fisher, S., Villa-Komanoff, L., Uster-Granite, M. and Neve, R.L. (1989) Science (Washington, D.C.) 245, 1417–1420.
74 Kowall, N.W., Beal, M.F., Busciglio, L.J., Duffy, L.K. and Yankner, B.A. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 7247–7251.
75 Podlisny, M.B., Stephenson, D.T., Frosch, M.P., Tolan, D.R., Lieberbury, I., Clemens, J.A. and Selkoe, D.J. (1993) Am. J. Pathol. 142, 17–24.
76 Whitson, J.S., Selkoe, D.J. and Cotman, C.W. (1989) Science (Washington, D.C.) 243, 1488–1490.
77 Whitson, J.S., Glabe, C.G., Shintani, E., Abcar, A. and Cotman, C.W. (1990) Neurosci. Lett. 110, 319–324.
78 Forloni, G. (1993) Funct. Neurol. 8, 211–225.
79 Mattson, M.P., Rydel, R.E., Lieberburg, I. and Smith-Swintosky, V.L. (1993) Ann. N.Y. Acad. Sci. 679, 1–21.
80 Barger, S.W., Smith-Swintosky V.L., Rydel, R.E. and Mattson M.P., (1993) Ann. N.Y. Acad. Sci. 695, 158–164.
81 Mattson, M.P., Barger, S.W., Cheng, B., Liederburg, I., Smith-Swintosky, V.L. and Rydel, R.E. (1993) Trends Pharmacol. Sci. 16, 409–414.
82 Pollard, H.B., Rojas, E. and Arispe, N. (1993) Ann. N.Y. Acad. Sci. 695, 165–168.
83 Kisilevsky, R., Lemieux, L.J., Fraser, P.E., Kong, X., Hultin, P.G. and Szarek, W.A. (1995) Nature Medicine, 1, 143–148.
84 Cordell, B. (1994) Annu. Rev. Pharmacol. Toxicol. 34, 69–89.
85 Brumback, R.A. and Leech, R.W. (1994) J. Okla. State Med. Assoc. 87, 103–111.
86 Maness, L.M., Kastin, A.J., Weber, J.T., Banks, W.A., Beckman, B.S. and Zadina, J.E. (1994) Neurosci. Biobehav. Rev. 18, 143–159.
87 Deckwerth, T.L. and Johnson, E.M. (1993) Ann. N.Y. Acad. Sci. 679, 121–131.
88 Shigeno, T., Mima, T., Takakura, K., Graham, D.I., Kato, G., Hashimoto, Y. and Furukawa, S. (1991) J. Neurosci. 11, 2914–2919.
89 Cheng, B. and Mattson, M.P. (1991) Neuron 7, 1031–1041.
90 Roush, W. (1995) Science (Washington, D.C.) 267, 793–794.
91 Strittmatter, W.J., Weisgraber, K.H., Goedert, M., and 13 other authors (1994) Exp. Neurol. 125, 163–171.
92 Allain, H., Belliard, S., De Certaines, J., Bentué-Ferrer, D., Bureau, M. and Lacroix, P. (1993) Dementia 4, 347–352.
93 Ohm, T.G. (1993) Hippocampus 3, 131–138.
94 Allain, H., Neuman, E., Wesnes, K., Gandon, J.M., Malbezin, M. and Guez, D. (1994) in Actualités sur la Maladie d'Alzheimer et les Syndromes Apparentés. (Poncet M., Michel B. and Nieoullon A., eds.) pp. 373–377, Editions Solal, Marseille.
95 Allain, H., Bentué-Ferrer, D., Saïag, B., Belliard, S. and Thaminy, S. (1993) Thérapie 48, 697–705.
96 Bentué-Ferrer, D., Philouze, V., Pape, D., Reymann, J.M., Allain, H. and Vandendriesche, J. (1989) Fundam. Clin. Pharmacol. 3, 323–328.
97 Belliard, S. and Allain, H. (1993) La Lettre du Pharmacol. 7, 18–20.
98 Allain, H. and Bellissant, E. (1993) La Lettre du Pharmacol. 7, 82–85

99 Drachman, D.A. and Sweater, J.M. (1993) in Guidelines for Drug Trials in Memory Disorders (Canal, N. Hachinski, V.C., McKhann, G. and Franceschi, M., eds.) Chap. 9, 71–86, Raven Press, New York.
100 Lader, M. and Curran, V. (1993) in Ref. 99, Chapter 10.
101 Fewster, P.H., Griffin-Brooks, S., MacGregor, J.A., Djalvo-Rose, E. and Baill, M.J. (1991) Dementia 2, 121–132.
102 Allain, H., Raoul, P., Lieury, A., Lecoz, F., Gandon, J.M. and Darbigny, P. (1993) Clin. Ther. 15, 549–557.
103 Allain, H., Lieury, A., Brunet-Bourgin, F., Mirabaud, C., Trebon, P., Lecoz, F. and Gandon, J.M. (1992) Psychopharmacology 106, S56-S61.
104 Naatanen, R., Ilmoniemi, R. and Alho, K. (1994) Trends Neurosci. 17, 389–395.
105 Allain, H., Le Coz, F. and Bureau, M. (1993) Thérapie 48, 239–242.
106 Lieury, A., Trébon, P., Boujon, C.H., Bernoussi, M. and Allain, H. (1991) Année Psychol. 91, 169–186.
107 Watson, F.L., Pasteur, M.A.L., Healy, D.T. and Hugues, E.A. (1994) Hum. Psychopharmacol. 9, 51–61.
108 Allain, H., Bernard, P.M., Dartigues, J.F., Dérouesné, Ch., Dubois, B. and 12 other authors (1994) L'Encéphale XX, 725–739.
108a Mohr, E., Feldman, H. and Gauthier, S. (1995) Can. J. Neurol. Sci. 22, 62–71.
109 Milgram, N.W., Racine, R.J., Nellis, P., Mendonea, A. and Ivy, G.O. (1990) Life Sci. 47, 415–420.
110 Delumeau, J.C., Bentué-Ferrer, D., Gandon, J.M., Amrein, R., Belliard, S. and Allain, H. (1994) J. Neural. Transm. Suppl. 41, 259–266.
111 Allain, H., Pollak, P. and Neukirch, H.C. (1993) Mov. Disord. 8, Suppl. 1, 36–40.
112 The Parkinson Study Group (1993) N. Engl. J. Med. 328, 176–183.
113 Knoll, J. (1989) Acta Neurol. Scand. 126, 83–91.
114 Heinonen, E.H., Karnani, A.M. and Lammintausta, R. (1994) Mov. Disord. 9, Suppl. 1 (Abstract no. P248).
115 Heinonen, E.H., Myllyla, V., Sotaniemi, K., Lammintausta, R., Salonen, J.S., Anttila, M., Savijarvi, M., Kotila, M. and Rinne, U.K. (1989) Acta Neurol. Scand. 126, 93–99.
116 Wiseman, L.R. and McTavish, D. (1995) CNS Drugs 31, 1–15.
117 Mangoni A., Grass, M.P., Frattola, L., Picolti, R., Bassi, S., Motta, A., Marcone, A. and Suirne, S. (1991) Eur. Neurol. 31, 100–107.
118 Burke, W.J., Ranno, A.E., Roccaforte, W.H., Wengel, S.P., Bayer, B.L. and Willcockson, N.K. (1993) J. Am. Geriatr. Soc. 41, 367–370.
119 Burke, W.J., Roccaforte, W.H., Wengel, S.P., Bayer, B.L., Ranno, A.E. and Willcockson, N.K. (1993) J. Am. Geriatr. Soc. 41, 1219–1225.
120 Schneider, L.S., Olin, J.T. and Pawluczyk, S. (1993) Am. J. Psychiatry 150, 321–323.
121 Berry, M.D., Juorio, A.V. and Paterson, I.A. (1994) Prog. Neurobiol. 42, 375–391.
122 Zernig, G. (1990) Trends Pharmacol. Sci. 11, 38–44.
123 Verity, M.A (1992) Neurotoxicology 13, 139–148.
124 Nicotera, P. and Orrenius, S. (1992) Ann. N.Y. Acad. Sci. 646, 17–27.
125 Siesjo, B.K. (1992) J. Neurosurg. 77, 337–354.
126 Zivin, J.A. and Choi, D.W. (1991) Sci. Am. 265, 56–63.
127 Delumeau, J. C., Bentué-Ferrer, D., Saiag, B. and Allain, H. (1989) Fundam. Clin. Pharmacol. 3, 89S-102S.
128 Tsien, R.W., Lipscombe, D., Madison, D., Bley, K. and Fox, A. (1995) Trends Neurosci. 18, 52–54.
129 Choi, D. (1995) Trends Neurosci. 18, 58–60.
130 Dunlap, K., Luebke, J.I. and Turner, T.J. (1995) Trends Neurosci. 18, 89–98.

131 Khachaturian, Z.S (1989) Aging 1, 17–34.
132 Deshpande, J., Berstedt, K. and Linden, T. (1992) Exp. Brain Res. 88, 91–105.
133 Heizmann C. and Braun, K. (1992) Trends Neurosci. 15, 259–269.
134 Koike, T., Martin, D.P. and Johnson, E.M. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 6421–6425.
135 Koh, J. and Cotman, C.W. (1992) Brain Res. 587, 233–240.
136 Soong, T.W., Stea, A., Hodson, C.D., Dubel, S.J., Vincent, S.R. and Snutch, T.P. (1993) Science (Washington, D.C.) 260, 1133–1136.
137 Saïag, B., Lacroix, P., Bellissant, E. and Bentué-Ferrer, D. (1994) Angéiologie 46, 41–51.
138 Middlemiss, D.N. and Spedding, M. (1985) Nature (London) 316, 94–96.
139 Kondoh, Y., Mizusawa, S., Murakami, M., Nagatu, K., Nakamichi, H. and Watanabe, K. (1991) Metab. Brain. Dis. 6, 111–124.
140 Hoffmeister, F., Benz, U., Heise, A., Krause, H.P. and Neuser, V. (1982) Arzneim.-Forsch. 32, 347–360.
141 Nomura, M. (1988) Arzneim.-Forsch. 38, 1282–1286.
142 Straube, K.T., Deyo, R.A., Moyer, J.R. and Disterhoft, F. (1990) Neurobiol. Aging. 11, 659–661.
143 Isaacson, R., Jonston, J.E. and Vargas, D.M. (1988) Physiol. Behav. 42, 447–452.
144 Izquierdo, I. (1990) Trends Pharmacol. Sci. 11, 309–310.
145 Finger, S., Green, L., Tarnoff, M.E., Mortman, K.D. and Andersen, A. (1990) Exp. Neurol. 109, 279–285.
146 Parnetti, L., Senin, U., Carosi, M. and 83 other authors (1993) Clin. Ther. 15, 394–406.
147 Ban, T.A., Morey, L., Aguglia, E. and 15 other authors (1990) Prog. Neuropsychopharmacol. Biol. Psychiatry 14, 525–551.
148 Tollefson, G.D. (1990) Biol. Psychiatry 27, 1133–1142.
149 Itil, T.M., Michael, S.T., Hoffmeister, F., Kunitz, A. and Eralp, E. (1984) Curr. Ther. Res. 35, 1–18.
150 Kanowski, S., Fischhof, P., Hiersemenzel, R., Röhmel, J. and Kern, U. (1989) in Diagnosis and Treatment of Senile Dementias (Kanowski, S., Fischhof, P., Malauzat, D., Michel, B. and 7 others) pp. 339–349, Springer-Verlag, Berlin.
151 Fischer, V. W., Siddiqui, A. and Yusufaly, Y. (1990) Acta Neuropathol. 79, 672–679.
152 Freeman, S.E. and Dawson, R.M. (1991) Prog. Neurobiol. 36, 257–277.
153 Nielsen, J.A., Mena, E.E., Williams, I.H., Nocerini, M.R. and Liston, D. (1989) Eur. J. Pharmacol. 173, 53–64.
154 Allain, H., Goulon, M. and Bentué-Ferrer, D. (1988) in Pharmacologie Clinique, Bases de la Thérapeutique (Giroud, J.P., Mathé, G. and Meyniel, G., eds.) Expansion Scientifique Française Paris, France.
155 Sims, N.R. and Bowen, D.M. (1983) in Alzheimer's Disease (Reisberg, B., ed.) pp. 88–92, Free Press, New York.
156 Drachman, D.A. and Leavitt, J.B. (1974) Arch. Neurol. 30, 113–121.
157 Dickey, W. and Morrow, J.I. (1990) Prog. Neurobiol. 34, 331–342.
158 Dekker, A.D., Connor, D.J. and Thal, L.J. (1991) Neurosci. Behav. Rev. 15, 299–317.
159 Dickson, C.T. and Vanderwolf, C.H. (1990) Behav. Brain Res. 41, 215–227.
160 Squire, L. and Zola-Morgan, S. (1988) Trends Neurosci. 11, 170–175.
161 Mesulman, M.M. and Moran, M.A. (1987) Ann. Neurol. 22, 223–228.
162 London, E.D., Ball, M.J. and Waller, S.B. (1989) Neurochem. Res. 14, 745–750.
163 Pomerleau, O.F. and Rosecrans, J. (1989) Psychoneuroendocrinology 14, 407–423.
164 Sahakian, B., Jones, J., Levy, R., Gray, J. and Warburton, D. (1989) Br. J. Psychiatry 154, 797–800.
165 Iversen, L.L. (1993) Prog. Brain Res. 98, 423–426.

166 Hershkowitz, N. and Rogawski, M.A. (1991) Mol. Pharmacol. 39, 592–598.
167 Davenport, C.J., Monyer, H. and Choi, D.W. (1988) Eur. J. Pharmacol. 154, 73–78.
168 Nordberg, A. (1993) Acta Neurol. Scand. Suppl. 149, 62–65.
169 Schneider, L.S. (1993) Neurology 43 (suppl 4), S64-S79.
170 Summers, W.K., Majovski, L.V., Marsh G.M., Tachiki, K. and Kling, A. (1986) N. Engl. J. Med. 315, 1241–1245.
171 Petit, H. and Pasquier, F. (1994) La Lettre du Pharmacologue 8 (Suppl), 21–25.
172 Growdon, J.H. (1992) N. Engl. J. Med. 327, 1306–1308.
173 Mohr, E., Mendis, T., Rusk, I.N. and Grimes, J.D. (1994) J. Psychiatry Neurosci. 19, 17–23.
174 Gauthier, S. and Gauthier L. (1993) Prog. Brain Res. 98, 427–429.
175 Knapp, M.J., Knopman, D.S., Solomon, P.R. and 103 other authors (1994) J. Am. Med. Assoc. 271, 985–991.
176 Winker, M.A. (1994) J. Am. Med. Assoc. 271, 1023–1024.
177 Watkins, P.B. (1994) J. Am. Med. Assoc. 272, 992–998.
178 Lamy, P.P. (1994) CNS Drugs 1, 146–165.
179 Fisher, A., Brandeis, R., Karton, I., Pittel, Z., Gurwitz, D., Haring, R., Sapir, M., Levy, A. and Heldman, E. (1991) J. Pharmacol. Exp. Ther. 257, 392–403.
180 Perry, E.K., Tomlinson, B.E., Blessed, G., Bergmann, K., Gibson, P.H. and Perry, R.H. (1978) Br. Med. J. 2, 1457–1459.
181 Warburton, D.M. (1992) Biol. Psychiatry 16, 181–191.
182 Wesnes, K. and Warburton, D.M. (1983) Pharmacol. Ther. 21, 189–208.
183 Newhouse, P.A., Sunderland, T., Tariot, P.N., Blumhardt, C.L., Weingartner, H., Mellow, A. and Murphy, D.L. (1988) Psychopharmacology 95, 171–175.
184 Jones, G.M.M., Sahakian, B.J., Levy, R., Warburton, D.M. and Gray, J.A. (1992) Psychopharmacology 108, 485–494.
185 Smith, K.M., Joseph, M.H. and Gray, J.A. (1991) in Effects of Nicotine on Biological Systems (Adlkofer, F. and Thurau, K., eds.), pp. 345–350, Birkhauser, Boston.
186 Arneric, S.P. and Williams, M. (1994) in Recent Advances in the Treatment of Neurodegenerative Disorders and Cognitive Dysfunction (Racagni, G., Brunello, N. and Langer, S.Z., eds.), Vol. 7, pp. 58–70, Int. Acad. Biomed Drug Res. Basel Karger.
187 Siak-Shie, N.N., Meyer, E.M. and Hunter, B.E. (1991) in Cholinergic Basis for Alzheimer Therapy (Becker, R. and Giacobini, E., eds.), pp. 379–385, Birkhauser, Boston.
188 Smith, C.J. and Giacobini, E. (1992) Rev. Neurosci. 3, 25–43.
189 Plassman, B.L., Helms, M.J., Welsh, K.A., Saunders, A.M. and Breitner, J.C.S. (1995) Lancet 345, 387.
190 Tilley, J.W. and Kramer, M.J. (1981) Prog. Med. Chem. 18, 1–44.
191 Rothstein, J.D., Tsai, G. and Kuncl, R.W. (1990) Ann. Neurol. 28, 18–25.
192 Lees, G.J. (1993) Neuroscience 54, 287–322.
193 Beal, M.F., Hyman, B.T. and Koroshetz, W. (1993) Trends Neurosci. 16, 125–131.
194 Young, A.B., Greenamyre, J.T., Hollingsworth, Z., Albin, R., Damato, C., Shoulson, I. and Penney, J.B. (1988) Science (Washington, D.C.) 241, 981–983.
195 Koh, S.Y., Yang, L.L. and Cotman, C.W. (1990) Brain Res. 533, 315–320.
196 Pomara, N., Singh, R., Deptula, D., Chou, J.C.Y., Schwartz, M.B. and Levitt, P.A. (1992) Am. J. Psychiatry 149, 251–254.
197 Coyle, J.T. and Puttfarcken, P. (1993) Science (Washington, D.C.) 262, 689–695.
198 Smirnova, T., Stinnakre, J. and Mallet, J. (1993) Science (Washington, D.C.) 262, 430–433.
199 Lawlor, B. and Davis, K.L. (1992) Biol. Psychiatry 31, 337–350.
200 Danbolt, N.C. (1994) Prog. Neurobiol. 44, 377–396.

201 Stutzmann, J.M. and Doble, A. (1994) in Neurodegenerative Diseases, 11, 205–214, Academic Press, London.
202 Nair, N.P.V. and McKeith, I. (1994) Neurobiol. Aging 15 (Suppl 1), S 137.
203 Wesemann, W., Sontag, K.H. and Maj, J. (1983) Arzneim.-Forsch. 33, 1122–1125.
204 Seif el Nasr, M., Peruche, B., Rossberg, C., Mennel, H.D. and Krieglstein, J. (1990) Eur. J. Pharmacol. 185, 19–24.
205 Fleischmacker, W.W., Bughgeher, A. and Schubert, H. (1986) Prog. Neuropsychopharmacol. Biol. Psychiatry 10, 87–93.
206 Lee, R.C. and Benfield, P. (1994) Drugs Aging 4, 257–273.
207 Service, R.F. (1994) Science (Washington, D.C.) 266, 218–219.
208 Doyle, E., Regan, C.M. and Shiotani, T. (1993) J. Neurochem. 61, 66–272.
209 Murphy, T.H., Miyamoto, M., Sastre, A., Schnaar, R.L. and Coyle, J.T. (1989) Neuron 2, 1547–1558.
210 Volterra, A., Trotti, D., Floridi, S. and Racagni, G. (1992) Soc. Neurosci. Abstr. 18, 647.
211 Siesjö, B.K., Agarah, C.D. and Bengtsson, F. (1989) Cerebrovasc. Brain Metab. Rev. 1, 165–211.
212 Hall, E.D. (1992) Ann. Neurol. 32, S137-S142.
213 Allain, H., Denmat, J., Bentué-Ferrer, D., Milon, D., Pignol, P., Reymann, J.M., Pape, D., Sabouraud, O. and Vandendriesche, J. (1988) Fundam. Clin. Pharmacol. 2, 1–11.
214 McLachlan, D.R., Dalton, A.J., Kruck, T.P.A., Bell, M.Y., Smith, W.L., Kalow, W. and Andrews, D.F. (1991) Lancet 337, 1304–1308.
215 Youdim, M.B.H., Ben-Shachar, D. and Riederer, P. (1993) Mov. Disord. 8, 1–12.
216 Poeggeler, B., Reiter, R.J., Tan, D.X., Chen, L.D. and Manchester, L.C. (1993) J. Pineal Res. 14, 151–168.
217 Benesova, O. (1994) Drugs Aging 4, 285–303.
218 Sarter, M. (1991) Trends Pharmacol. Sci. 12, 456–461.
219 Nicholson, C.D. (1990) Psychopharmacology 101, 147–159.
220 Allain, H., Boyer, P., Kossmann, L., Lepine, J.P. and Kanowski, S. (1990) Pharmacopsychiatry 23 (Suppl 2), 49–51.
221 Thompson, T.L., Filley, C.M., Mitchell, W.D., Culig, K.M., Loverde, M. and Byyny, R.L. (1990) N. Engl. J. Med. 323, 445–448.
222 Pettegrew, J.W., Panchalingam, K., Klunk, W.E., McClure, R.J. and Muenz, L.R. (1994) Neurobiol. Aging 15, 117–132.
223 Allain, H., Weanes, K., Neuman, E., Gandon, J.M., Salzmann, V., Malbezin, M. and Guez, D. (1994) Neurobiol. Aging 15 (suppl 1), S136.
224 Derouesné, C., Renault, B., Gueguen, B., Vanderlinden, M., Lacomblez, L., Homeyer, P., Ouss, L., Neuman, E., Malbezin, M., Barrandon, S. and Guez, D. (1994) Neurobiol. Aging 15 (suppl 1), S100.
225 Breitner, J.C.S., Gau, B.A., Welsh, K.A., Plassman, B.L., McDonald, W.M., Helms, M.J. and Anthony, J.C. (1994) Neurology 44, 227–232.
226 Rich, J.B., Rasmusson, D.X., Folstein, M.F., Carson, K.A., Kawas, C. and Brandt, J. (1995) Neurology 45, 51–55.
227 Rogers, J., Kirby, L.C., Hempelman, S.R. and 8 other authors (1993) Neurology 43, 1609–1611.
228 Sohrabji, B.F., Miranda, R.C. and Toran-Allerand, C.D. (1994) J. Neurosci. 14, 459–471.
229 Jaffe, A.B., Toran-Allerand, C.D., Greengard, P. and Gandy, S.E. (1994) J. Biol. Chem. 269, 13065–13068.
230 Henderson, V.W., Paganini-Hill, A., Emanuel, C.K., Dunn, M.E. and Buckwalter, J.G. (1994) Arch. Neurol. 51, 896–900.
231 Rose, C.D. and Henneberry, R.C. (1993) Neurodegeneration 2, 287–298.

232 Bonnaure, P. (1994) Futuribles 41–62.
233 Crozier, M. (1970) La société bloquée, Le Seuil, Paris.
234 Gagnepain, J. (1991) Du vouloir dire, Livre et Communication, Paris.

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Progress in Medicinal Chemistry – Vol. 34 edited by G.P. Ellis and D.K. Luscombe

2 Recent Advances in the Medicinal Chemistry and Pharmacology of Camptothecin

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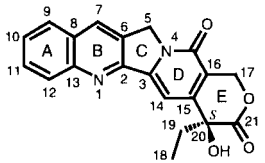
INTRODUCTION

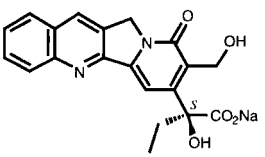
The discovery of new agents with novel mechanisms of action is the result of continual progress in the search for efficacious drugs for use in cancer chemotherapy. Although the rate of successful outcomes of chemotherapy is not yet satisfactory, especially in the treatment of malignant solid tumours, the discovery of epoch-making agents, such as doxorubicin (adriamycin) and cisplatin, has improved the clinical efficacy of chemotherapy. It has taken almost three decades for camptothecin to be recognised as a major cancer chemotherapeutic agent. Camptothecin (1) (*Chemical Abstracts* name: 4-ethyl-4-hydroxy-(*S*)-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinoline-3,14-(4*H*,12*H*)-dione [numbered as in (1a)]), was first isolated from the stem and bark of a Chinese tree *Camptotheca acuminata* and the structure of camptothecin was determined by Wall *et al.* in 1966 [1]. Preclinical studies demonstrated the remarkable antitumour activity of camptothecin against L1210 mouse leukaemia and the rat Walker 256 carcinosarcoma [1, 2], and its potent inhibition of DNA and RNA synthesis in L1210 mouse leukaemia cells [3–5].

In the late 1960's, demonstration of the impressive activity of camptothecin opened the door to clinical investigations. Clinical studies were initiated with the sodium salt of camptothecin (2) because of the lack of water-solubility of the parent compound. However, these studies were discontinued owing to the unpredictable occurrence of myelosuppression and severe haemorrhagic cystitis [6–9], in spite of the fact that promising antitumour responses were observed among patients with gastric and colon cancer. The discontinuation of these early clinical studies slowed down the progress of camptothecin research.

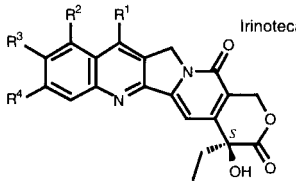
In the late 1980's, however, the discovery that type I DNA topoisomerase (topoisomerase I: Topo I) is an intracellular target of camptothecin and that camptothecin is a specific inhibitor of Topo I [10–13] paved the way for further progress in research on camptothecin. Most of the renewed interest in camptothecin focused on investigation of the antitumour activity of water-soluble analogues which were expected to reduce the unpredictable toxic effects of the parent compound. Based on detailed studies of the structure-activity relationships of camptothecin analogues synthesized during the 1970's and 1980's [14–20], further modification of the camptothecin molecule was explored in order to find an effective compound with clinical potential. As a result, irinotecan (CPT-11) (3) [21, 22] and topotecan (hycamtin, 5) [23], both semisynthetic, water-soluble analogues of camptothecin, were introduced into the clinic in the early 1990's [24–27], and, in 1994, irinotecan, the first anticancer agent derived from camptothecin was ap-



Camptothecin (1)



Camptothecin sodium salt (2)



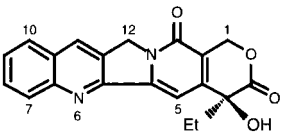
Irinotecan (CPT-11) (3) R^1=Et, R^2=R^4=H R^3=OOCN

SN-38 (4) R^1=Et, R^2=R^4=H, R^3=OH

Topotecan (5) R^1=R^4=H, R^2=CH_2NMe_2, R^3=OH

9-AC (6) R^1=R^3=R^4=H, R^2=NH_2

GG-211 (7) R^1=H_2CN NMe R^2=H, R^3=R^4=OCH_2CH_2O



(1a)

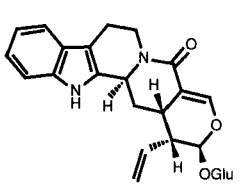
proved in Japan for the treatment of lung, cervical, and ovarian cancer. More recently, clinical studies of 9-aminocamptothecin (9-AC) (6) [28], orally-administered camptothecin [29], and GG-211 (7) [30] were also initiated. This review concentrates on advances in the medicinal chemistry and pharmacology of camptothecin and its analogues in the period 1990–1995. Several excellent reviews of research on camptothecin and Topo I have been published, and the reader is also referred to these [31–38].

CHEMISTRY OF CAMPTOTHECIN

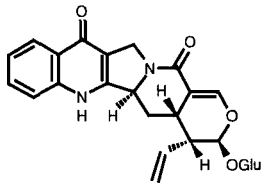
ISOLATION OF CAMPTOTHECIN

Camptothecin (1) was first isolated from *Camptotheca acuminata* Decne (Nyssaceae) [1, 39] which has been used for many years as a traditional Chinese medicine. Extensive studies of the natural distribution of this unique alkaloid led to its isolation from several plant families: *Nothapodytes foetida* [40–43], *Ophiorrhiza mungos* [44], *Ervatmia heyneana* [45], *Merrilliodendron*

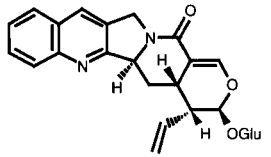
megacarpum [46], and *Ophiorrhiza pumila* [47, 48]. Regarding the biosynthetic pathway for camptothecin, the 'post-strictosamide biosynthetic events' were proposed by Hutchinson [49] to be preceded by oxidative transformation of the indole ring system of strictosamide (8) to a quinolone ring system to give the quinolone glycoside. In 1989, two new glucosidic alkaloids, pumiloside (9) and deoxypumiloside (10) were isolated from *O. pumila* together with camptothecin [47]. Pumiloside (9) has also been isolated independently from *C. acuminata* [39] and *N. foetida* [42]. The fact that both pumiloside (9) and deoxypumiloside (10) were isolated from the same plant as camptothecin provides strong evidence in support of Hutchinson's proposal for the biosynthesis of camptothecin.



Strictosamide (8)



Pumiloside (9)



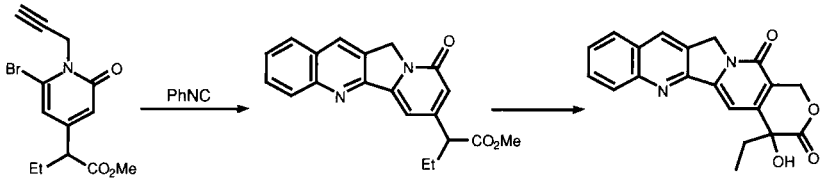
Deoxypumiloside (10)

TOTAL SYNTHESIS OF CAMPTOTHECIN

A number of synthetic strategies for the preparation of camptothecin were developed in the 1970's and early 1980's [49–55], and these efforts culminated in several successful total syntheses, the first by Stork and Schultz [56]. Discontinuation of the early clinical studies of camptothecin dramatically reduced the research effort devoted to investigating new methodologies for the total synthesis of this alkaloid. In the late 1980's, however, the discovery of the unique biological mode of action of camptothecin, and the development of water-soluble derivatives such as CPT-11 [21] and topotecan [23], revived interest in the development of practical methods for the synthesis of camptothecin.

In 1992, novel methodology for the construction of the camptothecin molecule was reported by Curran *et al.* [57–59]. They have applied a novel (4 + 1) radical annulation reaction to the construction of rings B and C of camptothecin. Reaction of *N*-propargylpyridone (11) with phenyl isonitrile and hexamethylditin under irradiation with a sunlamp gave the tetracyclic compound (12), which was readily converted to (20*RS*)-camptothecin (13) (*Scheme 2.1*) using Danishefsky's procedure [60]. The synthesis of key com-

pound (12) requires only six steps starting from dimethyl acetonedicarboxylate, and the overall yield is 13%.

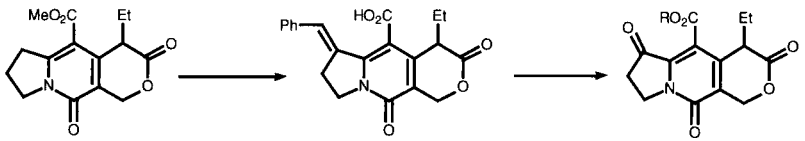


(11) (12) (20*RS*)-Camptothecin (13)

Scheme 2.1

Danishefsky *et al.* [61] reported an improved route to (20*RS*)-camptothecin in connection with their original total synthesis [60, 62–64]. Reaction of tricyclic compound (14) with sodium hexamethyldisilazide and benzaldehyde afforded a 90% yield of the benzylidene acid (15). A mechanism similar to a Stobbe condensation with participation of the methoxycarbonyl function on the pyridone ring is suggested for this reaction step. Ozonolysis of this compound (15) (*Scheme 2.2*) afforded a 96% yield of the acid (16) which, upon esterification, provided (17) in 81% yield. Compound (17) is readily converted to (20*RS*)-camptothecin.

As for the synthesis of racemic camptothecin, another convergent approach has been reported by Rao *et al.* [65].

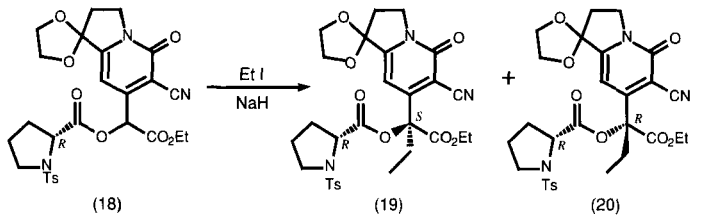


(14) (15) (16) R=H
(17) R=Me

Scheme 2.2

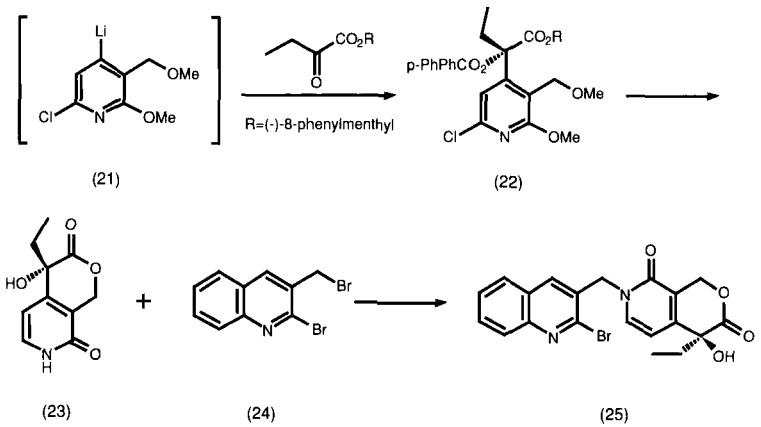
Although several enantioselective syntheses of camptothecin have been reported [66–69], there are some major limitations when considering a practical method for the preparation of the biologically active (20*S*)-isomer. In 1987, the first asymmetric synthesis of (20*S*)-camptothecin was reported by Ejima *et al.* [70]. They attempted an asymmetric alkylation of an indolizine derivative (18) (*Scheme 2.3*) employing (*R*)-tosylproline as a chiral auxiliary. Thus, ethylation of compound (18) with ethyl iodide in the presence of NaH [64% diastereo excess (*de*)], followed by treatment with propanol pro-

vided the optically pure (*S*)-isomer (19) in 56% yield. (20*S*)-Camptothecin is easily obtained from compound (19) in several steps [67].



Scheme 2.3

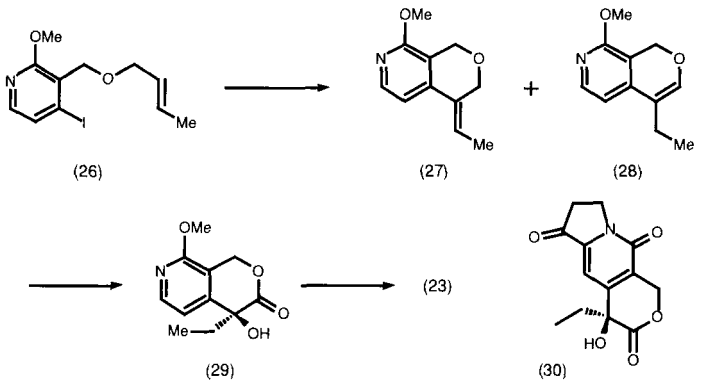
In 1992, a more efficient asymmetric synthesis of (20*S*)-camptothecin was reported by Comins *et al.* [71]. The key reaction of their procedure is addition of the lithiated pyridine derivative (21) to an α-keto-phenylmenthyl ester (*Scheme 2.4*). Trapping of the lithium alkoxide intermediate with 4-phenylbenzoyl chloride (87% *de*) and recrystallization gave diastereomerically pure compound (22) in 60% yield. The intermediate (23) was prepared from (22) in three steps, and (23) was coupled with quinoline derivative (24) to give (25). Intramolecular Heck reaction of (25) gave (20*S*)-camptothecin in 12% overall yield from the starting material, 2-chloro-6-methoxypyridine. The same authors have also reported a nine-step synthesis of (20*S*)-camptothecin [72] and a six-step convergent method for the preparation of (20*RS*)-camptothecin [73], using a modified version of their original method.



Scheme 2.4

The first asymmetric synthesis of (20*S*)-camptothecin using catalytic asymmetric induction was achieved by Fang *et al.* in 1994 [74]. They carried out a catalytic enantioselective synthesis of Comins's intermediate (23) in order to avoid the use of the expensive chiral auxiliary, 8-phenylmenthol, or similar compound. Intramolecular Heck reaction of pyridine derivative (26) gave the cyclic olefins (27) and (28) in a ratio 1 : 8. The allylic ether (27) can be isomerized to (28) upon treatment with Wilkinson's catalyst [75]. Asymmetric Sharpless dihydroxylation of (28) proceeded successfully when 2,5-diphenyl-4,6-bis(9-*O*-dihydroquinidyl)pyrimidine [$(DHQD)_2$-PYR] was used as the chiral catalyst [76], and subsequent oxidation gave (29) in 94% *ee*. Treatment of (29) with acid gave the target molecule (23, *Scheme 2.5*), which was converted to (20*S*)-camptothecin in 2 steps using the Comins's procedure [73].

Jew *et al.* [77] have also successfully applied the Sharpless catalytic asymmetric dihydroxylation to the preparation of the homochiral intermediate (30) in a synthesis of (20*S*)-camptothecin [67–69].



Scheme 2.5

ANALOGUES AND STRUCTURE-ACTIVITY RELATIONSHIPS

EFFECTS OF THE INTRODUCTION OF SUBSTITUENTS

Since the initial isolation and structure determination of camptothecin in 1966 [1], numerous studies of the synthesis of analogues of this potent alkaloid have been carried out in order to define structure-activity relationships. The history of advances in camptothecin research may be divided into two

periods, prior to and following the year 1985, when the molecular target of camptothecin was identified as a Topo I [10]. In the late 1970's and early 1980's, various camptothecin analogues were synthesized, both by semi-synthesis and total synthesis, in racemic and optically active form. Most of these analogues were water-insoluble and their biological activities were evaluated by *in vitro* cytotoxicity measurements and *in vivo* by their antitumour activity in mice. These early studies provided the basic information for the structure-activity relationships of camptothecin derivatives, which can be summarized briefly as follows: (i) the conjugated fused ring ABCD is required for both *in vitro* [78] and *in vivo* activity [52, 53, 79], (ii) the *S* configuration at position 20 is necessary for antitumour activity, because racemic (20*RS*)-camptothecin analogues have half the potency of corresponding (20*S*)-analogues [14, 15, 67, 68], (iii) the intact lactone ring E is essential for activity, because the sodium salt of camptothecin has one tenth of the antitumour activity of camptothecin [14, 80], (iv) substitution at position 9 or 10 in the A ring enhances biological activity [15, 16], (v) although substitution at position 11 results in either only moderate activity or total inactivity, substituents of limited size may be acceptable [17], (vi) substitution at position 12 decreases activity significantly [16].

In the late 1980's and early 1990's, based on the results of these early studies, a novel trend in camptothecin research became apparent, especially the search for a clinically useful analogue with aqueous solubility. Steady progress in the methodology for the synthesis of camptothecin derivatives has also facilitated this trend and made it possible to prepare a wide variety of optically pure analogues with an (20*S*)-configuration. The assay for Topo I inhibition has also become an important primary screen for evaluating the antitumour activity of camptothecin analogues. It has been shown that camptothecin produces protein-linked DNA breaks in the presence of purified Topo I [10], and does not bind to isolated DNA or to the isolated enzyme [10, 81–83]. An extensive investigation of the mechanism of inhibition of Topo I by camptothecin has revealed that camptothecin binds reversibly to a Topo I-DNA cleavable complex to form a stable ternary complex [82], although its exact structure is still under investigation.

A large number of camptothecin analogues are active in the assay for Topo I inhibition, indicating that Topo I is the major cellular target for camptothecin and that inhibition of this enzyme is responsible for the antitumour activity of camptothecin anlogues [11, 19, 84–86]. However, the potency of Topo I inhibition does not always correlate with cytotoxicity against tumour cells or *in vivo* antitumour activity. Jaxel *et al.* [19] showed that the correlation between Topo I inhibitory activity and *in vivo* antitumour activity against mouse leukaemia is good. However, other studies showed that

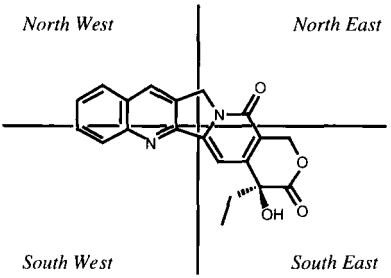


Figure 2.1

no quantitative relationship between Topo I inhibitory activity and cytotoxicity or *in vivo* antitumour activity seems to exist [22, 86]. These results indicate that cellular uptake or pharmacokinetics of the agent probably play a significant role in determining the observed cytotoxicity and *in vivo* antitumour activity, especially in the case of compounds with a hydrophilic or a labile substituent [86, 87].

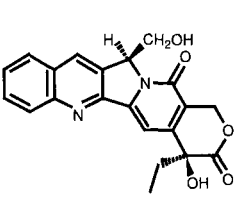
Introduction of a hydrophilic substituent into a potent pharmacophore, such as 10,11-methylenedioxycamptothecin, is the major trend in synthetic studies aimed at obtaining a water-soluble analogue of camptothecin with significant antitumour activity.

In order to summarize the structure-activity relationships of camptothecin analogues, we have divided the camptothecin molecule into four regions, i.e. north east (NE), north west (NW), south east (SE), and south west (SW) regions, and describe the distinctive features of each region.

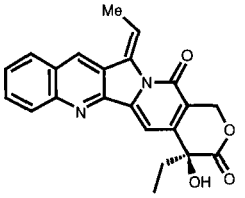
North East region (NE). The north east region includes positions 4, 5, and 16a. It is quite difficult to define a structure-activity relationship for this region, because there are relatively few examples of the modification of these positions. One study showed that hydroxymethylation of position 5 of camptothecin results in a drastic loss of potency for Topo I inhibition and cytotoxicity in both the α-isomer and β-isomer (31) [88]. The formation of a hydrogen bond between the hydroxymethyl substituent at position 5 and the pyridone moiety, or possibly steric hindrance may result in a loss of recognition by the binary Topo I-DNA complex. This result is consistent with the early observation that substitution at position 5 *via* a single bond, as when introducing groups such as methyl, methoxy or hydroxy, reduces the antitumour activity of camptothecin [83, 89]. These studies suggest that the subtituent at position 5 must have minimal steric bulk otherwise it will probably be perpendicular to the molecular plane of camptothecin, resulting in loss

of activity. However, if the substituent at position 5 is co-planar with the rest of the molecule, functionalization of this position may be tolerated. For example, introduction of an ethylidene group at position 5 of camptothecin, which avoids the generation of an α,β-epimer, gave compound (32) with the same order of potency as camptothecin in the P388 *in vitro* assay [90]. This result indicates that the enzyme-DNA complex might have some tolerance for substituents of moderate size at position 5, but only if they are in plane with the camptothecin core.

The NE region appears to be specifically recognized by a narrow groove on the binary enzyme-DNA complex, and both steric and electronic constraints of the indolizine ring system are therefore crucial for the potent Topo I inhibitory activity of camptothecin.



(31)



(32)

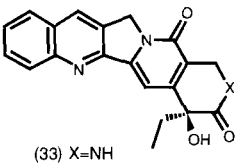
South East region (SE). The south east region, including positions 3, 14–19, and 20, is also highly sensitive to modification by a substituent. The lack of activity of compounds which have a lactam (33) [18, 84], thiolactone (34) [18], or *N*-aminolactam (35) [91] replacing the lactone in ring E provides strong support for the requirement for an intact α-hydroxy lactone for potent biological activity. The importance of a hydroxy group at position 20 is also supported by the low activity of 20-aminocamptothecin (36) [84, 92], which was initially supposed to be able to mimic the hydrogen-bonding characteristics of the α-hydroxy lactone. It is possible that hydrogen bonding with the enzyme-DNA complex, or with the lactone carbonyl of ring E is an essential function of the 20-hydroxy group, resulting in potent Topo I inhibition [18].

An ethyl group at position 20 also appears to be specifically recognized by the enzyme-DNA complex, because no modifications of this group have increased activity. It should be noted that compound (37), in which the exocyclic methylene group was expected to provide a more electrophilic alkylation target, exhibited potent camptothecin-like inhibition of Topo I activity [93].

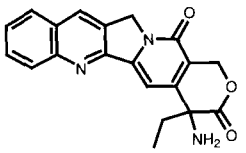
Although there is little information about the modification of position 14, the steric requirements of the group at this position for good Topo I inhibi-

tory activity have been partly explored in a study of the 14-methoxycarbonyl analogues of camptothecin [94]. It seems that a bulky substituent at position 14 tends to reduce activity.

In general, the south east region appears to be recognized by a specific region on the Topo I-DNA complex and is therefore not the most suitable part of the molecule for introduction of substituents which might enhance biological activity.



(33) X=NH
(34) X=S
(35) X=N-NH_2



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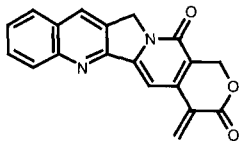
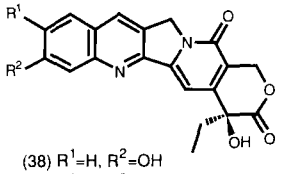


(37)

South West region (SW). The south west region, including positions 1, 2, 11, 12, and 13, is the most sensitive to modification. It has been reported that substitution at either position 11 or 12 results in dramatically decreased activity [14, 17]. In particular, most of the analogues which are modified at position 12 show entire loss of activity, regardless of the properties of the substituent. The relatively high potencies of 11-hydroxycamptothecin (38) and 11-cyanocamptothecin (39) in the *in vivo* L1210 mouse leukaemia assay suggest that a substituent with low steric bulk is acceptible at position 11 [17]. Introduction of a fluorine atom at position 11 significantly enhances the biological activity of camptothecin [95]. The difference in potency between the highly active 10,11-methylenedioxycamptothecin (40) and the inactive 10,11-dimethoxycamptothecin (41) [16] is remarkable and may be explained by the intrusion of 11-methoxy group into the south west region.

From these observations, it can be concluded that the south west region is mostly buried in the Topo I-DNA complex and that positions 1, 2, and 12 are on the inside of the binary complex while position 11 is probably at the edge of the complex.



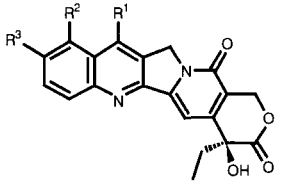
(38) R^1=H, R^2=OH
(39) R^1=H, R^2=CN
(40) R^1R^2=OCH_2O
(41) R^1=OMe, R^2=OMe

North West region (NW). The major conclusion of research into the structural requirements for biological activity of camptothecin is that the north west region, including positions 6, 7, 8, 9 and 10, appears to be the most suitable for manipulating the substituents whilst retaining potent antitumour activity. For example, substitution at position 7, 9 or 10 with amino, methyl, hydroxy, or halogeno groups provides highly active analogues in the Topo I inhibition assay [16, 19, 86, 94]. These results suggest that the Topo I-DNA binary complex forms a relatively large and flexible binding pocket around this region and therefore tolerates a variety of substituents.

The majority of studies of camptothecin analogues during the late 1980's and early 1990's focused on the introduction of hydrophilic substituents at position 7, 9 or 10 in efforts to improve the aqueous solubility of the molecule without loss of biological activity [23, 87, 96, 97]. Thus, substitution at position 7, 9 or 10 by various amino groups has been explored in order to improve aqueous solubility by permitting the formation of a salt with an appropriate acid, which is chosen so as to avoid opening of the lactone ring E. In a comparison of Topo I inhibitory activity among the 7-, 9- and 10-aminoalkylcamptothecins, both compound (42) and compound (43) are more potent than the 10-aminomethyl analogue (44) [23]. These results show that there is a larger degree of tolerance for a bulky group at position 7 or 9 than at position 10, and that a larger substituent in position 10 as well as position 11 probably perturbs the south west region, hence diminishing biological activity. Tanizawa *et al.* reported that the stability of the Topo I-cleavable complexes, conferred by camptothecin derivatives, may be important for drug activity [98]. Comparison of the stability among the Topo I-cleavable complexes conferred by camptothecin itself, SN-38 (4) (an active metabolite of CPT-11), and 10-hydroxycamptothecin, reveals that both the hydroxy group at position 10 and the ethyl group at position 7 enhance the stability of the cleavable complex. It is suggested that these sustituents may enhance interactions with the drug receptor site and drug affinity at the Topo I-DNA interface [98, 99].

In conclusion, these results suggest that a combination of the introduction of a hydrophilic substituent at position 7 or 9 to improve aqueous solubility and introduction of substituents at position 10 or 11 to increase activity is the best methodology for finding potent analogues with a good solubility profile.

Among the four regions, the north west region is the most suitable for the introduction of substituents which enhance both biological activity and aqueous solubility.



(42) R^1=CH_2CH_2NHMe, R^2=R^3=H

(43) R^1=R^3=H, R^2=CH_2NMe_2

(44) R^1=R^2=H, R^3=CH_2NH_2

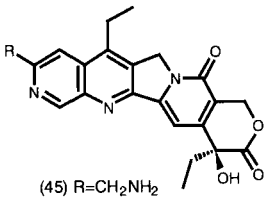
NEW CAMPTOTHECIN-LIKE STRUCTURES

Many attempts at modification of rings A, B, C, D, and E of camptothecin have been made in order to try to define the minimum essential structural components for biological activity or to create novel structures which show favourable biological activity and solubility profiles [16, 18, 84, 94]. Most of these studies, however, only succeeded in demonstrating the necessity of the intact pentacyclic ring system for potent biological activity [90, 100]. However, some hexacyclic analogues of camptothecin, which have an additional fused ring adjacent to the original ring A or ring B, showed higher potency than camptothecin itself [16, 101, 102]. It should also be noted that the *des*-B ring analogues of camptothecin cause complete DNA fragmentation in the cleavable complex assay [103]. Studies of the modification of the ring system of camptothecin are summarized in this section.

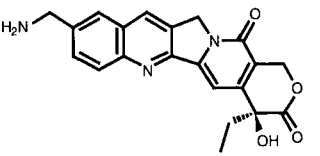
MODIFICATION OF RING A

Previous studies have shown that the planarity of rings A and B of camptothecin appears to be essential for antitumour activity [78]. It is proposed that ring A may have to fit in a narrow pocket in the enzyme-DNA complex [94]. Among the ring A modified compounds which have been reported, 10-, 11- and 12-azacamptothecins show modest activity in the mouse leukaemia assay [14, 16, 90]. Uehling *et al.* showed that 11-azacamptothecin is approximately two-fold more potent than camptothecin in the Topo I-cleavable complex assay [104]. Structure-activity relationship studies of 11-azacamptothecin analogues provide rather different information from that of camptothecin about substitution at position 10. The 10-aminomethyl-7-ethyl-11-azacamptothecin (45) is two-fold more potent than 7-ethyl-11-azacamptothecin (46), while 10-aminomethylcamptothecin (47) is about 12-fold less potent than camptothecin in the Topo I-cleavable complex assay [23]. It is possible that formation of a hydrogen bond between the 10-aminomethyl group and the nitrogen atom at position 11 maintains the co-planarity of the substituent in the 11-azacamptothecin series. These studies led to

the identification of the water-soluble 10-aminohydroximinomethyl derivative (48), which showed comparable *in vivo* antitumour activity to GG-211 (7) against HT-29 xenograft.



(45) R=CH_2NH_2
(46) R=H
(48) R=$C(NH_2)$=NOH



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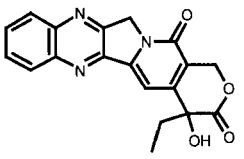
MODIFICATION OF RING B

Although ring B of camptothecin is also considered to be a critical moiety for biological activity, there are few examples of the modification of this ring. Replacement of quinoline ring AB with quinoxaline or indole results in either only moderately active or inactive analogues of camptothecin (49, 50) [90]. The oxodihydroindolylidene derivatives (51), which are B-ring-modified camptothecin analogues, were prepared and evaluated as Topo I inhibitors in the cleavable complex assay by Lackey *et al.* [103]. Maintaining both co-planarity and the *E*-geometry of the double bond appear to be critical for the retention of biological activity in this series of compounds.

(*RS*)-Isomer of compound (51) causes complete DNA fragmentation in the cleavable complex assay, but the (*S*)-isomer (51) showed slightly more potent activity (IC_{50} = 4.0 μM, IC_{50} for camptothecin is 0.7 μM) than the (*RS*)-isomer, while the (*R*)-isomer is inactive. Taken together, these results suggest that the mode of interaction of these oxodihydroindolylidene derivatives with Topo I is similar to that of camptothecin itself. Introduction of a fluorine atom into ring A gave compound (52), which showed comparable activity to camptothecin.



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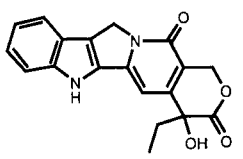

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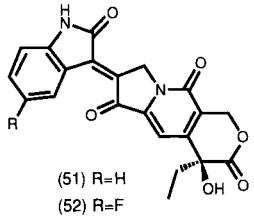
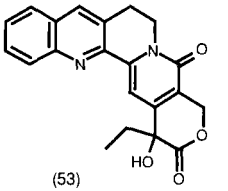


(51) R=H
(52) R=F

MODIFICATION OF RINGS C, D, AND E

Very little information about the modification of ring C or D is available, which probably reflects the difficulty of obtaining new analogues which show potent biological activity by modification of these rings. Both C-homo camptothecin (53) [90] and C-nor-4,6-secocamptothecin (54) [105] are significantly less active, probably because of the lack of planarity of camptothecin skeleton. Although the shape of the D-benzo derivative (55) is virtually identical with camptothecin in a molecular model, it is 60 times less potent than (20*RS*)-camptothecin in the assay for cytotoxicity against the 9KB cell line [84]. This study suggests that the pyridone carbonyl group at position 16a might play a pivotal role in the interaction of camptothecin with Topo I-DNA binary complex and that the pyridone ring D is essential for potent biological activity.

New types of ring E-modified camptothecin analogues (56) and (57) were found to be cytotoxic against L1210 mouse leukaemia cells [18]. However, the cytotoxicity of these compounds is not related to inhibition of Topo I, because DNA single strand breaks caused by these analogues apparently were not associated with the formation of Topo I covalent complexes.



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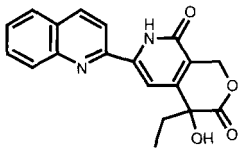

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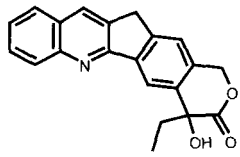

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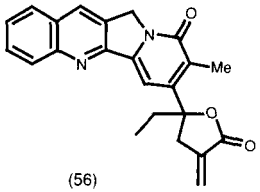

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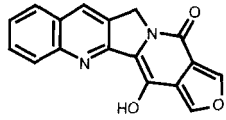

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HEXACYCLIC ANALOGUES OF CAMPTOTHECIN

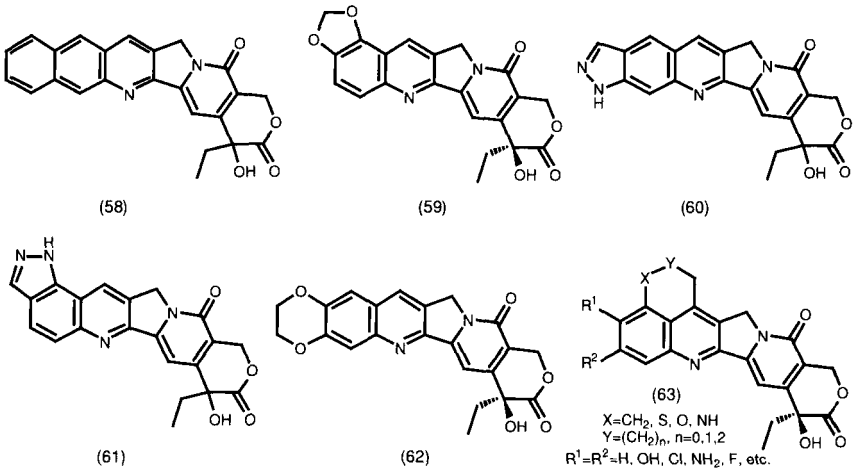
Most of the studies on modification of the camptothecin ring system have demonstrated the necessity of the intact skeleton of camptothecin for potent biological activity. However, by adding another ring to the intact pentacyclic

ring system, several potent hexacyclic analogues have been discovered [16, 101, 102]. The most suitable position for the fusion of an additional ring seems to be the site adjacent to ring A or B within the northwest region of the camptothecin molecule.

In an early study of hexacyclic analogues of camptothecin, benz[*j*]-(20*RS*)-camptothecin (58) demonstrated the same order of potency as camptothecin [14], while 10,11-methylenedioxycamptothecin (40) showed significantly greater antitumour activity than camptothecin in mouse leukaemia assays [16]. The relatively low activity of compound (58) may be due to steric effects on the south west region or alteration of the electronic distribution caused by the fused benzene ring.

Another study has revealed that in the cleavable complex assay, 10,11-methylenedioxycamptothecin (40) has about 25 times the Topo I inhibitory activity of camptothecin [86]. It is suggested that the fused 1,3-dioxolane ring system adjacent to ring A may stabilize the camptothecin molecule through hydrophobic interaction with the Topo I-DNA complex [99]. It is interesting to note that the activity varies depending on the location of the fusion of an additional ring in ring A. Thus, 10,11-methylenedioxycamptothecin (40) is 5 times more active than 9,10-methylenedioxycamptothecin (59) in the Topo I-cleavable complex assay [86], while pyrazolo[3,4-*j*]-(20*RS*)-camptothecin (60) shows only one-third the cytotoxicity of pyrazolo[4,5-*i*]-(20*RS*)-camptothecin (61) against P388 mouse leukaemia [90]. Co-planarity of the additional ring also appears to be crucial for increasing antitumour activity, because 10,11-ethylenedioxycamptothecin (62) is seven-fold less potent than (40) in the Topo I-cleavable complex assay [86].

As mentioned before, the north west region, especially position 7 or 9, appears to have a relatively high degree of tolerance for bulky substituents. However, larger substituents at these positions tend to decrease the antitumour activity. It is likely that the lower activity shown by these compounds is not only due to the size but also the flexibility of the substituent and therefore conformational restriction of substituents at positions 7 and 9 is necessary for biological activity. These considerations led us to synthesize the novel hexacyclic analogues of camptothecin (shown in 63), because cyclization at positions 7 and 9 was expected to define the position of the substituents [101]. All kinds of hexacyclic analogues, which contain additional, fused 5-, 6-, or 7-membered rings, with or without a hetero atom, have since been demonstrated to show superior activity to camptothecin. Structure-activity correlations for these hexacyclic analogues are very similar to those for the pentacyclic series, although the potency is somewhat higher. For example, the introduction of a fluorine atom at R^2 significantly enhanced both Topo I inhibitory activity and cytotoxicity [106].



WATER-SOLUBLE ANALOGUES OF CAMPTOTHECIN

The major strategy of structural modification, with the objective of enhancing the aqueous solubility of a compound, is the introduction of a highly polar function, preferably one which has the ability to form a water-soluble salt. In the case of camptothecin, however, the introduction of an acidic function such as a carboxylic acid or a sulphonic acid group is not feasible, because under basic conditions opening of the lactone ring E readily occurs to produce the salt of a hydroxy acid, which is considerably less potent than the closed lactone form [14, 80]. The majority of synthetic efforts to prepare camptothecin analogues with increased solubility have focused on the introduction of a substituent with a basic amino group, in a position chosen so as to retain the potent biological activity. These amino analogues generally have enhanced aqueous solubility under acidic conditions which maintain the closed lactone ring E. Positions 7 and 9 of camptothecin, the north west region, are considered to be the most suitable for the introduction of an amino group because they are relatively free of steric and electronic constraints. The scope for variations at other positions is severely limited. For example, in addition to substantially lower tolerance for large groups, induction of the inhibition of acetylcholinesterase also occurs when an amino substituent is introduced at position 10 [107] (see following section).

The conclusion from these studies was the identification of GG-211 (7), and a novel hexacyclic analogue of camptothecin, DX-8951 (64) which has

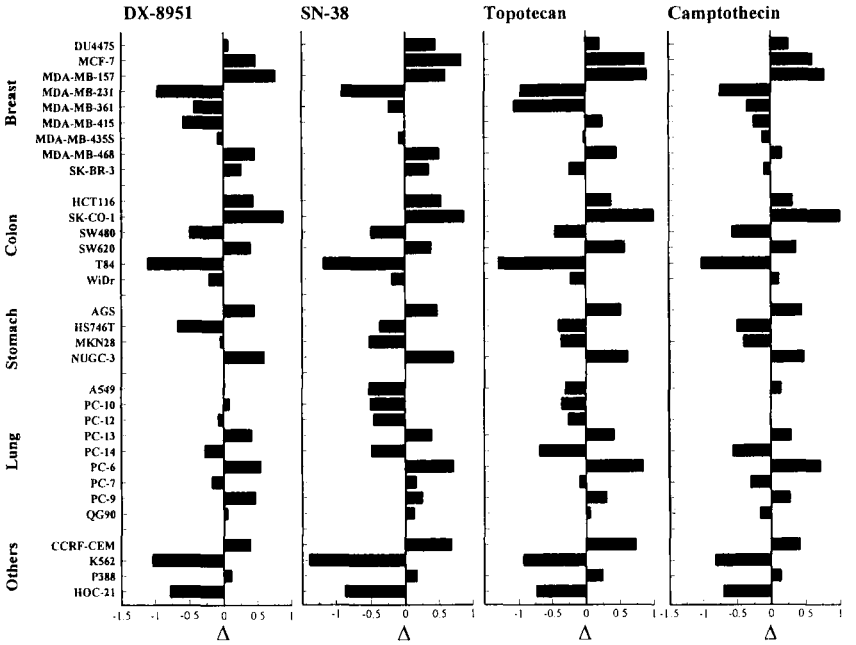


Figure 2.2

lipophilic substituents in ring A and an amino group at position 1, corresponding to position 7 in a pentacyclic ring system. Comparison of the biological activity of both compounds with topotecan shows that GG-211 is 2.5 times more potent in the Topo I cleavable complex assay and about 5 times more potent in the cytotoxicity assay against five tumour cell lines [108], while DX-8951 is 10 times more potent in the Topo I inhibition and is 28 times more potent in the cytotoxicity assay against 32 tumour cell lines [109]. Both compounds are unaffected by the multidrug resistance P-glycoprotein, and exihibited significant *in vivo* antitumour activity [108, 109]. The pattern of anticellular activities of DX-8951 against 32 malignant cell lines was almost similar to that of SN-38, topotecan, or camptothecin itself (*Figure 2.2*) [109]. This suggests that these compounds might have the same mechanism of action.

In the course of our investigation of more water-soluble hexacyclic analogues, we found that the Topo I inhibitory activity does not always correlate with cytotoxicity as was found in the case of pentacyclic analogues [106, 110]. Thus, the 3-amino-4-hydroxy-analogue (73) shows more potent Topo I

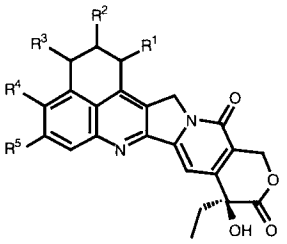


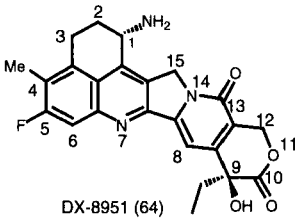
Table 2.1. CYTOTOXICITY AND TOPO I INHIBITORY ACTIVITY OF HEXACYCLIC CAMPTOTHECIN ANALOGUES

						Cytotoxicity (P388)	*Topo I inhibition*
Compound	R^1	R^2	R^3	R^4	R^5	IC_{50} *(ng/ml)*	IC_{50} *(μg/ml)*
(64)*	NH_2	H	H	Me	F	0.58	0.12
(65)	NH_2	H	H	Cl	H	1.77	0.50
(66)	NH_2	H	H	H	F	0.98	0.54
(67)	NH_2	H	H	Me	H	2.36	0.56
(68)	NH_2	H	H	OH	H	112	0.59
(69)	NH_2	H	H	NH_2	H	29.1	0.37
(70)	H	NH_2	H	Me	H	8.46	1.60
(71)	H	NH_2	H	OH	H	275	0.80
(72)	H	H	NH_2	Me	H	2.86	0.43
(73)	H	H	NH_2	OH	H	94.7	0.80
(74)	H	H	NH_2	H	H	5.52	1.39

* DX-8951

inhibitory activity than the 3-amino-analogue (74), while the cytotoxic potency of (73) is much lower than that of (74). These phenomena were observed in almost all analogues which have two or more hydrophilic polar substituents, such as hydroxy or amino groups at any of the positions 1 through 5.

Microinjection of compounds (73) and (74) into human SW-480 cells showed that both compounds have the same order of potency for inhibition of DNA synthesis, while the cytotoxic potency against SW-480 of (73) is less than that of (74) [106]. This result strongly supports the supposition that intracellular uptake plays a significant role in determining the level of antitumour activity shown by camptothecin analogues.



DX-8951 (64)

Table 2.2. EFFECTS OF MICROINJECTION OF COMPOUNDS (73) AND (74) INTO SW-480 CELLS UPON DNA SYNTHESIS

Compound	*Cytotoxicity (SW-480) IC_{50} (ng/ml)*	*Inhibition (%) of DNA synthesis* Concentration (mg/ml)*	
		0.1	*1*
(73)	11.7	47	58
(74)	0.763	29	58

* Inhibition (%) of DNA synthesis = $100 - \frac{\%\ \text{BrdU labelled microinjected cells}}{\%\ \text{BrdU labelled background cells}} \times 100$

PHARMACOLOGY OF CAMPTOTHECIN

CAMPTOTHECIN AND TYPE I DNA TOPOISOMERASE

For a long time, the mechanism of action of camptothecin was unclear. In 1985, almost 20 years after the discovery of camptothecin, Hsiang *et al.* first reported that this compound possesses inhibitory activity against type I DNA topoisomerase derived from calf thymus [10]. DNA topoisomerases are regarded as essential nuclear enzymes for resolving topological problems of DNA caused by DNA processes such as replication and transcription [111], and are classified into two major types (type I and type II). In high eukaryotic cells, type I DNA topoisomerase (topoisomerase I: Topo I) and type II DNA topoisomerase (topoisomerase II: Topo II) relax supercoiled DNA through transient DNA single-strand breaks and DNA double-strand breaks, respectively. In this transient state, broken DNA strand possesses 3′ and 5′ ends at the cleaved site, and Topo I covalently links to this 3′ end resulting in the formation of a Topo I-DNA cleavable complex. Camptothecin stabilizes this cleavable complex and inhibits DNA rejoining [10, 11, 82]. This inhibition of DNA rejoining is considered to cause DNA single-strand breaks in cultured cells [11, 85, 112]. Andoh *et al.* established a camptothecin-resistant RPMI 8402 human lymphoblastic leukaemia cell line and

Topo I purified from this cell line was highly resistant to camptothecin [113]. This mutation of Topo I might arise from the substitution of Asp^{533} (GAC) with Gly (GGC) [114]. Topo I-deficient yeast strains showed no sensitivity to camptothecin [115, 116]. Moreover, the growth inhibitory effects of camptothecin derivatives on tumour cell lines correlate with their inhibitory activities against Topo I-mediated DNA relaxation [19] and to their abilities to accumulate Topo I-DNA cleavable complex [12]. These discoveries confirmed that the antitumour effect of camptothecin and its derivatives result from Topo I inhibition.

Malignant tumours of colon [20] and ovary [117] have a higher level of activity of Topo I than benign tumours and normal parts of the same tissues. Ovarian cancer treated with cisplatin and cyclophosphamide possesses the same Topo I activty as the untreated one [117]. Acute leukaemia also highly expresses Topo I [118]. These observations give an advantage to camptothecin and its derivatives, because cancers with higher Topo I activity are regarded as being sensitive to the Topo I inhibitors.

CAMPTOTHECIN AND DRUG RESISTANCE

Drug resistance in cancer is one of the main therapeutic difficulties. The best-known mechanism of drug resistance is multidrug resistance (MDR) overexpressing *MDR1* gene encoding P-glycoprotein, and camptothecin derivatives are little or not at all affected by MDR [119–123]. There is an idea that charged camptothecin derivatives may be more sensitive to *MDR1* than non-charged ones [120, 124]. However, to a MDR cell line, some non-charged camptothecin derivatives as well as topotecan (a charged derivative) are less toxic than to the wild-type cell line [122]. Furthermore, Mitsui *et al.* demonstrated that DX-8951 (64), a charged derivative, overcomes P-glycoprotein overproduction as much as SN-38, a non-charged one [109]. Some MDR cell lines overexpressing *MRP* (multidrug resistance-associated protein) gene showed no or little cross-resistance to camptothecins [125–128]. Camptothecin is also effective on another phenotype of MDR, atypical MDR (at-MDR), in which Topo II activity is depressed [129]. Topo II is inhibited by many kinds of anticancer agents such as amsacrine (*m*-AMSA) [130], doxorubicin [131] and epipodophyllotoxins (etoposide, teniposide) [132], and MDR or at-MDR cells are usually cross-resistant to these agents. Therefore, Topo I inhibitor camptothecin and its derivatives are expected to be active against cancer cells refractory to many other anticancer drugs.

Nowadays, several camptothecin derivatives such as CPT-11 and topotecan are launched or under development. Therefore, investigations of mechanisms of camptothecin resistance in mammalian cells (except for resist-

ant cells established by gene transfection) in comparison with parental cells are important to enhance their clinical efficacies. As mentioned above, it was reported that CPT-K5 cell line derived from RPMI 8402 human lymphoblastic leukaemia cell line was 300 times more resistant to CPT-11 than its parent cell line and Topo I from CPT-K5 was 125-fold less sensitive to camptothecin than that from RPMI 8402 [113]. An amino acid change at residue 533 from Asp to Gly was considered to be predominantly responsible for the difference in camptothecin sensitivity between parent and resistant cell lines [114]. Some other papers reported that Topo I from camptothecins-resistant mammalian cell lines were less sensitive to camptothecins than that from their parent cell lines [133–142]. When the sensitivity of Topo I in mammalian cells to camptothecin or its derivatives were reduced, amino acid mutations were often observed (Tyr^{729} to Ala [143], Gly^{505} to Ser [144], Phe^{361} to Ser [138], Asn^{722} to Ser [141]). Other mechanisms of resistance to camptothecin include the decrease in the amount or activity of Topo I [113, 133–137, 141, 143, 145–153] and the reduction of drug uptake [135, 154]. The suppression of conversion of CPT-11 to SN-38 was sometimes demonstrated in CPT-11-resistant cells [134, 140, 155].

CAMPTOTHECIN DERIVATIVES AND ACETYLCHOLINESTERASE INHIBITION

Early and delayed onset diarrhoea is often induced by CPT-11 [25, 156, 157], and can be a dose-limiting factor for this drug. Delayed onset diarrhoea is not a rare adverse effect when using anticancer agents, but the early onset observed during or immediately after injection is almost specific to CPT-11. It is important to clarify the mechanism(s) of this toxicity in order to obtain a less toxic camptothecin derivative.

It was reported that acetylcholine-like action is observed in pharmacological studies using guinea-pig ileal and tracheal preparations treated with CPT-11 [158]. Gastrointestinal motility [159] and secretion of chloride ion [160–162] are enhanced by cholinergic agents, and may result in diarrhoea [163]. Takasuna *et al.* demonstrated that CPT-11-induced early onset diarrhoea in rats was partially inhibited by atropine, an anticholinergic agent [164]. On the other hand, salivation and abdominal pain [24, 157, 165] were reported in early clinical studies and these symptoms possibly arise from a cholinergic mechanism. Therefore, CPT-11 probably possesses acetylcholine-like activity. In fact, it was shown that CPT-11 and some of camptothecin derivatives inhibit acetylcholinesterase activity with IC_{50} (concentration inducing 50% inhibition) values of 1.6 μM or less [107]. Inhibition of this enzyme causes an increase in acetylcholine level and induces cholinergic action. After a 90 min intravenous infusion of CPT-11 at 50–150 mg/m^2 in hu-

mans, the peak plasma concentration of this drug ranged from 0.6 to 3 μg/ml (from 0.8 to 4 μM) [25]. When patients were infused intravenously with 100 mg/m^2 of CPT-11 for 30 min, the plasma concentration maximally reached 1.87 μg/ml (ca. 2.5 μM) [24] and it was about 0.6 μM and about 0.15 μM at 8 h and 24 h after infusion, respectively. These values are greater than the Ki (inhibition constant) value of 0.2–0.3 μM for CPT-11 against acetylcholinesterase [107]. Therefore, it is likely that acetylcholinesterase inhibition by CPT-11 is one of the mechanisms which causes early onset diarrhoea and other cholinergic responses. Nowadays, as reported by Gandia *et al.* [157], it is known that anticholinergic agents such as atropine and scopolamine at least partially relieve some of the clinical acute side-effects induced by CPT-11, such as early diarrhoea and salivation. In this section, the profile of acetylcholinesterase inhibition by CPT-11 and the structure-activity correlations between camptothecin analogues and their acetylcholinesterase inhibitory effect are described.

In 1990, we reported [166] that CPT-11 inhibits acetylcholinesterase activity. In the paper, the rate of thiocholine (TCh) production from acetylthiocholine iodide (ATChI) by acetylcholinesterase was analyzed with Lineweaver-Burk plots (*Figure 2.3*). This kinetic analysis indicated that CPT-11 noncompetitively inhibited acetylcholinesterase. In this case, the apparent Michaelis constant (Km) and inhibition constant (Ki) ranged from 63 to 68 μM and from 0.221 to 0.300 μM, respectively (*Table 2.3*). The same analysis revealed that the Ki value of physostigmine (a potent acetylcholinesterase

Table 2.3. INHIBITORY EFFECT OF CPT-11, SN-38, AND PHYSOSTIGMINE ON ACETYLCHOLINESTERASE

Agent	*Concentration (μM)*	*Vmax*[a] *(ΔA/min)*	*Km*[b] *(μM)*	*Ki*[c] *(μM)*
CPT-11	0	0.312	67.2	–
	0.1	0.234	62.6	0.300
	0.2	0.177	65.9	0.262
	0.4	0.111	68.3	0.221
SN-38	0	0.309	64.7	–
	100	0.253	65.3	452
	250	0.211	67.2	538
Physostigmine	0	0.325	79.1	–
	0.02	0.224	76.8	0.0444
	0.05	0.143	82.3	0.0393

[a] Maximum velocity of production of TCh from ATChI by acetylcholinesterase.
[b] Michaelis constant.
[c] Inhibition constant calculated from Vmax.

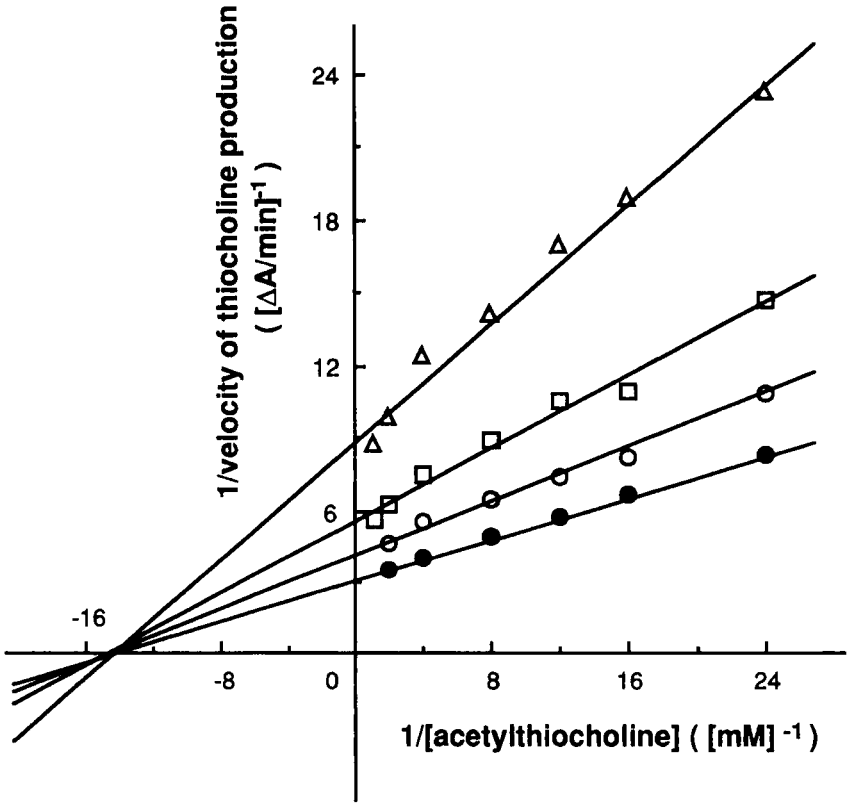
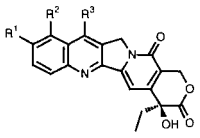


Figure 2.3

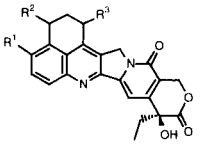
inhibitior) was about 0.04 μM (or 7 times more potent than CPT-11). SN-38, however, gave a Ki value of approximately 500 μM. This result indicates that the urethane group of physostigmine appears to be indispensable for its acetylcholinesterase inhibitory activity [167]; CPT-11 also has an urethane group at position 10 but SN-38 does not. Therefore, it is suggested that this structural difference between CPT-11 and SN-38 might be responsible for their differential effects on acetylcholinesterase. In order to confirm this point, the inhibitory activity against this enzyme of various camptothecin derivatives were examined.

Table 2.4. summarizes the acetylcholinesterase inhibitory activity (%) of each analogue at a concentration of 1 μg/ml. As shown in this table, compound (75) which has no urethane group at position 10 strongly inhibits

Table 2.4. INHIBITORY ACTIVITIES OF CAMPTOTHECIN DERIVATIVES AGAINST ACETYLCHOLINESTERASE



Compound	R^1	R^2	R^3	*AChE inhibition % at 1 μg/ml*[a]
CPT-11		H	Et	91-95
SN-38	OH	H	Et	7
Topotecan	OH	CH_2NMe_2	H	0
(75) (20*RS*)		H	Et	95
(76) (20*RS*)	H	H	CH_2NH_2	0



Compound	R^1	R^2	R^3	*AChE inhibition % at 1 μg/ml*[a]
(67)	Me	H	NH_2	38-48
(68)	OH	H	NH_2	9-17
(72)	Me	NH_2	H	19-25
(73)	OH	NH_2	H	14-24
(74)	H	NH_2	H	29-48
(77)	OMe	NH_2	H	30-34
(78)	H	H	NH_2	24-28
(79)	OMe	H	NH_2	17-24
(80)	Me	H	NHMe	9-15
(81)	Me	H	NMe_2	3-8
(82)	OH	H	H	0
(83)	CH_2NH_2	H	H	79-82
(84)	H_2NHMe	H	H	70
(85)	CH_2NMe_2	H	H	85
(86)	CH_2NMe_3	H	H	86
(87) (9*RS*)	$O(CH_2)_2NMe_2$	H	H	99
(88) (9*RS*)	[b]	H	H	72
(89) (9*RS*)	$O(CH_2)_2NH_2$	H	H	78

[a] Acetylcholinesterase (AChE) activity was determined by spectrophotometry using ATChI as a substrate.

[b] NHC = NH
|
NH_2

acetylcholinesterase activity. All compounds which have an amino substituent at position 10 or at position 4 of hexacyclic analogues exert a relatively strong inhibitory effect on acetylcholinesterase with inhibition values of 50% or greater. However, the other compounds tested inhibit this enzyme by less than 50% at 1 μg/ml, even if they possess an amino substituent at any position other than position 10 or position 4 of hexacyclic series. For ex-

ample, inhibiton of acetylcholinesterase is 0% for topotecan and compound (76).

In hexacyclic derivatives with an amino group at position 1 or 3, a hydroxy group at position 4 tends to reduce inhibitory activity against acetylcholinesterase in comparison with a hydrogen, a methoxy or a methyl group. Among the derivatives which have a methyl group at position 4 and an amino group at position 1, the primary amino compound (67) is the strongest inhibitor, followed by the secondary amino derivative (80) and the tertiary one (81) in order of potency.

CPT-11 and 4-aminomethyl hexacyclic analogue (83) induced defecation within 4 h after intravenous administration to dogs, whereas dogs treated with 4-hydroxy analogue (82) never showed such an adverse effect [107]. These results suggest that the inhibition of acetylcholinesterase by camptothecin analogues which have an amino substituent at position 10 or at position 4 of hexacyclic analogues possibly relates to the early onset of defecation or diarrhoea. With regard to inhibitory activity against Topo I, compound (82) is more potent than CPT-11 or (83). It is demonstrated that there is no correlation between inhibition of acetylcholinesterase and inhibition of Topo I [107]. Therefore, it is expected that we will be able to synthesize more potent tumouricidal derivatives than CPT-11, without the severe gastrointestinal tract side-effects, which appear during and immediately after CPT-11 injection.

MECHANISM AND TREATMENTS OF DELAYED ONSET DIARRHOEA INDUCED BY CPT-11

In early clinical trials in Japan, the dose regimen of CPT-11 was 200 mg/m^2 once every 3 weeks, but later, dose regimens using 100 mg/m^2 once every week and 150 mg/m^2 once every 2 weeks were introduced. These new regimens were expected to reduce the peak plasma concentration of CPT-11 and to decrease the incidence of early onset diarrhoea. Prolongation of CPT-11 infusion time also appears to decrease the incidence of diarrhoea [168]. These changes in dosage resulted in relief of early onset diarrhoea in clinical study in Japan. At the present time, the more critical side-effect is delayed onset diarrhoea. This type of diarrhoea is usually considered to be a typical adverse event associated with some anticancer agents and is hardly avoidable. However, some on-going investigations are attempting to define the CPT-11-specific mechanisms of delayed onset diarrhoea and consequently to reduce this adverse effect.

SN-38, the main active metabolite of CPT-11, has significantly more potent cytocidal activity than CPT-11 [169, 170] and is likely to damage the in-

testinal mucosa. This active metabolite is glucuronized once and excreted in the bile [169, 171, 172], then hydrolysed by intestinal microflora to regenerate SN-38 [171]. Recently, it has been demonstrated that more severe diarrhoea seems to correlate with a higher ratio of SN-38 to glucuronized SN-38, and total CPT-11 [173], and that the AUC of SN-38 correlates with episodes of diarrhoea better than that of CPT-11 [174]. Therefore, SN-38, especially the non-glucuronized compound, is regarded as a dominant cause of delayed onset diarrhoea. Narita *et al.* showed that β-glucuronidase is responsible for this hydrolysis and that this enzyme is inhibited by some natural glucuronides found in Chinese herbal medicines [175]. In a clinical combination study of Hange-shashinto (TJ-14), a Chinese herbal medicine, and CPT-11, nine of 19 evaluable patients developed no or weak symptoms of diarrhoea [176]. Although this approach is highly original, further studies are required in order to clarify the value of such herbal medicines. In rats, it was demonstrated that baicalin, an inhibitor of β-glucuronidase, TJ-14, and TJ-114, another herbal medicine, significantly decreased the delayed onset of diarrhoea symptoms, and the herbal medicines exerted protective and healing effects on intestinal epithelium [177].

Abigerges *et al.* suggested that the combined use of loperamide, a synthetic opioid considered to be an enteric μ opioid receptor agonist, with CPT-11 reduced the severity of diarrhoea and raised the dose intensity of CPT-11 [178, 179]. More recently, the same group reported that acetorphan, an enkephalinase inhibitor, dramatically reduced the incidence of diarrhoea during and after treatment with CPT-11 and also that it seemed to potentiate the anti-diarrhoeal effect of loperamide [180]. Because the modulation of a secretory system(s) is regarded as one of the dominant mechanisms of anti-diarrhoeal action of loperamide and acetorphan, it is supposed that delayed onset diarrhoea partly arises from abnormal secretion in the intestine.

Another study speculates on the attribution of diarrhoea induced by CPT-11 to effects on secretion. In this study, thromboxane synthase inhibitors such as indomethacin and a thromboxane A_2 receptor blocker depressed the short-circuit current caused by CPT-11 [181]. This current depends on the presence of Cl^- ions and is considered to reflect the amount of secretion of these ions. Therefore, it was postulated that CPT-11 induces secretion of Cl^- ions mediated by eicosanoid and this secretion may be related to the occurrence of diarrhoea.

Recently, it was also suggested that CPT-11 induced apoptosis and cell differentiation in the mucosa of mouse ileum and caecum, and thus causing diarrhoea [182]. As shown above, CPT-11 may induce diarrhoea through complex mechanisms and these findings will contribute to the relief of the diarrhoea.

Table 2.5. RECENT RESULTS OF PHASE I STUDIES ON CAMPTOTHECINS

Agents[a)]	*Route*	*Schedule*[b)]	*DLT*[c)]	*MTD or RD*[d)]	*Reference*
Camptothecin	p.o.	qd × 21d q4w	Diarrhoea	8.7 mg/m^2/d	29
Topotecan	i.v.	21d C.I. q4w	Myelosuppression	0.53 mg/m^2/d	189
	i.v.	120h C.I. q3w	ditto	0.87 mg/m^2/w	190
	i.v.	72h C.I. q3w	ditto	1.08 mg/m^2/w	190
	i.v.	24h C.I. q3w	ditto	8.4 mg/m^2/d	191
	p.o.	Single	ditto	14 mg/m^2	192
	p.o.	b.i.d. × 21d q4w	Diarrhoea	0.6 mg/m^2/d	193
GG211	i.v.	qd × 5 q3w	Myelosuppression	1.2 mg/m^2/d	194
	i.v.	72h C.I. q3w	ditto	1.2 mg/m^2/d*	194
9-AC (DMA)	i.v.	72h C.I. q2w	Neutropenia	0.84 mg/m^2/d	195
	i.v.	72h C.I. q3w	ditto	1.1 mg/m^2/d	195

In this table, the results on CPT-11 and on daily × 5 schedule of topotecan are omitted.
a) 9-AC (DMA): Dimethylacetamide formulation of 9-AC
b) qd: every day, qnw: every n weeks, d: days, C.I.: Continuous infusion, b.i.d.: twice a day
c) DLT: Dose limiting toxicity
d) MTD: Maximum tolerated dose, RD: Recommended dose,
*: 2.0 mg/m2/d for minimally pretreated or untreated patients.

CLINICAL SUMMARY

Table 2.5 summarizes the recent results of phase I studies on orally administered camptothecin, orally administered and continuously infused topotecan, GG-211, and a dimethylacetamide (DMA) formulation of 9-AC. Several excellent review articles on phase I studies of CPT-11 [183–185] and of topotecan [183, 185, 186] have been published and the reader is referred to these.

Up to the end of 1995, many results of phase II clinical studies on CPT-11 and topotecan administered intravenously have been presented and summarized [183, 184, 187, 188]. In phase II studies of CPT-11 against solid tumours, the most popular dosage schedules were 100 mg/m^2/day once weekly or 150 mg/m^2/day once every 2 weeks in Japan, 125 mg/m^2/day once weekly in U.S.A., and 350 mg/m^2/day once every 3 weeks in Europe. On the other hand, topotecan was infused for 30 min at doses of 1.25–2.0 (dominantly 1.5) mg/m^2/day once for 5 consecutive days every 3 weeks. Usually, the dose-limiting toxicity of CPT-11 is myelosuppression (mainly neutropenia)

Table 2.6. EFFICACY OF CPT-11 (IRINOTECAN) IN PHASE II STUDIES

Tumour[a]	*Prior chemo*[b]	*CR+MR/pts.*[c]	*Response rate (%)*	*Dosage schedule*[d] (mg/m^2/day)	*Reference*
NSCLC	N	0+4/13	31	200 q3-4w	196
	Y	0+3/22	14	ditto	196
	N	0+23/72	32	100 qw	196a
	Y	0+0/26	0	ditto	197
	N	0+4/11	36	350 q3w	198
SCLC	N	0+4/8	50	100 qw	197
	Y	2+7/27	33	ditto	197
	Y	0+7/15	47	ditto	199
	Y (ref)	0+8/16	50	ditto (pilot study)	200
Cervix	N(19 pts.)+Y	5+8/55 (6/19)	N 32, Y 19	100 qw	201
	N	1+4/34	15	350 q3w	202
	CDDP-ref.	0+0/14	0	125 qw × 4+2w rest	203
Ovary	N	0+1/3	33	150 q2w	201
	Y	0+12/52	23	ditto	201
Stomach	N	0+5/15	33	100 qw, 150 q2w	204
	Y	0+9/45	20	ditto	204
Colorectal	N	0+4/12	33	100 qw, 150 q2w	205
	Y	0+13/51	25	ditto	205
	Recurrent	0+4/10	40	100 qw	206
	N(48 pts.)+Y	4+28/178	N 19, Y 18	350 q3w	207
	N	0+6/19	32	125 qw × 4+2w rest	208
	N	(2/13)	15	ditto	209
	Y	(5/21)	24	ditto	209
	5-FU-ref.	1+10/44	25	125 or 150 qw × 4+2w rest	210
Pancreas	N	0+4/21	19	100 qw, 150 q2w	211
	Y	0+0/14	0	ditto	211
	N	0+3/32	9	350 q3w	212
Breast	N	0+1/4	25	100 qw	213
	Y	1+13/61	23	ditto	213
	Y	1+0/12	8	350 q3w	214
SCC(skin)	N+Y	2+11/33	39	100 qw	215
MM	N+Y	0+3/32	9	100 qw	215
Renal cell	N+Y	0+0/23	0	100 qw × 3-4+2w rest 150 q2w × 2-3+3w rest	Unpublished
NHL	Y	9+17/62	42	(40 × 3d) qw	216
HL	Y	0+0/4	0	ditto	216

Table 2.6. (CONTINUED)

Tumour[a]	*Prior chemo*[b]	*CR+MR/pts.*[c]	*Response rate (%)*	*Dosage schedule*[d] (mg/m^2/day)	*Reference*
ATL	Y	1+4/13	39	ditto	217
ALL	Y	0+2/17	12	(15 or 20 × 2/d × 7d) q2-4w	216
AML	Y	0+0/24	0	(15 or 20 × 2/d × 7d) q2-4w	216

Most of the results of early phase II studies in Japan are omitted.
[a] NSCLC: Non-small cell lung cancer, SCLC: Small cell lung cancer, SCC: Squamous cell carcinoma, MM: Malignant melanoma, NHL: Non-Hodgkin's lymphoma, HL: Hodgkin's lymphoma, ATL: Adult T-cell leukaemia-lymphoma, ALL: Acute lymphocytic leukaemia, AML: Acute myelogenous leukaemia
[b] Y: treated with prior chemotherapy, N: untreated with prior chemotherapy, ref: refractory
[c] CR: Complete response, PR: Partial response, pts.: No. of evaluable patients
[d] qw: every week, qnw: every n weeks, d: day(s)

Table 2.7. EFFICACY OF TOPOTECAN IN PHASE II STUDIES

Tumour[a]	*Prior chemo*[b]	*CR+MR/pts.*[c]	*Response rate (%)*	*Dosage schedule*[d] (mg/m^2/day)	*Reference*
NSCLC	N	0+0/20	0	(2 × 5d) q3w	218
	N	0+5/38	13	(1.5 × 5d) q3w	219
	N	0+2/37	5	(1.3 × 3d C.I.) q4w	219
	N	0+5/37	14	(1.5 × 5d) q3w	220
Ad	N	0+1/17	6	ditto	220
SCC	N	0+4/11	36	ditto	220
SCLC	N	0+7/18	39	(2 × 5d) q3w	221
	Y	2+5/20	35	(1.5 × 5d) q3w	222
	Y	5+7/57	21	ditto	223
	Etoposide-ref.	0+3/25	12	(1.25 × 5d) q3w	224
	N	(18/47)	38	(1.5 × 5d) q3w	*
	Y (sensitive)	5+12/43	40	ditto	*
	Y (resistant)	1+2/43	7	ditto	*
Ovary	CDDP/CBDCA-ref.	0+4/28	14	ditto	225
	Y	1+3/16	25	ditto	226
	Y	1+14/77	19	ditto	*
	Y	1+5/24	25	ditto	*
	Y	5+24/65	45	ditto	*

Table 2.7. (CONTINUED)

Tumour[a]	*Prior chemo*[b]	*CR+MR/pts.*[c]	*Response rate (%)*	*Dosage schedule*[d] (mg/m^2/day)	*Reference*
H & N	N	0+4/15	27	ditto	227
Colorectal	Y	0+0/19	0	ditto	228
	N	0+4/57	7	ditto	228a
	N	1+0/16	6	(0.6 × 21d C.I.) q4w	229
Pancreas	N	0+0/15	0	(1.5 × 5d) q3w	230
	N	0+4/34	12	ditto	*
Breast	N+Y	0+5/14	36	ditto	231
MM	N	1+0/17	6	ditto	232
Renal cell	N	0+0/14	0	(1.5 × 5d) q4w	233
Prostate	Hormone-ref.	0+2/28	7	(1.5 × 5d) q3w	234
MG	N(3pts.)+Y(9pts.)	1+1/12	17	ditto	235
STS	N	0+2/16	13	ditto	235
PM	N	0+0/22	0	ditto	236
CLL	Y	0+0/12	0	(2 × 5d) qm	237

[a] NSCLC: Non-small cell lung cancer, Ad: Adenocarcinoma, SCC: Squamous cell carcinoma, SCLC: Small cell lung cance, H & N: Head and neck, MM: Malignant melanoma, MG: Malignant glioma, STS: Soft tissue sarcoma, PM: Pleural mesothelioma, CLL: Chronic lymphocytic leukaemia

[b] Y: treated with prior chemotherapy, N: no prior chemotherapy, ref: refractory

[c] CR: Complete response, PR: Partial response, pts.: No. of evaluable patients

[d] qnw: every n weeks, qm: every month, d: days, C.I.: Continuous infusion

* Data presented by Verweij, J. (1995) [6th Conference on DNA Topoisomerases in Therapy]

and/or diarrhoea, and that of topotecan is myelosuppression (neutropenia and thrombocytopenia). Their efficacies in phase II studies are listed in *Table 2.6* and *Table 2.7*. Also now in progress are phase II studies of GG-211 given daily for 5 consecutive days every 3 weeks, phase I study of this agent administered orally, and some combination studies of these camptothecin derivatives with conventional anticancer drugs such as cisplatin, etoposide, and 5-FU.

CONCLUSION

In 1995, irinotecan (CPT-11) was approved in Japan for the treatment of colorectal, stomach and breast cancer, squamous cell carcinoma and non Hodgkin's lymphoma, in addition to first approval for the treatment of lung, cervical, and ovarian cancer. This reveals that camptothecin essentially has broad spectrum antitumour activity and that Topo I is the attractive target for cancer therapy. Moreover, the search for other uses of Topo I inhibitors possibly as an anti-retroviral [238] or an anti-trypanosomal agents [239, 240] is under investigation. Many efforts devoted to the research on camptothecins and Topo I have clarified the structure-activity relationships and mechanistic differences of camptothecin analogues, i.e. DNA sequence selectivity for cleavable complex formation, stability of cleavable complex [98, 99], and the stability of lactone ring E with respect to the binding to human serum albumin [241–243]. It is expected that determination of the three-dimensional structure of drug-DNA-Topo I ternary complex will offer more effective methodology for the design of novel Topo I inhibitors.

REFERENCES

1 Wall, M.E., Wani, M.C., Cook, C.E., Palmer, K.H., McPhail, A.T. and Sim, G.A. (1966) J. Am. Chem. Soc. 88, 3888–3890.
2 Perdue, R.E., Jr., Smith, R.L., Wall, M.E., Hartwell, J.L. and Abott, B.J. (1970) U.S. Dep. Agri. Tech. Bull. 1415, 1–26.
3 Kessel, D. (1971) Biochem. Biophys. Acta 246, 225–232.
4 Hacker, B., Keude, A.S. and Hall, T.S. (1971) Fed. Proc. 30, 2409.
5 Kahn, H.E., Jr., Snyder, A.L. and Kohn, K.W. (1971) Proc. Am. Assoc. Cancer Res. 12, 59.
6 Gottlieb, J.A., Guarino, A.M., Call, J.B., Oliverio, V.T. and Block, J.B. (1970) Cancer Chemother. Rep. 54, 461–479.
7 Moertel, C.G., Schutt, A.J., Reitemeier, R.J. and Hahn, R.G. (1972) Cancer Chemother. Rep. 56, 95–101.
8 Gottlieb, J.A. and Luce, J.K. (1972) Cancer Chemother. Rep. 56, 103–105.
9 Muggia, F.M., Creven, P.J., Hansen, H.H., Cohen, M.H. and Selawry, O.S. (1972) Cancer Chemother. Rep. 56, 515–521.
10 Hsiang, Y.-H., Hertzberg, R.P., Hecht, S. and Liu, L.F. (1985) J. Biol. Chem. 260, 14873–14878.
11 Hsiang, Y.-H. and Liu, L.F. (1988) Cancer Res. 48, 1722–1726.
12 Hsiang, Y.-H., Liu, L.F., Wall, M.E., Wani, M.C., Nicholas, A.W., Manikumar, G., Kirschenbaum, S., Silber, R. and Potmesil, M. (1989) Cancer Res. 49, 4385–4389.
13 Hsiang, Y.-H., Lihou, M.G. and Liu, L.F. (1989) Cancer Res. 49, 5077–5082.
14 Wani, M.C., Ronman, P.E., Lindley, J.T. and Wall, M.E. (1980) J. Med. Chem. 23, 554–560.
15 Wall, M.E., Wani, M.C., Natschke, S.M. and Nicholas, A.W. (1986) J. Med. Chem. 29, 1553–1555.

16 Wani, M.C., Nicholas, A.W., and Wall, M.E. (1986) J. Med. Chem. 29, 2358–2363.
17 Wani, M.C., Nicholas, A.W., Manikumar, G. and Wall, M.E. (1987) J. Med. Chem. 30, 1774–1779.
18 Hertzberg, R.P., Caranfa, M.J., Holden, K.G., Jakas, D.R., Gallagher, G., Mattern, M.R., Mong, S.-M., Bartus, J.O., Johnson, R.K. and Kingsbury, W.D. (1989) J. Med. Chem. 32, 715–720.
19 Jaxel, C., Kohn, K.W., Wani, M.C., Wall, M.E. and Pommier, Y. (1989) Cancer Res. 49, 1465–1469.
20 Giovanella, B.C., Stehlin, J.S., Wall, M.E., Wani, M.C., Nicholas, A.W., Liu, L.F., Silber, R. and Potmesil, M. (1989) Science (Washington D.C.) 246, 1046–1048.
21 Kunimoto, T., Nitta, K., Tanaka, T., Uehara, N., Baba, H., Takeuchi, M., Yokokura, T., Sawada, S., Miyasaka, T. and Mutai, M. (1987) Cancer Res. 47, 5944–5947.
22 Sawada, S., Okajima, S., Aiyama, R., Nokata, K., Furuta, T., Yokokura, T., Sugino, E., Yamanouchi, K. and Miyasaka, T. (1991) Chem. Pharm. Bull. 39, 1446–1454.
23 Kingsbury, W.D., Boehm, J.C., Jakas, D.R., Holden, K.G., Hecht, S.M., Gallagher, G., Caranfa, M.J., McCabe, F.L., Faucette, L.F., Johnson, R.K. and Hertzberg, R.P. (1991) J. Med. Chem. 34, 98–107.
24 Taguchi, T., Wakui, A., Hasegawa, K., Niitani, H., Furue, H., Ohta, K. and Hattori, T. (1990) Jpn. J. Cancer Chemother. 17, 115–120.
25 Negoro, S., Fukuoka, M., Masuda, N., Takada, M., Kusunoki, Y., Matsui, K., Takifuji, N., Kudoh, S., Niitani, H. and Taguchi, T. (1991) J. Natl. Cancer Inst. 83, 1164–1168.
26 Ettinger, D., McGuire, W., Forastiere, A., Hurowitz, L., Easter, V. and Donehower, R. (1991) Proc. Am. Soc. Clin. Oncol. 10, 93.
27 Rowinsky, E.K., Grochow, L.B., Hendricks, C.B., Ettinger, D.S., Forastiere, A., Hurowitz, L.A., McGuire, W.P., Sartorius, S.E., Lubejko, B.G., Kaufmann, S.H. and Donehower, R.C. (1992) J. Clin. Oncol. 10, 647–656.
28 Rubin, E., Wood, V., Bharti, A., Trites, D., Lynch, C. and Kufe, D. (1994) Proc. Am. Assoc. Cancer Res., 35, 245.
29 Stehlin, J.S., Natelson, E.A., Hinz, H.R., Giovanella, B.C., de Ipolyi, P.D., Fehir, K.M., Trezona, T.P., Vardeman, D.M., Harris, N.J., Marcee, A.K., Kozielski, A.J. and Ruiz-Razura, A. (1995) in Camptothecins: New Anticancer Agents (Potmesil, M. and Pinedo, H., eds.), pp. 59–65, CRC Press, Boca Raton.
30 Luzzio, M.J., Besterman, J.M., Evans, M.G., Lackey, K., Leitner, P.P., Peel, M., Sisco, M., Sternbach, D.D., Tong, T. and Uehling, D.E. (1993) Proc. Am. Assoc. Cancer Res., 34, 332.
31 Slichenmyer, W.J., Rowinsky, E.K., Donehower, R.C. and Kaufmann, S.H. (1993) J. Natl. Cancer Inst. 85, 271–291.
32 Chen, A.Y. and Liu, L.F. (1994) Annu. Rev. Pharmacol. Toxicol. 34, 191–218.
33 Muggia, F.M. and Burris, H.A. (1994) Adv. Pharmacol. 29B, 1–31.
34 Costin, D. and Potmesil, M. (1994) Adv. Pharmacol. 29B, 51–72.
35 Pommier, Y., Tanizawa, A. and Kohn, K.W. (1994) Adv. Pharmacol. 29B, 73–92.
36 Potmesil, M. and Pinedo, H. (1995) in Ref. 29, pp. 1–142
37 Wall, M.E. and Wani, M.C. (1995) Cancer Res. 55, 753–760.
38 Rivory, L.P. and Robert, J. (1995) Bull. Cancer 82, 265–285.
39 Carte, B.K., DeBrosse, C., Eggleston, D., Hemling, M., Mentzner, M., Poehland, B., Troupe, N. and Westley, J.W. (1990) Tetrahedron 46, 2747–2760.
40 Govindachari, T.R. and Viswanathan, N. (1972) Phytochemistry 11, 3529–3531.
41 Roja, G. and Heble, M.R. (1994) Phytochemistry 36, 65–66.
42 Wu, T.S., Leu, Y.L., Hsu, H.C., Ou, L.F., Chen, C.C., Chen, C.F., Ou, J.C. and Wu, Y.C. (1995) Phytochemistry 39, 383–385.

43 Pirillo, A., Verotta, L., Gariboldi, P., Torregiani, E. and Bombardelli, E. (1995) J. Chem. Soc., Perkin Trans. 1 583–587.
44 Tafur, S., Nelson, J.D., Delong, D.C. and Svoboda, G.H. (1976) Lloydia 39, 261–262.
45 Gunasekera, S.P., Badawi, M.M., Cordell, G.A., Farnsworth, N.R. and Chitnis, M. (1979) J. Nat. Prod. 42, 475–477.
46 Arisawa, M., Gunasekera, S.P., Cordell, G.A. and Farnsworth, N.R. (1981) Planta Med. 43, 404–415.
47 Aimi, N., Nishimura, M., Hoshino, H., Sakai, S. and Haginiwa, J. (1989) Tetrahedron Lett. 30, 4991–4994.
48 Aimi, N., Hoshino, H., Nishimura, M., Sakai, S. and Haginiwa, J. (1990) Tetrahedron Lett. 31, 5169–5172.
49 Hutchinson, C.R. (1981) Tetrahedron 37, 1047–1065.
50 Schultz, A.G. (1973) Chem. Rev., 73, 385–405.
51 Shamma, M. and Georgiev, V. St. (1974) J. Pharm. Sci. 63, 163–183.
52 Cai, J.C. and Hutchinson, C.R. (1983) in The Alkaloids: Chemistry and Pharmacology (Brossi, A., ed.), Vol. 21, p. 101, Academic Press, New York.
53 Cai, J.C. and Hutchinson, C.R. (1983) Chem. Heterocycl. Compd. 25, 753–781.
54 Earl, R.E. and Vollhardt, K.P.C. (1984) J. Org. Chem. 49, 4786–4800.
55 Ihara, M., Noguchi, K., Ohsawa, T., Fukumoto, K. and Kametani, T. (1983) J. Org. Chem. 48, 3150–3156.
56 Stork, G. and Schultz, A.G. (1971) J. Am. Chem. Soc. 93, 4074–4075.
57 Curran, D.P. and Liu, H. (1992) J. Am. Chem. Soc. 114, 5863–5864.
58 Curran, D.P. and Liu, H. (1991) J. Am. Chem. Soc. 113, 2127–2132.
59 Curran, D.P., Sisko, J., Yeske, P.E. and Liu, H. (1993) Pure Appl. Chem., 65, 1153–1159.
60 Volkmann, R., Danishefsky, S., Eggler, J. and Soloman, D.M. (1971) J. Am. Chem. Soc. 93, 5576–5577.
61 Shen, W., Coburn, C.A., Bornmann, W.G. and Danishefsky, S.J. (1993) J. Org. Chem. 58, 611–617.
62 Danishefsky, S.J., Etheredge, S.J., Volkmann, R., Eggler, J. and Quick, J. (1971) J. Am. Chem. Soc. 93, 5575–5576.
63 Danishefsky, S.J., Volkmann, R. and Horwitz, S.B. (1973) Tetrahedron Lett. 2521–2524.
64 Danishefsky, S.J. and Etheredge, S.J. (1974) J. Org. Chem. 39, 3430–3432.
65 Rama Rao, A.V., Yadav, J.S. and Muralikrishna, V. (1994) Tetrahedron Lett. 21, 3613–3616.
66 Corey, E., Crouse, D.N. and Anderson, J.E. (1975) J. Org. Chem. 40, 2140–2141.
67 Ejima, A., Terasawa, H., Sugimori, M. and Tagawa, H. (1990) J. Chem. Soc., Perkin Trans. 1 27–31.
68 Wani, M.C., Nicholas, A.W. and Wall, M.E. (1987) J. Med. Chem. 30, 2317–2319.
69 Terasawa, H., Sugimori, M., Ejima, A. and Tagawa, H. (1987) J. Pharm. Sci. 76, S228, M 03-Y-10; U. S. Patent (1988) 4778891.
70 Ejima, A., Terasawa, H., Sugimori, M. and Tagawa, H. (1987) J. Pharm. Sci. 76, S228, M 03-Y-09.
71 Comins, D.L., Baevsky, M.F. and Hong, H. (1992) J. Am. Chem. Soc. 114, 10971–10972.
72 Comins, D.L., Hong H. and Jianhua, G. (1994) Tetrahedron Lett. 35, 5331–5334.
73 Comins, D.L., Hong H., Saha, J.K. and Jianhua, G. (1994) J. Org. Chem. 59, 5120–5121.
74 Fang, F.G., Xie, S. and Lowery, M.W. (1994) J. Org. Chem. 59, 6142–6143.
75 Grieco, P.A., Nishizawa, M., Marinovic, N. and Ehrmann, W.J. (1976) J. Am. Chem. Soc. 98, 7102–7104.
76 Crispino, G.A., Jeong, K.-S., Kolb, H.C., Wang, Z.-M., Xu, D. and Sharpless, K.B. (1993) J. Org. Chem. 58, 3785–3786.

77 Jew, S., Ok, K., Kim, H., Kim, M.G., Kim, J.M., Hah, J.M. and Cho, Y. (1995) Tetrahedron: Asymmetry 6, 1245–1248.
78 Horwitz, S.B. (1975) in Antibiotics III. Mechanism of Action of Antimicrobial and Antitumor Agents (Corcoran, J.W. and Hahn, F.E., eds.), p. 48, Springer Verlag, New York.
79 Wall, M.E. (1969) in Fourth International Symposium on the Biochemistry and Physiology of Alkaloids (Mothes, K., Schreiber, K. and Schutte, H. R., eds.), pp. 77–87, Akad. Verlag, Berlin.
80 Suffness, M. and Cordell, G.A. (1985) in The Alkaloids: Chemistry and Pharmacology (Brossi, A., ed.), Vol. 25, p. 73, Academic Press, Orland, FL.
81 Kuwahara, J., Suzuki, T., Funakoshi, K. and Sugiura, Y. (1986) Biochemistry 25, 1216–1221.
82 Hertzberg, R.P., Caranfa, M.J. and Hecht, S.M. (1989) Biochemistry 28, 4629–4638.
83 Fukada, M. (1985) Biochem. Pharmacol. 34, 1225–1230.
84 Nicholas, A.W., Wani, M.C., Manikumar, G., Wall, M.E., Kohn, K.W. and Pommier, Y. (1990) J. Med. Chem., 33, 972–978.
85 Mattern, M.R., Mong, S.M., Bartus, H.F., Mirabelli, C.K., Crooke, S.T. and Johnson, R.K. (1987) Cancer Res. 47, 1793–1798.
86 Wall, M.E., Wani, M.C., Nicholas, A.W., Manikumar, G., Tele, C., Moore, L., Truesdale, A., Leitner, P. and Besterman, J.M. (1993) J. Med. Chem. 36, 2689–2700.
87 Luzzio, M.J., Besterman, J.M., Emerson, D.L., Evans, M.G., Lackey, K., Leitner, P.L., McIntyre, G., Morton, B., Myers, P.L., Peel, M., Sisco, J.M., Sternbach, D.D., Tong, W.-Q., Truesdale, A., Uehling, D.E., Vuong, A. and Yates, J. (1995) J. Med. Chem. 38, 395–401.
88 Wang, H.-K., Liu, S. -Y., Hwang, K. -M., McPhail, A. T. and Lee, K. -H. (1995) Bioorg. Med. Chem. Lett. 5, 77–82.
89 Miyasaka, T., Sawada, S. and Nokata, K. (1981) Heterocycles 16, 1713–1717.
90 Sugimori, M., Ejima, A., Ohsuki, S., Matsumoto, K., Kawato, Y., Yasuoka, M., Tagawa, H. and Terasawa, H. (1994) Heterocycles 38, 81–94.
91 Yaegashi, T., Sawada, S., Furuta, T., Yokokura, T., Yamaguchi, K. and Miyasaka, T. (1993) Chem. Pharm. Bull. 41, 971–974.
92 Ejima, A., Terasawa, H., Sugimori, M., Ohsuki, S., Matsumoto, K., Kawato, Y., Yasuoka, M. and Tagawa, H. (1992) Chem. Pharm. Bull. 40, 683–688.
93 Snyder, L., Shen, W., Bornmann, W.G. and Danishefsky, S.J. (1994) J. Org. Chem. 59, 7033–7037.
94 Crow, R.T. and Crothers, D.M. (1992) J. Med. Chem., 35, 4160–4164.
95 Yaegashi, T., Sawada, S., Nagata, H., Furuta, T., Yokokura, T. and Miyasaka, T. (1994) Chem. Pharm. Bull. 42, 2518–2525.
96 Wang, H.-K., Liu, S.-Y., Hwang, K.-M., Taylor, G. and Lee, K.-H. (1994) Bioorg. Med. Chem. Lett. 4, 579–582.
97 Xie, Z.-F., Ootsu, K. and Akimoto, H. (1995) Bioorg. Med. Chem. Lett. 5, 2189–2194.
98 Tanizawa, A., Fujimori, A., Fujimori, Y. and Pommier, Y. (1994) J. Natl. Cancer Inst. 86, 836–842.
99 Tanizawa, A., Kohn, K.W., Kohlhagen, G., Leteurtre, F. and Pommier, Y. (1995) Biochemistry 34, 7200–7206.
100 Wall, M.E. and Wani, M.C. (1991) in DNA Topoisomerases in Cancer (Potmesil, M. and Kohn, K.W., eds.), pp. 93–102, Oxford University Press, New York.
101 Sugimori, M., Ejima, A., Ohsuki, S., Uoto, K., Mitsui, I., Matsumoto, K., Kawato, Y., Yasuoka, M., Sato, K., Tagawa, H. and Terasawa, H. (1994) J. Med. Chem. 37, 3033–3039.

102 Peel, M.R., Milstead, M.W., Sternbach, D.D., Besterman, J.M., Leitner, P., Morton, B., Wall, M.E. and Wani, M.C. (1995) Bioorg. Med. Chem. Lett. 5, 2129–2132.
103 Lackey, K., Besterman, J.M., Fletcher, W., Leitner, P., Morton, B. and Sternbach, D.D. (1995) J. Med. Chem. 38, 906–911.
104 Uehling, D.E., Nanthakumar, S.S., Croom, D., Emerson, D.L., Leitner, P.P., Luzzio, M.J., McIntyre, G., Morton, B., Proteta, S., Sisco, J., Sternbach, D.D., Tong, W.-Q., Vuong, A., and Besterman, J. M. (1995) J. Med. Cem., 38, 1106–1118.
105 Kurihara, T., Tanno, H., Takemura, S., Harusawa, S. and Yoneda, R. (1993) J. Heterocycl. Chem. 30, 643–652.
106 Ohsuki, S., Ejima, A., Sugimori, M., Sato, K., Mitsui, I., Hirota, Y., Uoto, K., Takenoshita, H., Tagawa, H. and Terasawa, H. (1993) Proc. 2nd. Internat. Symp. Bioorg. Chem. June 6–10, Fukuoka, 60.
107 Kawato, Y., Sekiguchi, M., Akahane, K., Tsutomi, Y., Hirota, Y., Kuga, H., Suzuki, W., Hakusui, H. and Sato, K. (1993) J. Pharm. Pharmacol. 45, 444–448.
108 Emerson, D.L., Besterman, J.M., Brown, H.R., Evans, M.G., Leitner, P.P., Luzzio, M.J., Shaffer, J. E., Sternbach, D.D., Uehling, D. and Vuong, A. (1995) Cancer Res. 55, 603–609.
109 Mitsui, I., Kumazawa, E., Hirota, Y., Aonuma, M., Sugimori, M., Ohsuki, S., Uoto, K., Ejima, A., Terasawa, H. and Sato, K. (1995) Jpn. J. Cancer Res. 86, 776–782.
110 Terasawa, H., Ejima, A., Sugimori, M., Ohsuki, S., Kawato, Y., Aonuma, M., Sato, K., Mitsui, I., Hirota, Y., Uoto, K. and Tagawa, H. (1992) Proc. 12th Internat. Symp. Med. Chem. September 13–17, Basel, 370.
111 D'Arpa, P. and Liu, L.F. (1989) Biochim. Biophys. Acta 989, 163–177.
112 Covey, J.M., Jaxel, C., Kohn, K.W. and Pommier, Y. (1989) Cancer Res. 49, 5016–5022.
113 Andoh, T., Ishii, K., Suzuki, Y., Ikegami, Y., Kusunoki, Y., Takemoto, Y. and Okada, K. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 5565–5569.
114 Tamura, H., Kohchi, C., Yamada, R., Ikeda, T., Koiwai, O., Patterson, E., Keene, J.D., Okada, K., Kjeldsen, E., Nishikawa, K. and Andoh, T. (1991) Nucleic Acids Res. 19, 69–75.
115 Nitiss, J. and Wang, J.C. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 7501–7505.
116 Eng, W.-K., Faucette, L., Johnson, R.K. and Sternglanz, R. (1988) Mol. Pharmacol. 34, 755–760.
117 van der Zee, A.G.J., Hollema, H., de Jong, S., Boonstra, H., Gouw, A., Willemse, P.H.B., Zijlstra, J.G. and de Vries, E.G.E. (1991) Cancer Res. 51, 5915–5920.
118 Gekeler, V., Frese, G., Noller, A., Handgretinger, R., Wilisch, A., Schmidt, H., Mulleer, C.P., Dopfer, R., Klingebiel, T., Diddens, H., Probst, H. and Niethammer, D. (1992) Br. J. Cancer 66, 507–517.
119 Tsuruo, T., Matsuzaki, T., Matsushita, M., Saito, H. and Yokokura, T. (1988) Cancer Chemother. Pharmacol. 21, 71–74.
120 Chen, A.Y., Yu, C., Potmesil, M., Wall, M.E., Wani, M.C. and Liu, L.F. (1991) Cancer Res. 51, 6039–6044.
121 Hendricks, C.B., Rowinsky, E.K., Grochow, L.B., Donehower, R.C. and Kaufmann, S.H. (1992) Cancer Res. 52, 2268–2278.
122 Mattern, M.R., Hofmann, G.A., Polsky, R.M., Funk, L.R., McCabe, F.L. and Johnson, R.K. (1993) Oncol. Res. 5, 467–474.
123 Jensen, P.B., Christensen, I.J., Shehested, M., Hansen, H.H. and Vindelov, L. (1993) Br. J. Cancer 67, 311–320.
124 Chen, A.Y. and Liu, L.F. (1994) Cancer Treat. Res. 73, 263–281.
125 Schneider, E., Horton, J.K., Yang, C.H., Nakagawa, M. and Cowan, K.H. (1994) Cancer Res. 54, 152–158.

126 van der Graaf, W.T.A., Mulder, N.H., Meijer, C. and de Vries, E.G.E. (1995) Cancer Chemother. Pharmacol. 35, 345–348.
127 Hasegawa, S., Abe, T., Naito, S., Kotoh, S., Kumazawa, J., Hipfner, D.R., Deely, R.G., Cole, S.P.C. and Kuwano, M. (1995) Br. J. Cancer 71, 907–913.
128 Binaschi, M., Supino, R., Gambetta, R.A., Giaccone, G., Prosperi, E., Capranico, G., Cataldo, I. and Zunino, F. (1995) Int. J. Cancer 62, 84–89.
129 Ganapathi, R., Grabowski, D., Ford, J., Heiss, C., Kerrigan, D. and Pommier, Y. (1989) Cancer Commun. 1, 217–224.
130 Nelson, E.M., Tewey, K.M. and Liu, L.F. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 1361–1365.
131 Tewey, K.M., Rowe, T.C., Yang, L., Halligan, B.D. and Liu, L.F. (1984) Science (Washington, D.C.) 226, 466–468.
132 Ross, W., Rowe, T., Glisson, B., Yalowich, J. and Liu, L. (1984) Cancer Res. 44, 5857–5860.
133 Gupta, R.S., Gupta, R., Eng. B., Lock, R.B., Ross, W.E., Hertzberg, R.P., Caranfa, M.J. and Johnson, R.K. (1988) Cancer Res. 48. 6404–6410.
134 Kanzawa, F., Sugimoto, Y., Minato, K., Kasahara, K., Bungo, M., Nakagawa, K., Fujiwara, Y., Liu, L.F. and Saijo, N. (1990) Cancer Res. 50, 5919–5924.
135 Chang, J.-Y., Dethlefsen, L.A., Barley, L.R., Zhou, B.-S. and Cheng, Y.-C. (1992) Biochem. Pharmacol. 43, 2443–2452.
136 Tanizawa, A. and Pommier, T. (1992) Cancer Res. 52, 1848–1854.
137 Madelaine, I., Prost, S., Naudin, A., Riou, G., Lavelle, F. and Riou, J. F. (1993) Biochem. Pharmacol. 45, 339–348.
138 Rubin, E., Pantazis, P., Bharti, A., Toppmeyer, D., Giovanella, B. and Kufe, D. (1994) J. Biol. Chem. 269, 2433–2439.
139 Prost, S. and Riou, G. (1994) Biochem. Pharmacol. 48, 975–984.
140 Saijo, N., Nishio, K., Kubota, N., Kanzawa, F., Shinkai, T., Karato, A., Sasaki, Y., Eguchi, K., Tamura, T., Ohe, Y., Oshita, F. and Nishio, M. (1994) Cancer Chemother. Pharmacol. 34, S112-S117.
141 Fujimori, A., Harker, W.G., Kohlhagen, G., Hoki, Y. and Pommier, Y. (1995) Cancer Res. 55, 1339–1346.
142 Matsumoto, Y., Fujiwara, T. and Nagao, S. (1995) J. Neurooncol. 23, 1–8.
143 Kubota, N., Kanzawa, F., Nishio, K., Takeda, Y., Ohmori, T., Fujiwara, Y., Terashima, Y. and Saijo, N. (1992) Biochem. Biophys. Res. Commun. 188, 571–577.
144 Tanizawa, A., Bertrand, R., Kohlhagen, G., Tabuchi, A., Jenkins, J. and Pommier, Y. (1993) J. Biol. Chem. 268, 25463–25468.
145 Tan, K.B., Mattern, M.R., Eng, W.-K., McCabe, F.L. and Johnson, R.K. (1989) J. Natl. Cancer Inst. 81, 1732–1735.
146 Eng, W.-K., McCabe, F.L., Tan, K.B., Mattern, M.R., Hofmann, G.A., Woessner, R.D., Hertzberg, R.P. and Johnson, R.K. (1990) Mol. Pharmacol. 38, 471–480.
147 Sugimoto, Y., Tsukahara, S., Oh-hara, T., Isoe, T. and Tsuruo, T. (1990) Cancer Res. 50, 6925–6930.
148 Woessner, R.D., Eng, W.-K., Hofmann, G.A., Rieman, D.J., McCabe, F.L., Hertzberg, R.P., Mattern, M.R., Tan, K.B. and Johnson, R.K. (1992) Oncol. Res. 4, 481–488.
149 Kijima, T., Kubota, N. and Nishio, K. (1994) Anticancer Res. 14, 799–803.
150 Pantazis, P., Mendoza, J.T., DeJesus, A., Rubin, E., Kufe, D. and Giovanella, B.C. (1994) Eur. J. Haematol. 53, 135–144.
151 Nagai, S., Yamauchi, M., Andoh, T., Nishizawa, M., Satta, T., Kodera, Y., Kondou, K., Akiyama, S., Ito, K. and Takagi, H. (1995) J. Surg. Oncol. 59, 116–124.
152 Sorensen, M., Sehested, M. and Jensen, R.B. (1995) Br. J. Cancer 72, 399–404.

153 Kapoor, R., Slade, D.L., Fujimori, A., Pommier, Y. and Harker, W.G. (1995) Oncol. Res. 7, 83–95.
154 Yang, C.J., Horton, J.K., Cowan, K.H. and Schneider, E. (1995) Cancer Res. 55, 4004–4009.
155 Niimi, S., Nakagawa, K., Sugimoto, Y., Nishio, K., Fujiwara, Y., Yokoyama, S., Terashima, Y. and Saijo, N. (1992) Cancer Res. 52, 328–333.
156 Ohno, R., Okada, K., Masaoka, T., Kuramoto, A., Arima, T., Yoshida, Y., Ariyoshi, H., Ichimaru, M., Sakai, Y., Oguro, M., Ito, Y., Morishima, Y., Yokomaru, S. and Ota, K. (1990) J. Clin. Oncol. 8, 1907–1912.
157 Gandia, D., Abigerges, D., Armand, J.-P., Chabot, G., da Costa, L., de Forni, M., Mathieu-Boue, A. and Herait, P. (1993) J. Clin. Oncol. 11, 196–197.
158 Takayanagi, I., Koike, K., Tagawa, M. and Mitsuhashi, E. (1989) Gen. Pharmacol. 20, 763–766.
159 Gerring, E.L. (1989) Vet. Clin. North Am. Equine Pract. 5, 283–294.
160 Isaacs, P.E.T., Corbett, C.L., Riley, A.K., Hawker, P.C. and Turnberg, L.A. (1976) J. Clin. Invest. 58, 535–542.
161 Browning, J.G., Hardcastle, J., Hardcastle, P.T. and Sanford, P.A. (1977) J. Physiol. 272, 737–754.
162 Donowitz, M. (1983) Am. J. Physiol. 245, G165-G177.
163 Ooms, L. and Degryse, A. (1986) Vet. Res. Commun. 10, 355–397.
164 Takasuna, K., Kasai, Y., Kitano, Y., Mori, K., Kakihata, K., Hirosaki, M. and Nomura, M. (1995) Folia Pharmacol. Jpn. 105, 447–460.
165 Fukuoka, M., Negoro, S., Niitani, H., Furue, H., Hasegawa, K., Hara, Y., Hara, N. and Taguchi, T. (1990) Jpn. J. Cancer Chemother. 17, 993–997.
166 Kawato, Y., Tsutomi, Y., Akahane, K., Sekiguchi, M. and Sato, K. (1990) The Clinical Report [Kiso To Rinsho] 24, 7407–7412.
167 Hemsworth, B.A. and West, G.B. (1970) J. Pharm. Sci. 59, 118–120.
168 de Forni, M., Bugat, R., Chabot, G.G., Culine, S., Extra, J.M., Gouyette, A., Madelaine, I., Marty, M.E. and Mathieu-Boue, A. (1994) Cancer Res. 54, 4347–4354.
169 Kaneda, N., Nagata, H., Furuta. and Yokokura, T. (1990) Cancer Res. 50, 1715–1720.
170 Kawato, Y., Aonuma, M., Hirota, Y., Kuga, H. and Sato, K. (1991) Cancer Res. 51, 4187–4191.
171 Kaneda, N. and Yokokura, T. (1990) Cancer Res. 50, 1721–1725.
172 Suzuki, W., Atsumi, R. and Hakusui, H. (1991) Xenobio. Metabol. Dispos. [Yakubutsudoutai] 6, 97–104.
173 Gupta, E., Lestingi, T.M., Mick, R., Ramirez, J., Vokes, E.E. and Ratain, M.J. (1994) Cancer Res. 54, 3723–3725.
174 Sasaki. Y., Hakusui, H., Mizuno, S., Morita, M., Miya, T., Eguchi, K., Shinkai, T., Tamura, T., Ohe, Y. and Saijo, N. (1995) Jpn. J. Cancer Res. 86, 101–110.
175 Narita, M., Nagai, E., Hagiwara, H., Aburada, M., Yokoi, T. and Kamataki, T. (1993) Xenobiotica 23, 5–10.
176 Sakata, Y., Suzuki, H. and Kamataki, T. (1994) Jpn. J. Cancer Chemother. 21, 1241–1244.
177 Takasuna, K., Kasai, Y., Kitano, Y., Mori, K., Kobayashi, R., Hagiwara, T., Kakihata, K., Hirosaki, M., Nomura, M., Nagai, E. and Kamataki, T. (1995) Jpn. J. Cancer Res. 86, 978–984.
178 Abigerges, D., Armand, J.-P., Cabot, G.G., da Costa, L., Fadel, E., Cote, C., Herait, P. and Gandia, D. (1994) J. Natl. Cancer Inst. 86, 446–449.
179 Abigerges, D., Chabot, G.G., Armand, J.-P., Herait, P., Gouyette, A. and Gandia, D. (1995) J. Clin. Oncol. 13, 210–221.

180 Goncalves, E., da Costa, L., Abigerges, D. and Armand, J. -P. (1995) J. Clin. Oncol. 13, 2144–2146.
181 Sakai, H., Diener, M., Gartmann, V. and Takeguchi, N. (1995) Naunyn-Schmiedeberg's Arch. Pharmacol. 351, 309–314.
182 Ikuno, N., Soda, H., Watanabe, M. and Oka, M. (1995) J. Natl. Cancer Inst. 87, 1876–1883.
183 Creemerst, G.J., Lund, B. and Verweij, J. (1994) Cancer Treat. Rev. 20, 73–96.
184 Taguchi, T. (1995) in Ref. 29, pp. 67–74.
185 Burris, H.A., III., Fields, S.M., Kuhn, J.G. and Von Hoff, D.D. (1995) in Ref. 29, pp. 113–121.
186 Hochster, H.S. (1995) in Ref. 29, pp. 93–104.
187 Armand, J.P., Ducreux, M., Mahjoubi, M., Abigerges, D., Bugat, R., Chabot, G., Herait, P., de Forni, M. and Rougier, P. (1995) Eur. J. Cancer 31A, 1283–1287.
188 Ardizzoni, A. (1995) Lung Cancer 12, S177-S185.
189 Hochster, H., Liebes, L., Speyer, J., Sorich, J., Taubes, B., Oratz, R., Wernz, J., Chachoua, A., Raphael, B., Vinci, R.Z. and Blum, R.H. (1994) J. Clin. Oncol. 12, 553–559.
190 Burris, H.A., III., Awada, A., Kuhn, J.G., Eckardt, J.R., Cobb, P.W., Rinaldi, D.A., Fields, S., Smith, L. and Von Hoff, D.D. (1994) Anticancer Drugs 5, 394–402.
191 van Warmerdam, L.J.C., ten Bokkel Huinink, W.W., Rodenhuis, S., Koier, I., Davies, B.E., Rosing, H., Maes, R.A.A. and Beijnen, J.H. (1995) J. Clin. Oncol. 13, 1768–1776.
192 Kuhn, J. Rizzo, J., Eckardt, J., Cobb, P., Rodriquez, G., Rinaldi, D., Drengler, R., Smith, L., Peacock, N., Thurman, A., DeLaCruz, P., Hodges, S., Von Hoff, D. and Burris, H. (1995) Proc. Am. Soc. Clin. Oncol. 14, Abstr. 1538.
193 Schellens, J.H.M., Eckardt, J.R., Creemers, G.J., Loos, W.J., McDonald, M., Broom, C., Davies, B.E., Verweij, J. and Von Hoff, D.D. (1995) Proc. Am. Soc. Clin. Oncol. 14, Abstr. 1472
194 Paz-Ares, L. (1995) 6th Conference on DNA Topoisomerases in Therapy. Abstr. S36.
195 Arbuck, S.G. and Takimoto, C. (1995) in Ref. 194, Abstr. S36a.
196 Nakai, H., Fukuoka, M., Furuse, K., Nakao, I., Yoshimori, K., Ogura, T., Hara, N., Sakata, Y., Saito, H., Hasegawa, K., Kuriyama, T., Ogawa, M., Ariyoshi, Y., Irie, K., Kimura, I., Niitani, H. and Taguchi, T. (1991) Jpn. J. Cancer Chemother. 18, 607–612.
196a Fukuoka, M., Niitani, H., Suzuki, A., Motomiya, M., Hasegawa, K., Nishiwaki, Y., Kuriyama, T., Ariyoshi, Y., Negoro, S., Masuda, N., Nakajima, S. and Taguchi, T. for the CPT-11 Lung Cancer Study Group (1992) J. Clin. Oncol. 10, 16–20.
197 Negoro, S., Fukuoka, M., Niitani, H., Suzuki, A., Nakabayashi, T., Kimura, M., Motomiya, M., Kurita, Y., Hasegawa, K., Kuriyama, T., Nishiwaki, Y., Ogawa, M., Nakao, I., Saijo, N., Obo, K., Furue, H., Ariyoshi, Y., Shimokata, K., Furuse, K., Nakajima, S., Irie, K., Kimura, I., Ogura, T., Fujii, M., Hara, N., Hara, Y., Nakano, S., Araki, J., Miyata, Y. and Taguchi, T. (1991) Jpn. J. Cancer Chemother. 18, 1013–1019.
198 Douillard, J.Y., Ibrahim, N., Riviere, A., Spaeth, D., Chomy, P., Soussan, K. and Mathieu-Boue, A. (1995) Proc. Am. Soc. Clin. Oncol. 14, Abstr. 1118.
199 Masuda, N., Fukuoka, M., Kusunoki, Y., Matsui, K., Takifuji, N., Kudoh, S., Negoro, S., Nishioka, M., Nakagawa, K. and Takada, M. (1992) J. Clin. Oncol. 10, 1225–1229.
200 Fujita, A., Takabatake, H., Tagaki, S. and Sekine, K. (1995) Jpn. J. Cancer Chemother. 22, 889–893.
201 Takeuchi, S., Dobashi, K., Fujimoto, S., Tanaka, K., Suzuki, M., Terashima, Y., Hasumi, K., Akiya, K., Negishi, Y., Tamaya, T., Tanizawa, O., Sugawa, T., Umesaki, N., Sekiba, K., Aono, T., Nakano, H., Noda, K., Shiota, M., Yakushiji, M., Sugiyama, T., Hashimoto, M., Yajima, Y., Takamizawa, H., Sonoda, T., Takeda, Y., Tomoda, Y., Ohta, M., Ozaki,

M., Hirabayashi, K., Hiura, M., Hatae, M., Nishigaki, K. and Taguchi, T. (1991) Jpn. J. Cancer Chemother. 18, 1681–1689.

202 Chevallier, B., Lhomme, C., Dieras, V., Vennin, Ph., Chauvergne, J., Rebattu, P., Fumoleau, P., Roche, H., Krakowski, Y., Lentz, M.A., Mathieu-Boue, A. and Van Glabbecke, M. (1995) Proc. Am. Soc. Clin. Oncol. 14, Abstr. 737.

203 Potkuj, R.K., Price, F.V., Bailey, H., Gelder, M., Rosenbluth, R. and Durivage, H.J. (1995) Proc. Am. Soc. Clin. Oncol. 14, Abstr. 785.

204 Futatsuki, K., Wakui, A., Nakao, I., Sakata, Y., Kambe, M., Shimada, Y., Yoshino, M., Taguchi, T. and Ogawa, N. (1994) Jpn. J. Cancer Chemother. 21, 1033–1038.

205 Shimada, Y., Yoshino, M., Wakui, A., Nakao, I., Futatsuki, K., Sakata, Y., Kambe, M., Taguchi, T. and Ogawa, N. (1993) J. Clin. Oncol. 11, 909–913.

206 Nishimura, G., Satou, T., Yoshimitsu, Y., Kurosaka, Y., Fujimura, T., Sugiyama, K., Kanno, M., Yonemura, Y., Miwa, K., Miyazaki, I. and Yamaguchi, A. (1995) Jpn. J. Cancer Chemother. 22, 93–97.

207 Rougier, P., Cujine, S., Bugat, R., Sue, E. and Douillard, J.Y. (1995) 6th Conference on DNA Topoisomerases in Therapy Abstr. 74.

208 Conti, J.A., Kemeny, N., Saltz, L., Tong, W., Chou, T.C. and Pulliam, M. (1994) Proc. Am. Soc. Clin. Oncol. 13, Abstr. 565.

209 Pitot, H.C., Wender, D., O'Connell, M.J., Wieand, H.S. and Maillard, J.A. (1994) Proc. Am. Soc. Clin. Oncol. 13, Abstr. 573.

210 Rothenberg, M.L., Eckardt, J.R., Burris, H.A., Nelson, J., Kuhn, J.G., Chen, S.F., Hilsenbeck, S.G., Clark, G.M., Fields, S.M., Rodriguez, G.I., Weiss, G.R., Smitn, L.S., Thurman, A.M., Evkhards, S.G., Rinaldi, D.A., Perez, E. and Von Hoff, D.D. (1994) Proc. Am. Soc. Clin. Oncol. 13, Abstr. 578.

211 Sakata, Y., Shimada, Y., Yoshino, M., Kambe, M., Futatsuki, K., Nakao, I., Ogawa, N., Wakui, A. and Taguchi, T. (1994) Jpn. J. Cancer Chemother. 21, 1039–1046.

212 Wagener, D.J., Verdonk, H.E., Dirix, L.Y., Catimel, G., Siegenthaler, P., Buitenhuis, M., Mathieu-Boue, A. and Verweij, J. (1995) Ann. Oncol. 6, 129–132.

213 Taguchi, T., Tominaga, T., Ogawa, M., Ishida, T., Morimoto, K. and Ogawa, N. (1994) Jpn. J. Cancer Chemother. 21, 1017–1024.

214 Bonneterrre, J., Pion, J.M., Adenis. A., Tubiana-Hulin, M., Tursz, T., Marty, M. and Mathieu-Boue, A. (1993) Proc. Am. Soc. Clin. Oncol. 12, Abstr. 179.

215 Ikeda, S., Ishihara, K., Ohura, T., Takahashi, M., Ohtsuka, F., Tamaoki, K., Kitamura, K., Yanagawa, S., Ishibashi, Y., Morishima, T., Takahashi, H., Ohhara, K., Nakajima, H., Saida, T., Mori, S., Matsunaka, M., Arata, J., Shimano, S., Yamamato, S., Hori, Y., Nonaka, S., Ogawa, N. and Taguchi, T. (1993) Skin Cancer 8, 503–513.

216 Ota, K., Ohno, R., Shirakawa, S., Masaoka, T., Okada, K., Ohashi, Y. and Taguchi, T. (1994) Jpn. J. Cancer. Chemother. 21, 1047–1055.

217 Tsuda, H., Takatsuki, K., Ohno, R., Masaoka, T., Okada, K., Shirakawa, S., Ohashi, Y. and Ota, K. (1994) Br. J. Cancer 70, 771–774.

218 Lynch, T.J., Jr., Kalish, L., Strauss, G., Elias, A., Skarin, A., Shulman, L.N., Posner, M. and Frei, E., 3rd. (1994) J. Clin. Oncol. 12, 347–352.

219 Weitz, J.J., Jung, S.-H., Marschke, R.F., Jr., Fitch, T.R. and Jett, J.R. (1995) Proc. Am. Soc. Clin. Oncol. 14, Abstr. 1053.

220 Perez-Soler, R., Glisson, B.S., Kane, J., Lee, J., Raber, M.N. and Hong, W.K. (1994) Proc. Am. Soc. Clin. Oncol. 13, Abstr. 1223.

221 Schiller, J.H., Kim, K. and Johnson, D. (1994) Proc. Am. Soc. Clin. Oncol. 13, Abstr. 1093.

222 Ardizzoni, A., Hansen, H., Dombernowsky, P., Postmus, P., Buitenhuis, M., Wanders, J.,

McDonald, M., Giaccone, G. and Verweij, J. (1994) Proc. Am. Soc. Clin. Oncol. 13, Abstr. 1116.
223 Wanders, J., Ardizzoni, A., Hansen, H.H., Dombernowsky, P., Postmus, P.E., Buitenhuis, M., McDonald, M., Giaccone, G. and Verweij, J. (1995) Proc. Am. Assoc. Cancer Res. 36, Abstr. 1415.
224 Perez-Soler, R., Glisson, B.S., Lee, J.S., Fossella, F.V., Lippman, S.M., Huber, M.H., Shin, D.M., Murphy, W.K. and Hong, W.K. (1995) Proc. Am. Soc. Clin. Oncol. 14, Abstr. 1078.
225 Kudelka, A., Edwards, C., Freedman, R., Wallin, B., Hord, M., Howell, E., Harper, K., Raber, M. and Kavanagh, J. (1993) Proc. Am. Soc. Clin. Oncol. 12, Abstr. 821.
226 Armstrong, D., Rowinsky, E., Donehower, R., Rosenshein, N., Walczak, J. and McGuire, W. (1995) Proc. Am. Soc. Clin. Oncol. 14, Abstr. 769.
227 Robert, F., Wheeler, R.H., Molthrop, D.C., Greene, P. and Chen, S. (1994) Proc. Am. Soc. Clin. Oncol. 13, Abstr. 905.
228 Sugarman, S.M., Ajani, J.A., Daugherty, K., Winn, R., Lanzotti, V., Bearden, J.D. and Abbruzzese, J.L. (1994) Proc. Am. Soc. Clin. Oncol. 13, Abstr. 686.
229 Greemers, G.J., Schellens, J.H.M., Planting, A.S.Th., Burg, M.E.L.v.d., de Boer-Dennert, M., Harteveld, M., McDonald, M., Stoter, G. and Verweij, J. (1995) Proc. Am. Soc. Clin. Oncol. 14, Abstr. 354.
229a Creemers, G.J., Wanders, J., Gamucci, T., Vallentin, S., Dirix, L.Y., Schöffski, P., Hudson, I. and Verweij, J. (1995) Ann. Oncol. 6, 844–846.
230 Sugarman, S.M., Pazdur, R., Daugherty, K., Evans, D., Winn, R., Dubovsky, D., Goodwin, J.W. and Abbruzzese, J.L. (1994) Proc. Am. Soc. Clin. Oncol. 13, Abstr. 684.
231 Chang, A.Y., Garrow, G., Boros, L., Asbury, R., Pandya, K. and Keng, P. (1995) Proc. Am. Soc. Clin. Oncol. 14, Abstr. 118.
232 Kraut, E.H., Staubus, A., Mayernick, D., King, G. and Balcerzak, S.P. (1995) Proc. Am. Assoc. Cancer Res. 36, Abstr. 1420.
233 Law, T.M., Ilson, D.H. and Motzer, R.J. (1994) Invest. New Drugs 12, 143–145.
234 Giantonio, B.J., Kosierowski, R., Ramsey, H.E., Fox, S.C., McAleer, C.A., Roethke, S., Ozol, R.F. and Hudes, G.R. (1993) Proc. Am. Soc. Clin. Oncol. 12, Abstr. 774.
235 Eisenhauer, E.A., Wainman, N., Boos, G., McDonald, D. and Bramwell, V. (1994) Proc. Am. Soc. Clin. Oncol. 13, Abstr. 488.
236 Maksymiuk, A.W., Jung, S.-H., Marschke, R.F., Jr., Nair, S. and Jett, J.R. (1995) Proc. Am. Soc. Clin. Oncol. 14, Abstr. 1383.
237 O'Brien, S., Kantarjian, H., Ellis, A., Zwelling, L., Estey, E. and Keating, M. (1995) Cancer 75, 1104–1108.
238 Priel, E., Showalter, S.D. and Blair, D.G. (1991) AIDS Res. Hum. Retroviruses 7, 65.
239 Bodley, A.L. and Shapiro, T.A. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 3726–3730.
240 Bodley, A.L., Wani, M.C., Wall, M. and Shapiro, T.A. (1995) Biochem. Pharmacol. 50, 937–942.
241 Burke, T.G. and Mi, Z. (1993) Anal. Biochem. 212, 285–287.
242 Burke, T.G. and Mi, Z. (1993) J. Med. Chem. 36, 2580–2582.
243 Burke, T.G. and Mi, Z. (1994) J. Med. Chem. 37, 40–46.

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Progress in Medicinal Chemistry – Vol. 34 edited by G.P. Ellis and D.K. Luscombe

3 Prodrug Design for Phosphates and Phosphonates

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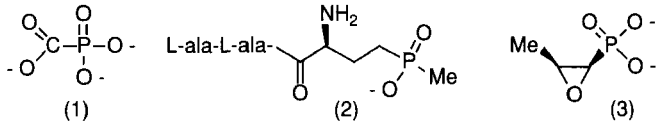
INTRODUCTION

The phosphate group has an important role in virtually all of the major metabolic pathways in nature. However, there is little therapeutic scope for underivatized phosphate and phosphonate analogues because they are doubly ionized at physiological pH and are unable to cross cell membranes by passive diffusion. Only a few compounds can utilize active transporter pathways: these include the antiviral phosphonoformate (1) which shows some affinity for inorganic phosphate transporters [1, 2], the herbicide bialaphos (2) which is transported by a peptide carrier [3], and the antibiotic fosfomycin (3) which utilizes the 3-phosphoglycerate transport pathway [4]. Phosphate monoesters are also prone to rapid hydrolysis by phosphatases, another property which makes them unsuitable drugs. However, this instability coupled with their high aqueous solubility makes phosphate monoesters ideal prodrug modifications of drugs which have low aqueous solubility. Although tetracationic compounds with two diquaternary 1,4-diaza[2,2,2]bicyclo-octane units form a 1:1 complex with nucleotide 5′-triphosphates and transport the triphosphates across liquid organic membranes, the cationic carriers break the liposomal structure, by their detergent action, rather than facilitate transport across liposomes [5]; therefore, lipophilic cations have limited uses in drug delivery. Hence, there is interest in designing prodrugs by covalent modification to mask the negative charges of phosphates or phosphonates to facilitate passive transport through cell membranes.



(1) (2) (3)

A prodrug is described as a pharmacologically inactive derivative of the parent drug; it has improved delivery properties and spontaneously or enzymatically releases the active drug within the body [6] *(Figure 3.1)*. Ideally, a prodrug should be quantitatively converted to the parent drug, specifically at the site where the drug is required.

Prodrug design is concerned with overcoming some undesirable element of the parent drug, for example:

(a) enhancement of bioavailability and passage through various biological barriers

(b) increased duration of pharmacological effects

(c) increased site-specificity

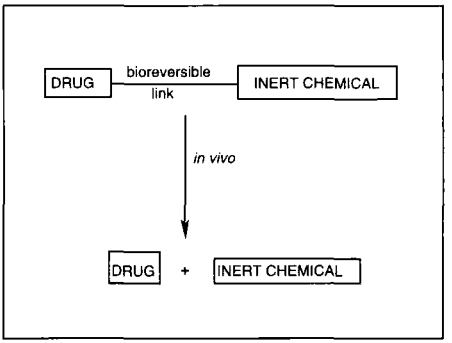


Figure 3.1

(d) decreased toxicity and adverse reactions

(e) improvement of stability and solubility properties.

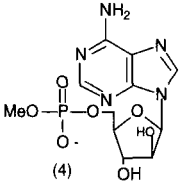
Recent advances towards the design of prodrugs of phosphates and phosphonates are discussed in this review. One such class of therapeutically important compounds are the anti-viral and anti-cancer nucleosides, for example, AZT,* ddC, ddI and FdU. For inhibition of HIV, the antiviral nucleosides are successively phosphorylated by host kinases to yield the nucleoside triphosphate which inhibits HIV reverse transcriptase. The first phosphorylation step can be rate-limiting for these nucleosides [7, 8], and therefore there is interest in delivery of the nucleoside monophosphate. Three recent reviews have discussed progress in the area of prodrugs of AZT [9] and nucleotides [10, 11], and part of this review covers similar material.

*The following abbreviations are used in this review: A, adenine; acyclovir, 9-[(2-hydroxyethoxy)methyl]guanine; ADEPT, antibody-directed enzyme prodrug therapy; ADP, adenosine 5′-diphosphate; AIDS, acquired immune deficiency syndrome; ara-A, adenine β-D-arabinofuranoside; ara-C, cytosine β-D-arabinofuranoside; ATP, adenosine 5′-triphosphate; AZT, 3′-azido-3′-deoxythymidine; BVDU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; c-AMP, adenosine 3′:5′-cyclic monophosphate; c-GMP, guanosine 3′:5′-cyclic monophosphate; CMV, cytomegalovirus; ddA, 2′,3′-dideoxyadenine; ddC, 2′,3′-dideoxycytidine; ddI, 2′,3′-dideoxyinosine; ddT, 3′-deoxythymidine; ddU, 2′,3′-dideoxyuridine; d4T, 2′,3′-didehydro-3′-deoxythymidine; dT, thymidine; DTE, dithiodiethanol; FdT, 2′,3′-dideoxy-3′-fluorothymidine; FdU, 5-fluoro-2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine 5′-monophosphate; 5-FU, 5-fluorouridine; HIV, human immunodeficiency virus; HPMPC, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; HSV, herpes simplex virus; Nu, appropriate nucleoside without 5′-H; PAF, platelet-activating factor; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; RPMI, Roswell Park Memorial Institute; SATE, *S*-acylthioethanol. Nucleosides are attached through the 5′-position, and therefore the 5′-H is absent.

LIPOPHILIC PRODRUGS

MONOALKYL ESTERS

In 1975, Revankar and coworkers synthesized the monomethyl phosphate ester of the antiviral nucleoside ara-A (4) which showed activity comparable with that of the parent drug [12]. Rosowsky and coworkers [13] then prepared a range of 5′-phosphate diester analogues of the anti-cancer nucleoside ara-C. The simple alkyl derivatives (5, R = Et, n-Bu, n-hexyl, n-octyl or n-hexadecyl) were not active against the kinase-deficient L1210 leukaemic cells in culture, whereas the 5′-glyceryl analogue (5, R = $CH_2CH(OH)$-CH_2OH, EC_{50} 0.35 μM) showed activity comparable with ara-C (EC_{50} 0.65 μM).



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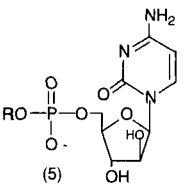

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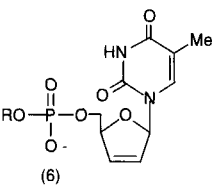
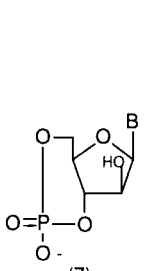


(6)

The hexadecyl and cholesteryl esters of d4T monophosphate (6, R = $(CH_2)_{15}Me$ and cholesterol-3β-OCH_2CH_2) have been synthesized [14]. The palmitoyl ester showed little activity, whereas the cholesteryl ester (EC_{50} 0.02 μM) showed greater inhibition of HIV replication in CEM-C113 cells than D4T (EC_{50} 0.08 μM).

Cyclic 3′,5′-phosphates of ara-A (7, B = adenyl) [12], ara-C (7, B = cytosyl) [15] and thioinosine (8, R = H) have been synthesized [16]. The acylated thioinosine derivative (8, R = $Me(CH_2)_{14}CO$, EC_{50} 6 μM) has been shown to readily enter S49 mouse lymphoma cells and then be metabolized to thioinosine 5′-monophosphate catalyzed by phosphodiesterase and deacylation. More recently, the cyclic phosphonate analogue (9) of the antiviral (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine (HPMPC) has been prepared and shown to have comparable activity against HSV-2, both *in vitro* in MA-104 and MRC-5 cells and *in vivo* in a mouse encephalitis model, to that of the parent drug [17]. The cyclic phosphonate (9) was converted to HPMPC inside the cells, before phosphorylation to the active diphosphate.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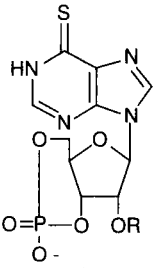
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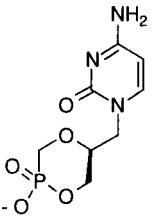
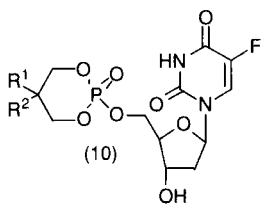

(9)

DIALKYL ESTERS

Jones and coworkers first prepared some 6-membered ring cyclic triesters of FdU (10). Analogues (10, $R^1 = R^2 = H$) and (10, $R^1 = H$, $R^2 = OH$ or OCH_2Ph) were found to be too stable towards chemical hydrolysis, whereas the difluoro analogue (10, $R^1 = R^2 = F$) was easily hydrolyzed at room temperature. The difluoro analogue was the most active prodrug against L1210 leukaemia (ID_{50} 0.003 μg/ml), showing comparable activity to FdU (ID_{50} 0.001 μg/ml) and its 5-monophosphate (ID_{50} 0.002 μg/ml). However, the difluoro analogue was less active against L1210 leukaemia in a thymidine kinase-deficient cell line, suggesting that the prodrug releases the nucleoside and not its 5′-monophosphate [18].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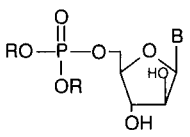
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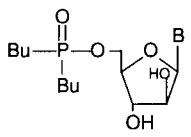
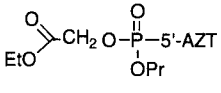

(12)

McGuigan and coworkers [19] prepared a range of acyclic 5′-dialkyl phosphate triester derivatives of the anticancer drug ara-C (11, B = cytosyl, R = Et, n-Pr, n-Bu, n-pentyl or n-hexyl). These triesters inhibited DNA synthesis and their efficacy improved with increasing lipophilicity. This result suggested that these triesters were membrane permeable; however, their mechanism of inhibition of DNA synthesis was unresolved. In a similar study, inhibition of DNA synthesis also increased as the lipophilicity of the phosphate triester of the anti-viral nucleoside ara-A (11, B = adenyl, R = Et, n-Pr, n-Bu or n-pentyl) increased [20]. The dibutyl phosphinate analogue

(12, B = adenyl), bearing more stable C-P bonds, also inhibited DNA synthesis, although it was less active than the isosterically similar di(n-propyl) phosphate triester analogue [21]. The phosphinate is unable to degrade to the 5′-monophosphate, therefore its inhibitory properties have been attributed to the release of ara-A. In contrast, the higher activity of the phosphate triester can be accounted for by some metabolism to the 5′-monophosphate.

Simple dialkyl phosphate triesters of AZT were inactive against HIV [22], whereas the di(2,2,2-trihaloethyl) phosphate analogues (halo = F or Cl) were active [23]. The finding that functionalized alkyl groups increased activity led McGuigan and coworkers to prepare phosphate triesters of AZT which contained both a simple alkyl group and either an ethyl glycolyl or a methyl lactyl group. The most active analogues were (13) and (14), with ED_{50} values of 0.5–0.6 μM against HIV-1 [24]. Triesters containing two methyl lactyl groups (15, $R^1 = R^2 = Me$), or one methyl lactyl and one ethyl glycolyl group (15, $R^1 = H$, $R^2 = Et$) were 100-fold more active against HIV-1 replication than the triesters bearing only one substituted ester; however, they are 10-fold less active than AZT. Although their mechanism of action is unclear, the antiviral and hydrolysis studies are consistent with intracellular formation of AZT 5′-monophosphate [25].



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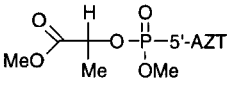

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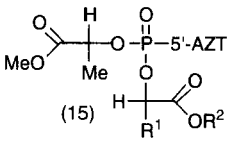


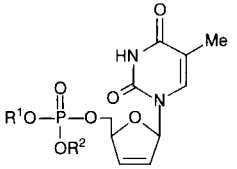
(15)

Sergheraert and coworkers [14] prepared some d4T phosphate triesters (16, R^1 = cholesteryl or n-hexadecyl, $R^2 = CH_2CH_2CN$). The cyanoethyl group of the triesters was susceptible to hydrolysis catalyzed by plasma enzymes, showing half-lives in the region of 12 hours. Although the triesters showed an increased cell penetration, there was no increase in antiviral activity of these compounds, presumably because the R^1 group of the diester metabolite was too stable.

Huynh-Dinh and coworkers have prepared phosphate triesters of the anticancer nucleosides, 5-FU and ara-C, bearing a lipophilic hexadecyl chain and a hydrophilic mannose residue (17, Nu = 5-FU or ara-C). These prodrugs were designed to interact with the membrane bilayer to facilitate their transport by passive diffusion, prior to their hydrolysis to the nucleoside 5′-monophosphates [26]. The triester analogue of AZT (17, Nu = AZT) has also been prepared [27]. In mice, the prodrug had a half-life of 24 h giving

rise to AZT 5′-monophosphate, mannose and hexadecanol. This prodrug modification is potentially useful for delivery to the brain, as high levels of AZT 5′-monophosphate were detected in the CNS [28].

The combination of antiviral and antibiotic drugs may be useful in the treatment of AIDS, and simple esters (18, R = hexyl or hexadecyl) of nucleotide dimers containing both AZT and the antibiotic cordycepin have been prepared as potential prodrugs. For the hexadecyl derivative, the diastereoisomers were separated, and the S_p isomer was slightly more active than R_p against HIV-1; however, all of the compounds were substantially less active than AZT [29].



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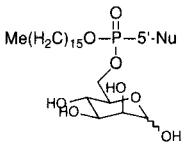

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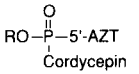

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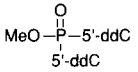


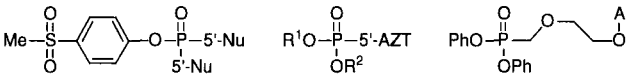
(19)

In many studies, the metabolic fate of the prodrug is not investigated and the proposed mechanism of action is based solely on antiviral results. In contrast, Imbach and coworkers [30] have thoroughly evaluated the fate of the prodrugs in biological media. By HPLC, they showed that the methyl ester of the 5′,5′-dimer of ddC (19) hydrolyzed slowly (6% in 5 days) in either culture medium or CEM cell extract at 3°C, to give only the nucleotide dimer. The dimer was stable in RPMI culture medium, but in the presence of 10% foetal calf serum, it hydrolyzed with a half-life of 22 h to give ddC and its 5′-monophosphate, the reaction being catalyzed by phosphodiesterase. The 5′-monophosphate of ddC was subsequently converted to ddC, catalyzed by phosphatases. In contrast, (19) was stable towards CEM cell extract, which led the authors to conclude that its anti-HIV activity can mainly be attributed to the extracellular release of ddC under the cell culture conditions. Many prodrug studies do not include hydrolysis information and Imbach and coworkers suggested that the antiviral activity observed in some studies may simply be attributed to release of the nucleoside in culture medium [30].

ARYL ESTERS

Aryl phosphate esters are more reactive towards chemical hydrolysis than simple alkyl esters, and their rate of degradation can be controlled by the presence of substituents in the aromatic ring [31]. The aryl group has there-

fore been used in prodrug design: Farrow and coworkers [32] evaluated a series of aryl bis(3′-*O*-acetylthymidin-5′-yl) phosphate derivatives designed to cleave to the nucleotide dimer under physiological conditions. The 4-(methylsulphonyl)phenyl substituent was selected, and the 5′,5′-linked nucleotide dimers (20) of BVDU (Nu = BVDU) and acyclovir (Nu = acyclovir) were shown to have comparable activity to BVDU and acyclovir, respectively, against HSV-1 and HSV-2, both *in vivo* and *in vitro*. The BVDU analogue (20, Nu = BVDU) was stable in human serum, giving sufficient time for cell penetration, but it hydrolyzed slowly, with a half-life of 17 h at pH 7.7 and 37°C, to the nucleotide dimer. This intermediate cleaved to BVDU, the hydrolyses being catalyzed by phosphodiesterases and phosphatases present in the serum. This study has been extended to the synthesis of the 4-(methylsulphonyl)phenyl derivatives of 5′,5′-nucleotide dimers containing d4T or ddA, which showed comparable activity to the parent nucleosides against HIV-1 and HIV-2 in MT-4 cells [33].



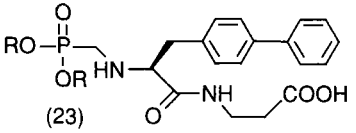
(20) (21) (22)

McGuigan and coworkers have prepared two phenyl phosphate esters of AZT, (21, R^1 = Ph, R^2 = CH_2CCl_3, IC_{50} 0.2 μM) and (21, R^1 = R^2 = Ph, IC_{50} 0.3 μM), however both were less active than AZT (IC_{50} 0.008 μM) against HIV-1 in C8166 cells. In contrast, triester (21, R^1 = R^2 = *p*-O_2N-Ph, IC_{50} 0.0032 μM), bearing the more reactive *p*-nitrophenyl leaving group, was three-fold more potent than AZT; however, it was significantly more toxic [34]. The other nitrophenyl isomers (21, R^1 = R^2 = *o*-O_2N-Ph or *m*-O_2N-Ph) have also been prepared and the meta isomer was the most active against HIV-1. However, all of the isomers showed little activity against HIV-1 in thymidine kinase-deficient cells, which suggested that these delivery forms gave the nucleoside rather than its 5′-monophosphate [35].

A range of substituted diphenyl prodrugs has been reported for the antiviral acyclic nucleoside analogue, 9-[2-(phosphonomethoxy)ethoxy]adenine, which shows only 2% oral bioavailability in rats [36]. The preferred prodrug was the crystalline hydrochloride salt of the diphenyl ester (22): this increased the oral bioavailability to 50% in rats and showed activity against Rauscher murine leukaemia virus in mice.

Phosphonate (23, R = H) is a potential antihypertensive agent as it is a potent inhibitor of neutral endopeptidase. However, it has low oral bioavailability, whereas the diphenyl analogue (23, R = Ph) has been shown to be an orally active prodrug [37]. The chemical hydrolysis of prodrug (23, R =

Ph) in potassium hydroxide (pH 13.65) or potassium carbonate (pH 12.01) was rapid at 25°C, giving only the monophenyl ester. Surprisingly, when the hydrolysis was conducted in potassium bicarbonate buffer (pH 8.78), removal of the first phenyl group (half-life 8.19 h) was significantly slower than removal of the second (half-life 0.52 h). Although this requires further investigation, it is speculated that at pH 8.78 the intermediate bearing one phenyl ester, exists predominantly in the ammonium form, which increases the susceptibility of phosphorus towards nucleophilic attack and forms an ion-pair with the nucleophilic bicarbonate anion. In human plasma, removal of the first phenyl group (half-life 1.4 h) was three-fold faster than removal of the second (half-life 4.2 h). Studies with heat-inactivated plasma and plasma from other species suggests that the hydrolyses are enzyme catalyzed. Prodrug (23, R = Ph) is currently being evaluated in clinical trials.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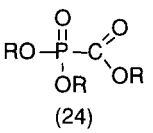
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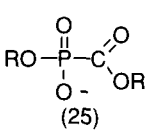
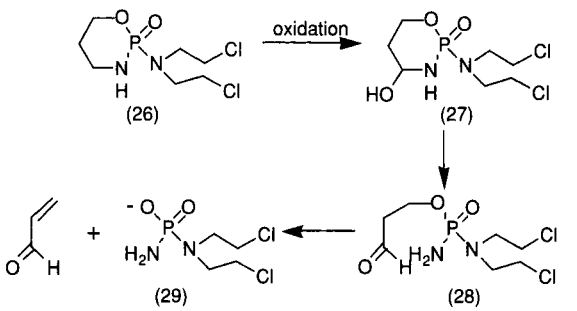

(25)

A range of triesters (24) and diesters (25) of the antiviral phosphonoformate has been synthesized. Compounds with simple alkyl groups showed no antiviral activity, which was attributed to their inability to undergo complete hydrolysis to phosphonoformate. In contrast, the more labile phenyl esters were active against HSV-1, with the triphenyl (24, R = Ph) and diphenyl (25, R = Ph) esters giving IC_{50} values of 39 and 52 μM, respectively [38].

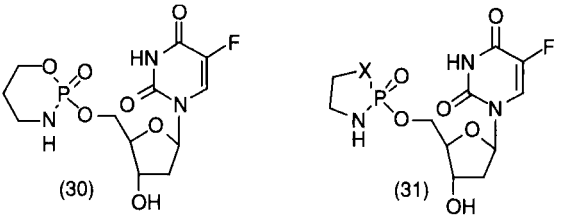
PHOSPHORAMIDATES

Phosphoramidates have been explored as prodrug modifications because of the potential cleavage of the P-N bond by chemical or enzymatic hydrolysis, or metabolic oxidation. Early research in this area was partly inspired by the mode of bioactivation of the anticancer drug, cyclophosphamide (26) [39] which undergoes metabolic oxidation to (27), cleavage to the aldehyde (28) and subsequent degradation to the alkylating agent (29) and acrolein *(Scheme 3.1)* [40]. This led Jones and coworkers to prepare the corresponding 5′-cyclic phosphoramidate of FdU (30), a potential prodrug of the 5′-monophosphate of the anticancer drug, 5-fluoro-2′-deoxyuridine [41]. Phosphoramidate (30) was not active against leukaemia L1210 and was only slightly active against sarcoma 180, suggesting either that (30) is not an enzyme substrate or that the enzymes which metabolize cyclophosphamide are not present. This result led the team to prepare the corresponding 5-



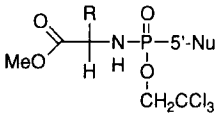
Scheme 3.1

membered ring phosphoramidate (31, X = O) and phosphorodiamidate (31, X = NMe) derivatives, and due to strain in the ring, one P-N bond cleaved readily by chemical hydrolysis under physiological conditions. The phosphorodiamidate (31, X = NMe) was active aginst L1210 leukaemia [42].



McGuigan and coworkers have prepared a range of acyclic phosphoramidates. Phosphoramidate (32, Nu = AZT) [43] and phosphorodiamidate (33, Nu = AZT) [44], analogues of AZT, are both good inhibitors of HIV. In contrast, the 2′,3′-dideoxy-3′-fluorothymidine (FdT) phosphoramidates (32, R = Pr^i or Me, Nu = FdT, ED_{50} both 0.3 μM) and phosphorodiamidate (33, Nu = FdT, ED_{50} 5 μM) were substantially less active than FdT (EC_{50} 0.005 μM) against HIV-1 in a human T-cell line, suggesting that prodrug modification will need optimization for each nucleoside [45]. The 2,2,2-trichloroethyl phosphoramidate analogue of d4T (32, Nu = d4T) was equally active (EC_{50} 0.2 μM) against HIV-1 in both C8166 cells and thymidine kinase-deficient JM cells. In contrast, d4T was more active in C8166 cells (EC_{50} 0.08 μM) than in the thymidine kinase-deficient JM cells (EC_{50} 0.8 μM), these data being consistent with intracellular release of d4T 5′-monophosphate from prodrug (32, Nu = d4T). The phosphoramidate (32,

Nu = d4T) also showed a four-fold increase in selectivity (2500), when compared with d4T (625) [46].



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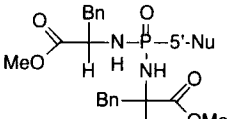
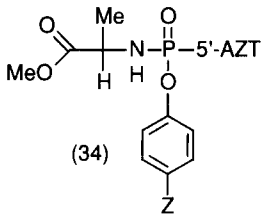


(33)

This study has been extended to similar phosphoramidate analogues of AZT, in which the 2,2,2-trichloroethyl group has been replaced by a substituted phenyl group (34) [47]. The compounds were screened against HIV-1 in thymidine kinase-deficient JM cells for which AZT is inactive (ED_{50} 100 μM). In contrast, phosphoramidates (34, Z = MeO, Et, F or H) were active, with ED_{50} values ranging from 0.32 to 0.8 μM, these results being consistent with hydrolysis of both the P-N bond and phenyl ester to give an intracellular release of AZT 5′-monophosphate. These compounds were also tested in another laboratory, where phosphoramidates (34) were an order of magnitude less active than AZT against HIV-1 and HIV-2 in CEM and MT-4 cells. However, in agreement with the previous testing data, the phosphoramidates were more active (EC_{50} 3.0–12 μM) than AZT (EC_{50} > 100 μM) in thymidine kinase-deficient CEM cells [48].



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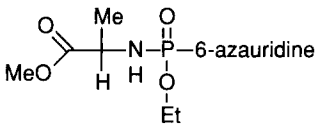


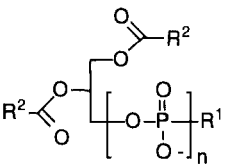
(35)

Orotidine 5′-monophosphate decarboxylase is a target for antiviral or antitumour chemotherapeutic agents, and 6-azauridine-5′-monophosphate has been shown to be a competitive inhibitor. Gabrielson and coworkers utilized McGuigan's phosphoramidate prodrug methodology in an attempt to obtain a membrane permeable analogue of this phosphate [49]. Prodrug 6-azauridine-5′-(ethyl methoxyalaninyl) phosphate (35) was less active than 6-azauridine *in vitro* against a range of RNA virus. However, prodrug (35) showed improved activity against Punta Toro virus in mice when compared with 6-azauridine, indicative of metabolism of (35) to 6-azauridine 5′-monophosphate [49].

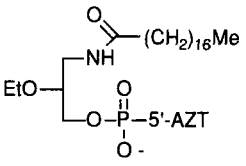
PHOSPHOLIPID ESTERS

A promising class of prodrugs for mono-, di- and tri-phosphates are based on the phospholipids and Hostetler's group have applied this prodrug approach to a range of antiviral compounds. Two prodrugs of AZT, AZT 5′-monophosphate 1,2-dimyristoylglycerol (36, R^1 = 5′-AZT, R^2 = $Me(CH_2)_{12}$) and AZT 5′-diphosphate 1,2-dipalmitoylglycerol (37, R^1 = 5′-AZT, R^2 = $Me(CH_2)_{14}$) [50] have been prepared. The 1,2-dimyristoylglycerol 5′-monophosphates of ddC and ddT (36) are also reported. In liposomes these compounds showed antiviral activity against HIV-1 in U937 and CEM cells with IC_{50} values ranging from 0.7 to 12 μM. Although their activities were lower than AZT (IC_{50} 0.2 μM) and ddC (0.04–0.60 μM), these lipid prodrugs provide a depot from which the drug is slowly released by cellular metabolism. Biodegradation of (36, R^1 = 5′-AZT, R^2 = $Me(CH_2)_{12}$) proceeded by deacylation to give glycero-3-phospho-5′-AZT catalyzed by phospholipases, followed by hydrolysis catalyzed by phosphodiesterases to give AZT and its 5′-monophosphate [51].

Prodrugs of AZT 5′-monophosphate have also been prepared by combination with an antiviral ether lipid [52], with the intention that the derivative would be active against two steps of the HIV replicative cycle. The most active compound was (38) with an IC_{50} of 0.03 mM and a selectivity of 1793. Although this prodrug is less active than AZT (IC_{50} 0.004 mM), the selectivity for AZT was lower (1281).



(36) n = 1
(37) n = 2
(39) n = 3



(38)

The 5′-diphosphate 1,2-dipalmitoylglycerol derivatives of ara-C, ara-A and tubercidin (37, R^1 = 5′-ara-C, 5′-ara-A or tubercidin, R^2 = $Me(CH_2)_{14}$) have been synthesized [53]. The ara-C analogue is more active against L1210 leukaemia in mice than ara-C. After uptake into cells, the diphosphate is metabolized to active ara-C 5′-monophosphate and natural L-α-phosphatidic acid.

In a related study, Hostetler and coworkers prepared acyclovir diphosphate dimyristoylglycerol (37, R^1 = acyclovir, R^2 = $Me(CH_2)_{12}$) [54], which

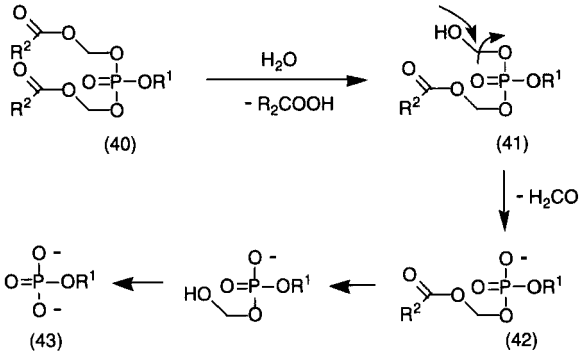
showed activity (IC_{50} 0.25 μM) against acyclovir-resistant thymidine kinase-deficient HSV strains to which acyclovir or its 5′-monophosphate was ineffective (IC_{50} >100 μM). This phospholipid prodrug is cleaved by a mitochondrial pyrophosphatase enzyme to give L-α-phosphatidic acid and the active acyclovir 5′-monophosphate. Similarly, ddT 5′-diphosphate dimyristoylglycerol (37, R^1 = 5′-ddT, R^2 = $Me(CH_2)_{12}$) has been shown to be 18- to 50-fold more effective than ddT against HIV-infected CEM and HT4–6C cells. Nucleoside ddT is only poorly phosphorylated by cellular thymidine kinase, and the higher activity of the phospholipid prodrug has been partly attributed to a by-pass of thymidine kinase. Indeed, the prodrug showed activity against HIV in thymidine kinase-deficient cells [55].

AZT 5′-triphosphate 1,2-distearoylglycerol (39, R = $Me(CH_2)_{16}$) inhibits HIV-1 replication in CEM cells (IC_{50} 0.33 mM). The triphosphate prodrug has been shown to give AZT and its 5′-monophosphate in a rat liver mitochondrial enzyme preparation [56].

α-ACYLOXYALKYL ESTERS

The rate of hydrolysis of prodrugs needs to be predictable and controlled by the use of suitable bioreversible protecting groups. The most common prodrugs are those requiring a hydrolytic cleavage mediated by enzymatic catalysis, such as cleavage of carboxyesters by esterases. In other cases, active drugs are regenerated by biochemical oxidative or reductive processes [6].

The first use of the acyloxymethyl substituent as a bioreversible phosphate protecting group was demonstrated by Farquhar and coworkers [57, 58] who prepared bis(acyloxymethyl) phosphate triesters of the model compounds, phenyl and benzyl phosphate (40, R^1 = Ph or Bn, R^2 = Me, Pr^i or Bu^t). After cell penetration, the triesters are designed to undergo esterase-catalyzed cleavage of one acyloxymethyl ester to give the hydroxymethyl analogue (41). This intermediate is labile and spontaneously eliminates formaldehyde to give diester (42). A repeat of this bioactivation gives phenyl or benzyl phosphate (43) *(Scheme 3.2)*. The rate of chemical hydrolysis of triesters (40, R^1 = Ph) in phosphate buffer at pH 7.4 and 37°C decreases as steric crowding of the acyl group increases, as exemplified by the half-lives of 3.2 h (R^2 = Me), 8.9 h (R^2 = Pr^i) and 14.3 h (R^2 = Bu^t). Slow chemical hydrolysis of the second acyloxymethyl ester group to give the phosphate monoester was also observed. Porcine liver carboxyesterase or plasma catalyzed hydrolyses of the triesters (40, R^1 = Ph) was also sensitive to the nature of the acyl group, and similarly the first acyloxymethyl group was cleaved at a rate substantially faster than the second. This bioreversible protection method has been evaluated for the delivery of a range of biologically active

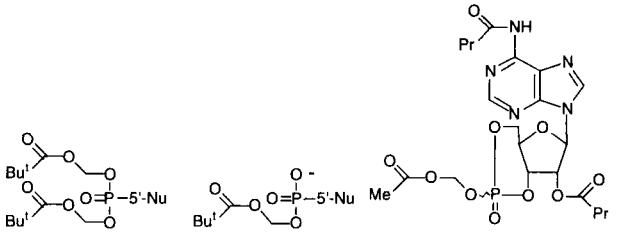


Scheme 3.2

compounds containing a phosphate or phosphonate group, and these studies are discussed.

Although the 5′-triphosphate of ddU is a potent inhibitor of reverse transcriptase, the parent nucleoside is inactive because it is a poor substrate for thymidine kinase. In an attempt to by-pass this phosphorylation step, the bis(pivaloyloxymethyl) triester of ddU 5′-monophosphate (44, Nu = 5′-ddU) was prepared [59]. This membrane-permeable prodrug is active against HIV-1 in either MT-4 or thymidine kinase-deficient CEM cells, and it has been shown to release the 5′-monophosphate of ddU in both cell lines.

In an approach to improve the delivery of the anticancer nucleotide, FdUMP, Farquhar and coworkers [60] have prepared bis(pivaloyloxymethyl) 2′-deoxy-5-fluorouridine 5′-monophosphate (44, Nu = 5′-FdU). In the presence of porcine liver carboxyesterase, the phosphate triester (44, Nu = 5′-FdU) was converted quantitatively to diester (45, Nu = 5′-FdU). However, the diester was a poor substrate for esterase and after 24 h only trace quantities of FdU 5′-monophosphate was observed. In contrast, in mouse plasma the triester was rapidly metabolized first to the diester and then to FdU 5′-monophosphate, suggesting that removal of the second pivaloyloxymethyl group may be catalyzed by phosphodiesterases. The prodrug showed similar cell growth-inhibitory properties to FdU in Chinese hamster ovary cells *in vitro*, and in mice implanted with P-388 leukaemia. Moreover, the prodrug was active against FdU-resistant cell lines both *in vitro* and *in vivo*, suggesting that triester (44, Nu = 5′-FdU) is an effective membrane-permeable prodrug of FdU 5′-monophosphate.



(44) (45) (46)

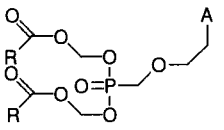
The hydrolysis of bis(pivaloyloxymethyl) AZT 5′-monophosphate (44, Nu = 5′-AZT) has also been evaluated [61]. In culture medium containing 10% foetal calf serum and in CEM-cell extract, the triester hydrolyzes to the diester with half-lives of 170 and 30 min, respectively, supporting intracellular release of the diester by esterase catalysis. Under the same conditions, the diester hydrolyses to AZT 5′-monophosphate with half-lives of 30 and 133 min. Anions are typically poor substrates for esterases, and therefore similar rates of hydrolysis for the triester and diester suggests that hydrolysis of the diester is catalyzed by phosphodiesterase.

Tsien and coworkers have isolated both diastereoisomers of N^6,2′-*O*-dibutyryl c-AMP acetyloxymethyl ester (46) as prodrugs of the second messenger, c-AMP [62]. At pH 7.4, the triesters had half-lives of 36h, whereas in the presence of carboxyesterase they degraded readily to c-AMP. These cell permeable analogues were shown to cause intracellular release of c-AMP in three biological systems.

The acyclic nucleoside phosphonate, PMEA, is a potent broad spectrum antiviral agent; however, its oral bioavailability is low (7.8% in rats). A range of esters and amides were evaluated as prodrugs of PMEA [63]. The most promising were the bis(acyloxymethyl) esters (47, R = Et, Pr^i or Bu^t) which gave improved oral bioavailabilities in rats of 15.4, 14.6 and 17.6%, respectively [63]. The bis(pivaloyloxymethyl) ester was 50- and 150-fold more active than PMEA against HSV-1 and HSV-2 in vero cells, respectively, whereas PMEA and the prodrug showed comparable activities against HIV and CMV [64].

Fridland and coworkers have shown that the bis(pivaloyloxymethyl) esters of three acyclic nucleoside phosphonates, including PMEA, have improved antiviral activity and selectivity when compared with the corresponding phosphonic acid [65]. Using radiolabelled material, in cells the PMEA prodrug (47, R = Bu^t) hydrolyzed rapidly to PMEA after which it was phosphorylated to the active diphosphate. In contrast, the prodrug had a half-life of over 24 h at pH 2.0, suggesting that it may be stable in gastric

juices. Indeed, oral administration of (47, R = Bu^t) to monkeys gave ~30% PMEA in circulation [66]. Oral administration of (47, R = Bu^t) to severe combined immune-deficiency mice has been shown to be as effective as subcutaneous PMEA in delaying Moloney murine-induced tumour formation [67] and in the inhibition of Friend leukaemia virus-induced splenomegaly [68]. Upon oral administration of prodrug (47, R = Bu^t), pharmacokinetic studies did not detect any prodrug, which suggested rapid hydrolysis to PMEA, 48% of which could be detected in the plasma [68].



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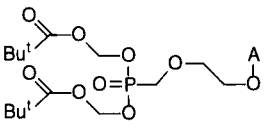

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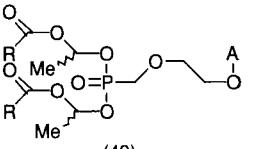
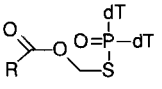


(49)

A range of prodrug modifications have also been reported on another antiviral acyclic nucleoside analogue, 9-[2-(phosphonomethoxy)ethoxy]adenine, which shows only 2% oral bioavailability in rats [69]. Amongst the most promising prodrug was the bis(pivaloyloxymethyl) derivative (48) which increased oral bioavailability to 30% in rats. The bis(1-acyloxyethyl) esters (49, R = Pr^i or Bu^t), bearing a methyl group on the α-carbon, were prepared as mixtures of diastereoisomers and these compounds showed oral bioavailabilities of 24 and 74%, respectively. Upon bioactivation with esterase these prodrugs would give acetaldehyde (rather than formaldehyde), which should have an improved toxicity profile. Although the Bu^t analogue showed the highest oral bioavailability of all analogues, it was not selected for further evaluation because of the presence of diastereoisomers and its physical properties.

With the intention of applying the methodology to the delivery of phosphorothioate oligonucleotides, the diastereoisomeric *S*-acyloxymethyl esters of the thymidine nucleotide dimer (50, R = Et, Pr^i or Bu^t) have been prepared [70]. In plasma, hydrolysis of the S_P and R_P diastereoisomers was stereospecific and gave rise to the S_P and R_P analogues of the nucleotide dimer, respectively, this being consistent with a mechanism proceeding by hydrolysis of the carboxyl ester. In plasma, hydrolysis of the S_P diastereoisomer was ~four-fold faster than hydrolysis of R_P, with half-lives of 335 and 1980 min, respectively, for the Bu^t analogue. In contrast, with porcine liver carboxyesterase, the R_P isomer hydrolyses faster than S_P. The three phosphorothioate diester linkages in a central stretch of a dodecathymidyl-

ate oligonucleotide analogue bearing terminal methylphosphonate residues have been derivatized with *S*-acyloxymethyl groups [71]. This analogue has a half-life of only 40 min in RPMI culture medium containing 10% foetal calf serum, and greater stability would be required for prodrugs of oligonucleotides.



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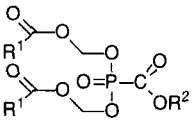

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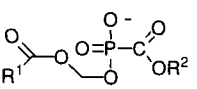
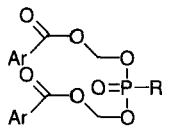


(52)

The antiviral phosphonoformate (Foscarnet) has poor transport through membranes, and bis(acyloxymethyl) alkoxycarbonylphosphonates (51, R^1 = Me, Pr^i or Bu^t, R^2 = Me, Et, Ph or 2,4-$Cl_2C_6H_3$) [72, 73] were prepared as potential prodrugs. Chemical hydrolysis of (51, R^1 = Me, R^2 = Et) was rapid giving diester (52). This diester was fairly stable in buffer (20% hydrolysis in 22 h), but in the presence of porcine liver carboxyesterase the second acyloxymethyl group was readily cleaved to give the monoester. Further hydrolysis of the ethyl carboxyester was not observed; in contrast, phosphonoformate was released from (51, R^1 = Me, R^2 = 2,4-$Cl_2C_6H_3$) bearing the more reactive 2,4-dichlorophenyl carboxyester, and this analogue showed activity against HIV-1 in H9 cells *in vitro*.

The bis(aroyloxymethyl) esters (53, Ar = Ph, 2-MeC_6H_4 or 2,4,6-$Me_3C_6H_2$) of the model compound, benzylphosphonate (R = Bn), and the methyl ester of the antiviral, phosphonoacetate (R = MeO_2CCH_2) have been prepared, with ortho-methyl substitution being introduced to control their rate of bioactivation [74]. All derivatives were stable towards chemical hydrolysis under physiological conditions. In the presence of porcine liver carboxyesterase, cleavage of a benzoyl or a 2-methylbenzoyl group from (53, R = Bn, Ar = Ph or 2-MeC_6H_4) was fast, with only slow hydrolysis of the second benzoyl group to give benzylphosphonate. For the phosphonoacetate analogues (53, R = MeO_2CCH_2, Ar = Ph or 2-MeC_6H_4), there was competition between hydrolysis of the aroyl and methoxycarbonyl ester groups. For the benzylphosphonate and phosphonoacetate analogues (53, R = Bn or MeO_2CCH_2) containing the hindered 2,4,6-trimethylbenzoyl group (Ar = 2,4,6-$Me_3C_6H_2$), esterase-catalyzed hydrolysis was not observed.



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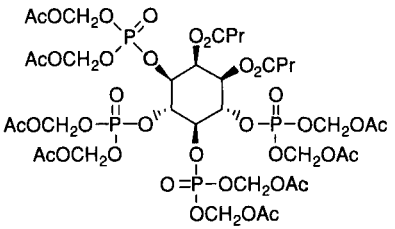


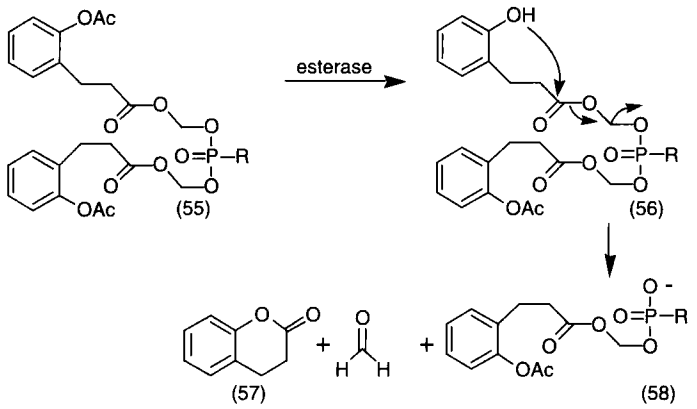
(54)

The role of D-*myo*-inositol 3,4,5,6-tetrakisphosphate [Ins(3,4,5,6)P_4] in the uncoupling of chloride anion secretion from intracellular calcium levels was confirmed with experiments using a membrane-permeable analogue. After cell penetration, derivative (54) with the four phosphate groups masked as acyloxymethyl esters and the two hydroxy groups as butanoyl esters, was cleaved by intracellular esterases to give Ins(3,4,5,6)P_4 which caused inhibition of chloride anion secretion [75].

One problem with the bis(α-acyloxyalkyl) prodrugs of phosphates and phosphonates is that the second α-acyloxyalkyl group is cleaved at a significantly slower rate than the first. This has been attributed to the mono(α-acyloxyalkyl) intermediate being a poor substrate for the esterase because of the close proximity of a P-O^- group to the site of hydrolysis. A successful approach to increase the distance of the P-O^- group from the carboxyester and make the cleavage of both bioreversible protecting groups proceed at similar rates has been observed with the bis[3-(2′-acetoxyphenyl)propionyl]oxymethyl esters of the model compounds phenyl phosphate (55, R = OPh) and phenylphosphonate (55, R = Ph). Hydrolysis of the acetyl group is catalyzed by esterase, and the resulting phenol (56) spontaneously cleaves to give the lactone, dihydrocoumarin (57), formaldehyde and the phospho anion (58), this being repeated to cleave the second group *(Scheme 3.3)* [76].

4-ACYLOXY-1,3,2-DIOXAPHOSPHORINANE ESTERS

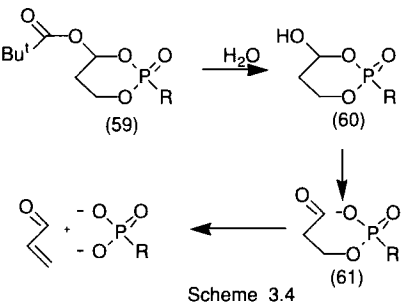
Farquhar and coworkers investigated the use of 2-phenoxy-2-oxo-4-pivaloyloxy-1,3,2-dioxophosphorinane (59, R = OPh) as a prodrug of the model compound phenyl phosphate [77]. The prodrug was reasonably stable at pH 7.4, with a half-life of 3.5 h, however in the presence of 20% human plasma, (59, R = OPh) was converted into phenyl phosphate with a half-life of 15 min. The decomposition is thought to require a single metabolic step as outlined in *Scheme 3.4*. This requires an esterase-catalyzed hydrolysis to



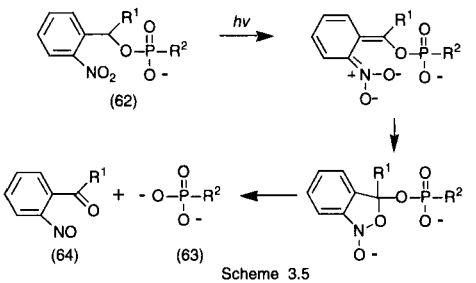
Scheme 3.3

give the hemi-acetal (60, R = OPh) which ring opens to aldehyde (61, R = OPh). Spontaneous elimination of acrolein gives phenyl phosphate.

This approach has been applied to the delivery of the 5′-monophosphate of anticancer nucleoside, 2′-deoxy-5-fluorouridine (FdU) [78, 79], in which prodrug (59, R = 5′-FdU) cleaves to FdU 5′-monophosphate and acrolein in the presence of carboxyesterase. The prodrug inhibited DNA synthesis in both Chinese hamster ovary cells and thymidine kinase-deficient mouse fibroblasts; this effect has been attributed to intracellular metabolism to FdU 5′-monophosphate and subsequent inhibition of thymidine monophosphate synthase. The prodrug was also as effective as 5-fluorouracil in prolonging the life-span of mice with P-388 leukaemia. The prodrug was tolerated better by mice when administered with 2-mercaptoethanesulphonic acid, a scavenger for the toxic by-product, acrolein.



Scheme 3.4

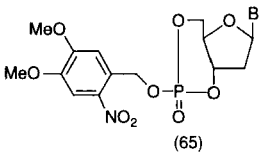


(62) (64) (63)

Scheme 3.5

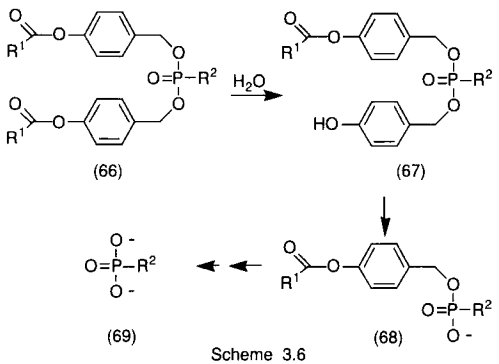
BENZYL ESTERS

o-Nitrobenzyl phosphate esters (62, R^1 = H or Me) have received considerable attention as 'caged' analogues of a range of biological phosphates. Upon photolysis there is rapid formation of a high concentration of phosphate (63) together with 2-nitrosobenzaldehyde (64, R^1 = H) or 2-nitrosoacetophenone (64, R^1 = Me) by the route outlined in *Scheme 3.5* [80, 81]. *o*-Nitrobenzyl esters are useful more as biochemical tools than for drug delivery, and applications have included the generation of ATP by photolysis of (62, R^1 = Me, R^2 = ADP) [82], c-AMP and c-GMP by the photolysis of (65, B = adenyl or guanyl) [83] and inositol 1,4,5-trisphosphate by the photolysis of a mono[1-(2-nitrophenyl)ethyl] ester at either the 1, 4 or 5-phosphate group (62, R^1 = Me, R^2 = $InsP_2$) [84, 85].



(65)

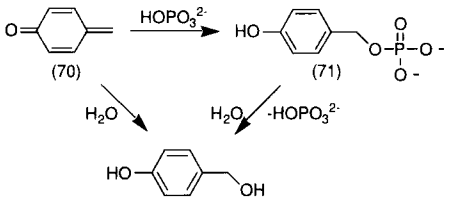
The bis(4-acetoxybenzyl) ester of the model compound, methylphosphonate (66, R^1 = R^2 = Me) [86] has been prepared with the rationale that the second 4-acyloxybenzyl protecting group may be removed more easily than a second acyloxymethyl substituent: compounds bearing a negative charge are poor substrates for esterases and the slow removal of the second acyloxymethyl group could be attributed to the presence of the P-O^- group in the active site. For the 4-acetoxybenzyl derivative, the site of esterase attack is 4Å (the length of an aromatic ring plus a C-C bond) further removed from the negative charge when compared with the acyloxymethyl analogue. The



Scheme 3.6

diester (66, $R^1 = R^2 = Me$) shows high chemical stability (half-life 55.4 h at 36.4°C), whereas in the presence of porcine liver carboxyesterase, hydrolysis of the acetyl group gives intermediate (67), the electron donating 4-hydroxybenzyl group of which promotes C-O bond cleavage leading to the formation of monoester (68). Hydrolysis of the second 4-acetoxybenzyl group was slower, but the formation of methylphosphonate (69, $R^2 = Me$) was observed *(Scheme 3.6)*. Hydrolysis of (66, $R^1 = R^2 = Me$) in $H_2{}^{18}O$ gave methylphosphonate (69, $R^2 = Me$) without incorporation of ^{18}O label; this evidence supports a mechanism by C-O bond cleavage without nucleophilic attack at phosphorus, as outlined in *Scheme 3.6* [87]. Although some of the quinone methide intermediate (70) reacts directly with water, the majority reacts first with the phosphate buffer to give 4-hydroxybenzyl phosphate (71) which hydrolyzes to 4-hydroxybenzyl alcohol with a half-life of 1 h *(Scheme 3.7)*.

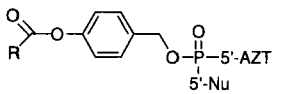
To explore the scope of the 4-acyloxybenzyl approach, bis(4-acyloxybenzyl) esters of the methyl carboxyester of the antiviral phosphonoacetate (66, $R^2 = MeO_2CCH_2$) [87] have been prepared. The acyl group has been varied (R^1 = Me, Et, Pr, Bu, Pr^i or Bu^t) to influence the esterase catalyzed



Scheme 3.7

hydrolysis to the parent drug, the Pr and Pr^i analogues being the most reactive. The formation of methoxycarbonylmethylphosphonate (69, $R^2 = MeO_2CCH_2$) was observed in porcine liver carboxyesterase, human plasma and porcine brain, but further hydrolysis to phosphonoacetate was not detected.

The 4-acyloxybenzyl delivery strategy has been applied to the antiviral nucleoside AZT, with the synthesis and evaluation of bis(4-acyloxybenzyl) AZT 5′-monophosphate (66, R^2 = 5′-AZT) [88]. Hydrolysis, catalyzed by porcine liver carboxyesterase, gives the 5′-monophosphate of AZT. Although as active as AZT in C8166 cells, these triesters were not active against HIV-1 in the thymidine kinase-deficient cell line, which suggests that their antiviral activity may be attributed to a release of AZT from its 5′-monophosphate catalyzed by phosphatases. The 4-acyloxybenzyl esters of the 5′,5′-nucleotide dimer of AZT (72, Nu = 5′-AZT), and the mixed nucleotide dimer between AZT and ddI (72, Nu = 5′-ddI) were also prepared [89]. Metabolism studies showed that the triesters degraded first to the nucleotide dimer, with further metabolism being catalyzed by phosphodiesterases and phosphatases, giving AZT and/or ddI and their 5′-monophosphates. The AZT nucleotide dimers were significantly more active than AZT against HIV-1 in a thymidine kinase-deficient JM cell-line, suggesting some intracellular delivery of AZT 5′-monophosphate.



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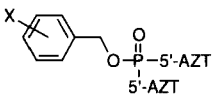
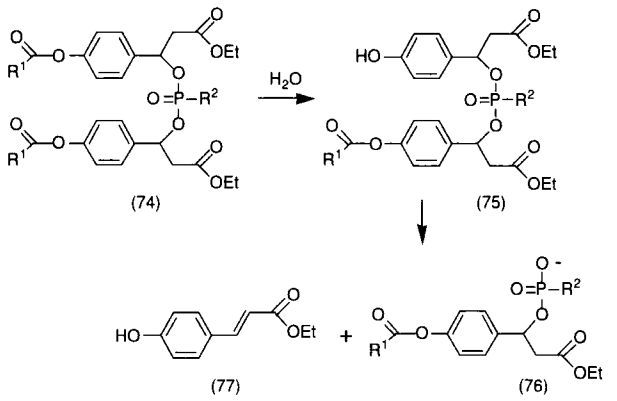


(73)

Meier and coworkers have prepared a range of substituted benzyl esters (X = 4-Me, H, 4-Cl, 4-CN or 3-NO_2) of the 5′,5′-nucleotide dimer of AZT (73) [90]. The triesters hydrolyzed cleanly to the nucleotide dimers in either phosphate buffer (pH 7.5) or RPMI culture medium/10% heat-inactivated foetal calf serum, at 37°C. The rate was dependent on the benzyl substituent, with half-lives ranging from 22 min (4-Me) to 9 days (3-NO_2) in the RPMI culture medium. The triesters showed activity comparable with AZT against HIV-1 and HIV-2 in CEM cells. However, the triesters were not active against HIV-2 in a thymidine kinase-deficient cell line, suggesting the release of AZT rather than its 5′-monophosphate.

Glazier and coworkers [91, 92] have prepared prodrugs of methylphosphonate bearing an α-substituted benzyl ester group (74, R^2 = Me) for which there is an internal mechanism for deactivation of the reactive, potentially toxic reactive quinone methide intermediate. Upon hydrolysis of a 4-



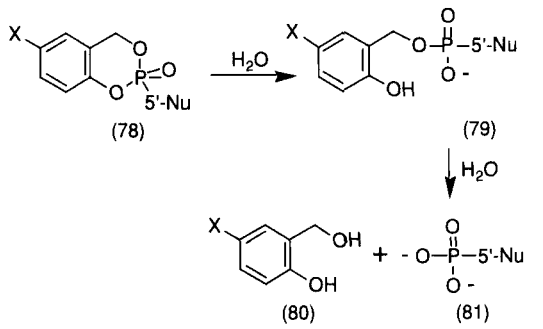
Scheme 3.8

acyloxy ester of (74), the 4-hydroxy substituent of intermediate (75) promotes cleavage of the C-O bond to give the phosphonate monoester (76) together with ethyl 4-hydroxycinnamate (77), a food preservative *(Scheme 3.8)*. Repeat of this bioactivation gives methylphosphonate. This prodrug system has been applied to AZT 5′-monophosphate, with the phosphate triester analogue (74, R^2 = 5′-AZT) showing activity against HIV-1 and hepatitis B.

Meier has prepared cyclic prodrugs (78, X = OMe, Me, H, Cl or NO_2, Nu = 5′-ddT or 5′-d4T), bearing both an aryl and a benzyl phosphate ester [93]. Chemical hydrolyses of (78) at pH 7.5 and 37°C proceeded by reaction of the aryl ester to give (79), the *o*-hydroxy substituent of which promotes cleavage of the benzyl ester to give the salicyl alcohols (80) and the nucleoside 5′-monophosphate (81) *(Scheme 3.9)*. With electron-donating substituents (X = OMe, Me), the hydrolyses of (78) were slow (half-lives 9.33 and 7.00 h, respectively) with cleavage of the aryl ester being rate -determining. In contrast, with electron-withdrawing substituents (X = Cl, NO_2), the hydrolyses of (78) were rapid (half-lives 1.56 and 0.2 h, respectively), and cleavage of the benzyl ester was rate-determining. The nature of the substituent, X, allows fine tuning of the rate of chemical hydrolysis of this prodrug modification.

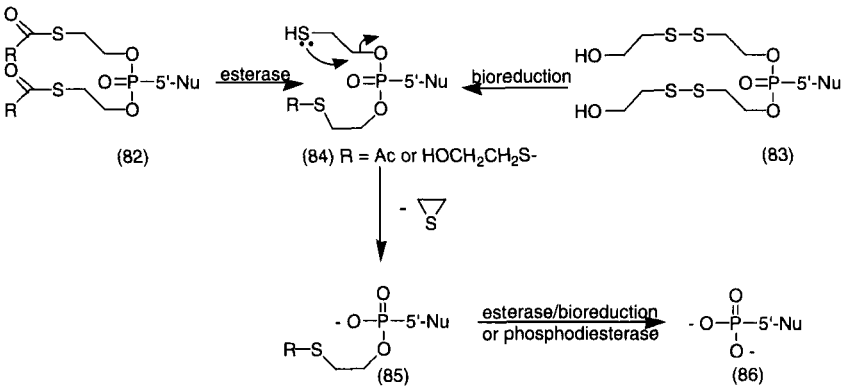
S-ACYLTHIOETHYL AND DITHIODIETHYL ESTERS

Imbach and coworkers [94] have reported two ways in which the unstable thioethyl phosphate ester can be formed following bioactivation with enzymes. Using ddU as a model nucleoside, the bis(*S*-acetylthioethanol)



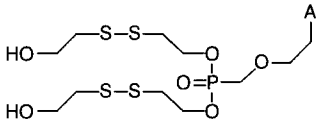
Scheme 3.9

(SATE, 82, R = Me, Nu = 5′-ddU) and bis(dithiodiethanol) (DTE, 83, Nu = 5′-ddU) derivatives have been prepared. The SATE prodrug undergoes bioactivation with esterase, whereas the DTE analogue requires a bioreductive reaction *(Scheme 3.10)*, each giving rise to the thioethyl phosphate triester (84). This intermediate is unstable and it spontaneously decomposes to the corresponding diester (85) and ethylene sulphide. Repetition of these bioactivation reactions or reaction with phosphodiesterases would give ddU 5′-monophosphate (86, Nu = 5′-ddU). Whereas ddU was inactive, the prodrugs (82, R = Me, Nu = 5′-ddU) and (83, Nu = 5′-ddU) were active against HIV-1 in both normal and thymidine kinase-deficient cells, and hydrolysis studies confirmed the formation of ddU 5′-monophosphate after cell penetration [95].

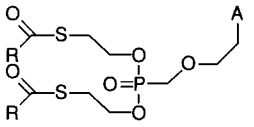


Scheme 3.10

These prodrug strategies have been reported for a range of nucleoside analogues. The bis(DTE) analogues of AZT (83, Nu = 5′-AZT) and the acyclic nucleotide phosphonate analogue PMEA (87) [95] have been evaluated. For AZT, the prodrug showed HIV-1 activity in a thymidine kinase-deficient cell line, whereas AZT was inactive, suggesting that AZT 5′-monophosphate is released intracellularly. For PMEA, prodrug (87) exhibited greater HIV-1 activity than the parent nucleotide, which was attributed to enhanced cellular uptake of the prodrug followed by intracellular release of PMEA. Bis-(SATE) esters of AZT 5′-monophosphate (82, Nu = 5′-AZT, R = Me, Pr^i, Bu^t or Ph) also inhibited HIV-1 in a thymidine kinase-deficient cell line, with the acetyl analogue being the most active (EC_{50} 0.049 μM). Hydrolysis studies showed that increasing the bulk of the acyl group decreased the rate of esterase-catalysed hydrolysis, which could be used to control the rate of intracellular delivery of AZT 5′-monophosphate [96, 97]. The bis(SATE) analogue of the antiviral nucleotide phosphonate PMEA (88) has also been prepared as an approach to improve its oral bioavailability [98].

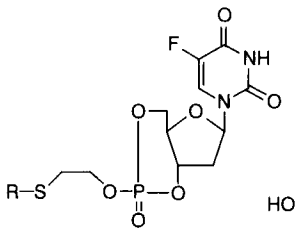


(87)

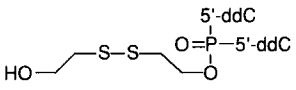


(88)

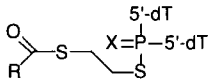
Similarly, the bis(SATE) ester of ddA 5′-monophosphate (82, Nu = 5′-ddA, EC_{50} 11 nM) was an effective prodrug of ddA 5′-monophosphate. Prodrug (82, Nu = 5′-ddA) showed activity comparable with AZT and was 1000-fold more active than ddA against HIV in MT-4 cells [99, 100]. In contrast, the DTE and SATE triesters of the 5′-monophosphate of the anti-cancer nucleoside FdU (82 and 83, Nu = 5′-FdU) and the 3′,5′-cyclic monophosphate of FdU (89, R = Ac or $HOCH_2CH_2S$-) showed lower or similar anti-tumour activities to FdU, suggesting that these prodrugs are ineffective at the intracellular delivery of FdU 5′-monophosphate [101].



(89)



(90)

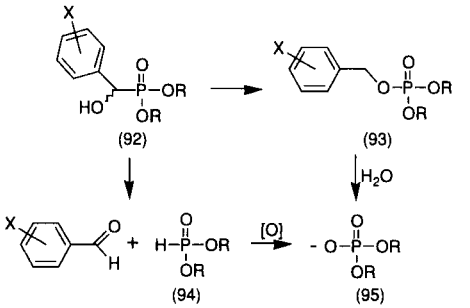


(91)

The DTE group has been applied to the nucleotide dimer of ddC (90) [95]. The triester had half-lives of 56 h and 50 h in phosphate buffer (pH 7.2) and culture medium, respectively, showing that it was reasonably stable under the antiviral assay conditions. In contrast, in CEM extract the triester had a half-life of only 0.5 h giving rise to the nucleotide dimer upon reductase-activation, which suggested that intracellular cleavage would occur. However, further metabolism of the nucleotide dimer was not observed. Similarly, the SATE esters of the phosphorothiolate and phosphorodithiolate nucleotide dimers of thymidine (91, R = Me or Bu^t, X = O or S) have been shown to degrade to the nucleotide dimer in CEM cell extracts, and this approach could be further extended to design prodrugs for the delivery of oligonucleotides [102, 103].

α-HYDROXYBENZYLPHOSPHONATES

A novel prodrug strategy based on the α-hydroxybenzylphosphonate group has recently been developed by Meier [104]. α-Hydroxybenzylphosphonate diesters (92) can either spontaneously rearrange to a benzyl phosphate triester (93) or cleave to give the H-phosphonate (94) and benzaldehyde under physiological conditions. The pathway choice can be controlled by the substituent on the aromatic ring: the former pathway is promoted by strongly electron-withdrawing substituents, whereas the latter pathway is promoted by weak electron-withdrawing or electron-donating substituents. The triester (93) can be cleaved by hydrolysis and the H-phosphonate (94) can be oxidized, both giving diester (95) *(Scheme 3.11)*, which can be cleaved to the phosphate monoester by phosphodiesterase enzymes. This approach has been applied to the delivery of nucleotide dimers of ddT [104] and AZT

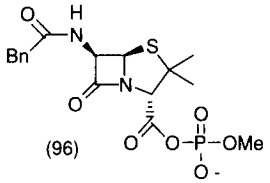


Scheme 3.11

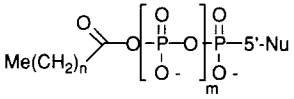
[105, 106], and chemistry has been developed to incorporate these into oligonucleotides [107].

ACYL PHOSPHATE ESTERS

The prodrug, acyl methyl phosphate benzylpenicillin (96), can release benzylpenicillin by hydrolysis of the acyl phosphate (half-life 90 h at pH 7.5 and 25°C). Moreover, (96) can also interact with β-lactamase by two mechanisms: it can either be a substrate, with the hydroxyl group of serine-70 of β-lactamase cleaving the lactam ring or can act as an irreversible inhibitor. The amino side-chain of Lys-234, which normally stabilizes the carboxylate group of the antibiotic, can react at the acyl group to form a covalent amide bond to the protein, with methyl phosphate as the by-product [108].



(96)



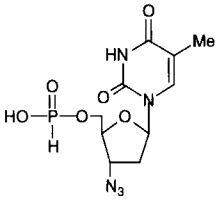
(97)

Nucleoside analogues require phosphorylation to the 5′-triphosphate by cellular kinases prior to their inhibition of DNA synthesis. However, most studies have focused upon prodrugs of monophosphates, whereas for some nucleosides (for example, AZT) the formation of the diphosphate is rate limiting. Huynh-Dinh and coworkers [109, 110] have recently prepared P-acyl esters of 5′-di- (m = 1) and tri-phosphates (m = 2) of both AZT and d4T (97, n = 6, 10, 12 or 14, Nu = 5′-AZT or 5′-d4T). The lipophilic acyl chain should facilitate passive diffusion across cellular membranes. Chemical hydrolyses at 37°C and physiological pH showed that the acyl phosphates were cleanly converted to the corresponding nucleoside di- and tri-phosphates.

H-PHOSPHONATES

5′-H-Phosphonates of nucleosides are only weakly acidic and they may diffuse through cell membranes before oxidation to the 5′-monophosphate. The H-phosphonate of AZT (98) has been evaluated by two groups. Imbach and coworkers [111] have shown that (98) was metabolized to AZT in both cell culture medium and CEM cell extract. Consistent with this observation,

they reported that (98) was inactive against HIV-1 in a thymidine kinase-deficient cell line. In contrast, Boal and coworkers found that (98) was metabolized to AZT 5′-monophosphate in U937 cells [112].

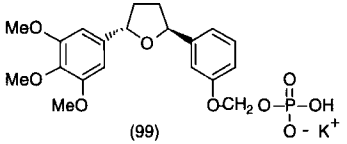


(98)

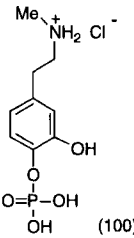
WATER SOLUBLE PRODRUGS

A simple and effective method of increasing the aqueous solubility of a drug containing an alcohol group is to derivatize it as a phosphate monoester. The parent drug is released by hydrolysis of the phosphate prodrug in the presence of phosphatases. This approach has been applied to a number of drugs. The water soluble 21-phosphates of a range of steroids, for example, the anti-inflammatory steroid cortisone, have been used for intravenous administration. Hydrolysis of the monophosphate to give the steroid is catalyzed by phosphatase [113]. Derivatization of the PAF antagonist (99) with a phosphate group increases the aqueous solubility from <100 μg/ml to >30 mg/ml, and gave a very active compound *in vivo* (ED_{50} 6.5 mg/kg, i.v. in rats) which could be administered intravenously [114]. A dopamine prodrug, *N*-methyldopamine 4-phosphate (100) has also been reported [115].

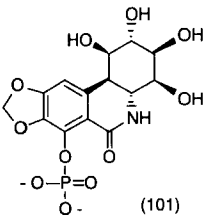
Pre-clinical evaluations of the anticancer drugs pancratistatin and combretastatin A-4 have been hampered by their very low aqueous solubilities. In each case phosphorylation of the phenolic position to give (101) and (102) substantially increased their solubilities in water [116, 117, 118] and these phosphate salts are currently in pre-clinical development.



(99)

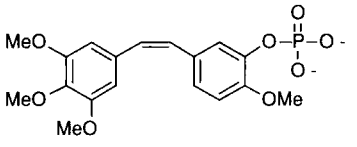


(100)

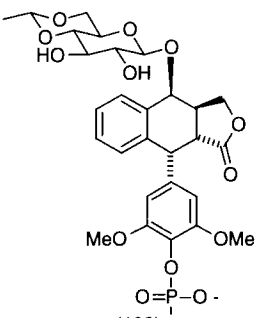


(101)

Similarly, etoposide 4′-phosphate (103) is a clinically active, water soluble prodrug of the clinically approved anticancer drug etoposide [119]. Phase 1 studies have shown that etoposide 4′-phosphate is rapidly converted into etoposide and that the maximum tolerated dose (150 mg/m^2/day) and toxicity (myelosuppression) are similar to those of etoposide [120].

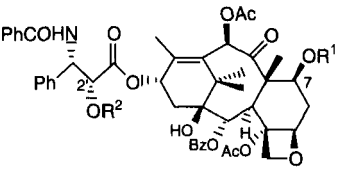


(102)



(103)

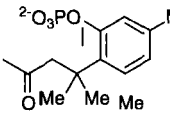
One of the major problems associated with the anticancer compound paclitaxel (Taxol®, 104) [121] is its low aqueous solubility and to overcome this, a variety of phosphate derivatives have been prepared. The group at Bristol-Myers Squibb first synthesized the simple phosphate derivatives of paclitaxel at C-7 (105) and C-2′ (106). These compounds were soluble in water, stable towards plasma and alkaline phosphatase, and showed no antitumour activity *in vivo* [122] or in a tubulin polymerization assay [123]. The finding that (105) and (106) were only poor substrates for phosphatase was attributed to the attachment of the phosphate group too close to the paclitaxel nucleus, and the next generation of compounds had a linker between paclitaxel and the phosphate group. Derivatives bearing the (2-phosphate phenyl)propionic ester group on either C-7 (107) or C-2′ (108) also showed good aqueous solubility and prodrug (107) exhibited antitumour activity comparable to that of paclitaxel against the M109 murine tumour model [124]. Both prodrugs generated paclitaxel in the presence of alkaline phosphatase by initial cleavage to the 2-hydroxy derivative (112) *(Scheme 3.12)*. Cyclization to the lactone, hydrocoumarin (113) is assisted by the presence of the 3,3-dimethyl substituents [124]. In the presence of phosphatase these prodrugs promoted and stabilized tubulin polymerization [123], consistent with the mode of action of paclitaxel.

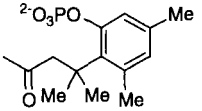


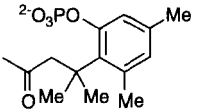
(104) $R^1 = R^2 = H$

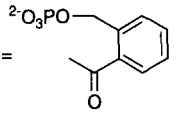
(105) $R^1 = PO_3^{2-}$ $R^2 = H$

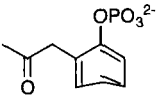
(106) $R^1 = H$ $R^2 = PO_3^{2-}$

(107) $R^1 =$  $R^2 = H$

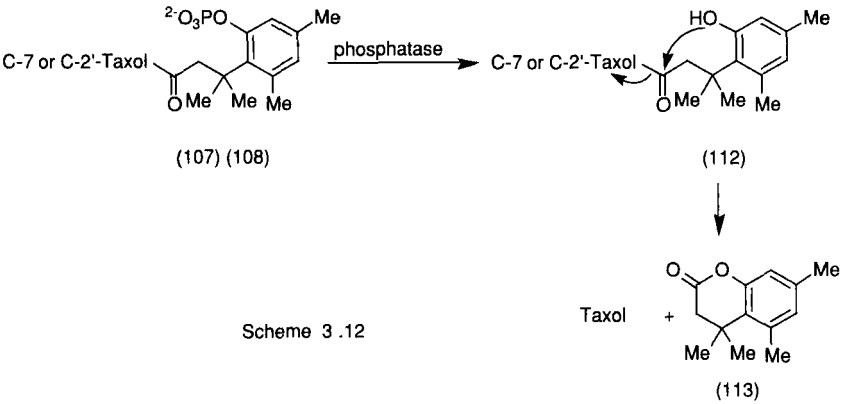
(108) $R^1 = H$ $R^2 =$ 

(109) $R^1 =$  $R^2 = -CO_2Et$

(110) $R^1 =$  $R^2 = -CO_2Et$

(111) $R^1 =$  $R^2 = -CO_2Et$

In a subsequent study, this prodrug modification was applied to paclitaxel-2′-ethylcarbonate with the synthesis of 7-phosphate (109). Related prodrugs (110) and (111) were also prepared. These derivatives cleaved in a similar way to (107), (108) and (109), with initial hydrolysis of the phosphate group catalyzed by phosphatase and subsequent lactonization to give phtha-

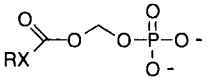


Scheme 3.12

(107) (108) (112) (113)

lide from (110) and 2-coumaranone from (111). Compounds (109), (110) and (111) were soluble in water, and (109) and (110) showed comparable activity to paclitaxel [125].

Phosphoryloxymethyl carbamates (114, X = NH) and carbonates (114, X = O) have also been evaluated as linkers to make phosphate derivatives of drugs containing amines and hindered alcohols, respectively. The carbonates were too unstable towards chemical hydrolysis, whereas the carbamates were more stable and should release the amine (for example, benzocaine) *in vivo* in the presence of phosphatase [126].



(114)

In an attempt to achieve higher tumour selectivity, antibody-directed enzyme prodrug therapy (ADEPT) has been applied to phosphate prodrugs of the anticancer drugs etoposide, doxorubicin, mitomycin and phenol mustard [127, 128]. The prodrugs release the parent drug at the tumour by hydrolysis catalyzed by alkaline phosphatase linked to a tumour-selective antibody.

CONCLUSION

A wide range of prodrug modifications have been utilized in the design of lipophilic triesters of phosphates or diesters of phosphonates. Cleavage to the parent drug can occur by chemical hydrolysis or be catalyzed by a range of enzymes, including carboxyesterases and phosphodiesterases. Esterase cleavage of the α-acyloxymethyl, 4-acyloxy-1,3,2-dioxaphosphorinane, 4-acyloxybenzyl and *S*-acylthioethyl phosphate and phosphonate esters give the toxic metabolites, formaldehyde, acrolein, quinone methide and ethylene sulphide, respectively, and therefore the toxicology of these prodrugs would need to be thoroughly evaluated. A variation of the 4-acyloxybenzyl prodrug has been reported in which the quinone methide intermediate is trapped as the non-toxic food preservative ethyl 4-hydroxycinnamate. Phospholipid esters are also promising prodrugs as the byproducts, for example, L-α-phosphatidic acid, are naturally occurring. Some of the prodrugs discussed have been used as biochemical tools, and others, for example, bis(pivaloyloxymethyl) PMEA will be evaluated in the clinic.

Phosphate derivatives of the anticancer drugs, for example, paclitaxel and etoposide, have been used to dramatically increase their water solubilities, and in the presence of phosphatase, the phosphate groups are cleaved. Tumour targeting is also possible with these analogues through ADEPT.

Prodrug design for phosphates and phosphonates will continue to be an active research area. Future objectives will focus on the design of new bioreversible moieties and on the development of prodrugs of oligonucleotides for gene therapy and of nucleoside triphosphates.

ACKNOWLEDGEMENTS

We thank BBSRC and Aston Molecules, Aston Science Park, Birmingham for a Total-Technology Studentship and the MRC AIDS-Directed Programme for a project grant.

REFERENCES

1 Tsuji, A. and Tamai, I. (1989) Biochem. Pharmacol. 38, 1022–1025.
2 Béliveau, R., Jetté, M., Demeule, M., Potier, M., Lee, J. and Tenenhouse, H.S. (1990) Biochim. Biophys. Acta 1028, 110–116.
3 Bayer, E., Gugel, K.H., Hägele, K., Hagenmaier, H., Jessipow, S., König, W.A. and Zähner, Z. (1972) Helv. Chim. Acta 55, 224–239.
4 Kahan, F.M., Kahan, J.S., Cassidy, P.J. and Kropp, H. (1974) Ann. N.Y. Acad. Sci. 235, 364–386.
5 Li, T., Krasne, S.J., Persson, B., Kaback, H.R. and Diederich, F. (1993) J. Org. Chem. 58, 380–384.
6 Bundgaard, H. (1991) in A Textbook of Drug Design and Development (Krogsgaard-Larsen, P. and Bundgaard, H., eds), pp. 113–191, Harwood Academic Publishers, Reading.
7 Norbeck, D.W. (1990) Annu. Rep. Med. Chem. 25, 149–158.
8 Mansuri, M.M. and Martin, J.C. (1991) Annu. Rep. Med. Chem. 26, 133–140.
9 Huynh-Dinh, T. (1993) Curr. Opin. Invest. Drugs 2, 905–915.
10 Jones, R.J. and Bischofberger, N. (1995) Antiviral Res. 27, 1–17.
11 Perigaud, C., Girardet, J.-L., Gosselin, G. and Imbach, J.-L. (1995) Adv. Antiviral Drug Design 2, 147–172.
12 Revankar, G.R., Huffman, J.H., Allen, L.B., Sidwell, R.W., Robins, R.K. and Tolman, R.L. (1975) J. Med. Chem. 18, 721–726.
13 Rosowsky, A., Kim, S.H., Ross, J. and Wick, M.M. (1982) J. Med. Chem. 25, 171–178.
14 Sergheraert, C., Pierlot, C., Tartar, A., Henin, Y. and Lemaitre, M. (1993) J. Med. Chem. 36, 826–830.
15 Long, R.A., Szekeres, G.L., Khwaja, T.A., Sidwell, R.W., Simon, L.N. and Robins, R.K. (1972) J. Med. Chem. 15, 1215–1218.
16 Meyer, R.B., Stone, T.E. and Ullman, B. (1979) J. Med. Chem. 22, 811–815.
17 Bischofberger, N., Hitchcock, M.J.M., Chen, M.S., Barkhimer, D.B., Cundy, K.C., Kent,

K.M., Lacy, S.A., Lee, W.A., Li, Z.-H., Mendel, D.B., Smee, D.F. and Smith, J.L. (1994) Antimicrob. Agents Chemother. 38, 2387–2391.
18 Hunston, R.N., Jones, A.S., McGuigan, C., Walker, R.T., Balzarini, J. and De Clercq, E. (1984) J. Med. Chem. 27, 440–444.
19 Colin, B., Jones, N.M., McGuigan, C. and Riley, P.A. (1989) Nucleic Acids Res. 17, 7195–7201.
20 McGuigan, C., Tollerfield, S.M. and Riley, P.A. (1989) Nucleic Acids Res. 17, 6065–6075.
21 McGuigan, C., Shackleton, J.M., Tollerfield, S.M. and Riley P.A. (1989) Nucleic Acids Res. 17, 10171–10177.
22 McGuigan, C., Nicholls, S.R., O'Connor, T.J. and Kinchington, D. (1990) Antiviral Chem. Chemother. 1, 25–33.
23 McGuigan, C., O'Connor, T.J., Nicholls, S.R., Nickson, C. and Kinchington, D. (1990) Antiviral Chem. Chemother. 1, 355–360.
24 McGuigan, C., Nickson, C., O'Connor, T.J. and Kinchington, D. (1992) Antiviral Res. 17, 197–212.
25 McGuigan, C., Nickson, C., Petrik, J. and Karpas, A. (1992) FEBS Lett. 310, 171–174.
26 Le Bec, C. and Huynh-Dinh, T. (1991) Tetrahedron Lett. 6553–6556.
27 Henin, Y., Gouyette, C., Schwartz, O., Debouzy, J.-C., Neumann, J.-M. and Huynh-Dinh, T. (1991) J. Med. Chem. 34, 1830–1837.
28 Namane, A., Gouyette, C., Fillion, M.-P., Fillion, G. and Huynh-Dinh, T. (1992) J. Med. Chem. 35, 3039–3044.
29 Meier, C., Neumann, J.-M., André, F., Henin, Y. and Huynh-Dinh, T. (1992) J. Org. Chem. 57, 7300–7308.
30 Puech, F., Pompon, A., Lefebvre, I., Gosselin, G. and Imbach, J.L. (1992) Bioorg. Med. Chem. Lett. 2, 603–606.
31 Bel'skii, V.E. (1977) Russ. Chem. Rev. 46, 828–841.
32 Farrow, S.N., Jones, A.S., Kumar, A., Walker, R.T., Balzarini, J. and De Clercq, E. (1990) J. Med. Chem. 33, 1400–1406.
33 Shimizu, S.I., Balzarini, J., De Clercq, E. and Walker, R.T. (1992) Nucleosides Nucleotides 11, 583–594.
34 McGuigan, C., Pathirana, R.N., Mahmood, N., Devine, K.G. and Hay, A.J. (1992) Antiviral Res. 17, 311–321.
35 McGuigan, C., Pathirana, R.N., Davies, M.P.H., Balzarini, J. and De Clercq, E. (1994) Bioorg. Med. Chem. Lett. 4, 427–430.
36 Serafinowska, H.T., Ashton, R.J., Bailey, S., Harnden, M.R., Jackson, S.M. and Sutton, D. (1995) J. Med. Chem. 38, 1372–1379.
37 De Lombaert, S., Singh, K., Blanchard, I. and Soliman, V.F. (1994) Bioorg. Med. Chem. Lett. 4, 899–902.
38 Norén, J.O., Helgstrand, E., Johansson, N.G., Misiorny, A. and Stening, G. (1983) J. Med. Chem. 26, 264–270.
39 Zon, G. (1992) Prog. Med. Chem. 19, 205–246.
40 Connors, T.A., Cox, P.J., Farmer, P.B., Foster, A.B. and Jarman, M. (1974) Biochem. Pharmacol. 23, 115–129.
41 Hunston, R.N., Jehangir, M., Jones, A.S. and Walker, R.T. (1980) Tetrahedron 36, 2337–2340.
42 Jones, A.S., McGuigan, C., Walker, R.T., Balzarini, J. and De Clercq, E. (1984) J. Chem. Soc., Perkin Trans. 1, 1471–1474.
43 McGuigan, C., Devine, K.G., O'Connor, T.J., Galpin, S.A., Jeffries, D.J. and Kinchington, D. (1990) Antiviral Chem. Chemother. 1, 107–113.

44 Jones, B.C.N.M., McGuigan, C., O'Connor, T.J., Jeffries, D.J. and Kinchington, D. (1991) Antiviral Chem. Chemother. 2, 35–39.
45 McGuigan, C., Jones, B.C.N.M., Devine, K.G., Nicholls, S.R., O'Connor, T.J. and Kinchington, D. (1991) Bioorg. Med. Chem. Lett. 1, 729–732.
46 McGuigan, C., Sheeka, H.M., Mahmood, N. and Hay, A. (1993) Bioorg. Med. Chem. Lett. 3, 1203–1206.
47 McGuigan, C., Pathirana, R.N., Mahmood, N. and Hay, A.J. (1992) Bioorg. Med. Chem. Lett. 2, 701–704.
48 McGuigan, C., Pathirana, R.N., Balzarini, J. and De Clercq, E. (1993) J. Med. Chem. 36, 1048–1052.
49 Gabrielsen, B., Kirsi, J.J., Kwong, C.D., Carter, D.A., Krauth, C.A., Hanna, L.K., Huggins, J.W., Monath, T.P., Kefauver, D.F., Blough, H.A., Rankin, J.T., Bartz, C.M., Huffman, J.H., Smee, D.F., Sidwell, R.W., Shannon, W.M. and Secrist, J.A. (1994) Antiviral Chem. Chemother. 5, 209–220.
50 Hostetler, K.Y., Stuhmiller, L.M., Lenting, H.B.M., van den Bosch, H. and Richman, D.D. (1990) J. Biol. Chem. 265, 6112–6117.
51 Hostetler, K.Y., Carson, D.A. and Richman, D.D. (1991) J. Biol. Chem. 266, 11714–11717.
52 Piantadosi, C., Marasco, C.J., Morris-Natschke, S.L., Meyer, K.L., Gumus, F., Surles J.R., Ishaq, K.S., Kucera, L.S., Iyer, N., Wallen, C.A., Piantadosi, S. and Modest, E.J. (1991) J. Med. Chem. 34, 1408–1414.
53 Matsushita, T., Ryu, E.K., Hing, C.I. and MacCoss, M. (1981) Cancer Res. 41, 2707–2713.
54 Hostetler, K.Y., Parker, S., Sridhar, C.N., Martin, M.J., Li, J.-L., Stuhmiller, L.M., van Wijk, G.M.T., van den Bosch, H., Gardner, M.F., Aldern, K.A. and Richman, D.D. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 11835–11839.
55 Hostetler, K.Y., Richman, D.D., Carson, D.A., Stuhmiller, L.M., van Wijk, G.M.T. and van den Bosch, H. (1992) Antimicrob. Agents Chemother. 36, 2025–2029.
56 Van Wijk, G.M.T., Hostetler, K.Y., Kroneman, E., Richman, D.D., Sridhar, C.N., Kumar, R. and van den Bosch, H. (1994) Chem. Phys. Lipids 70, 213–222.
57 Farquhar, D., Srivastva, D.N., Kuttesch, N.J. and Saunders, P.P. (1983) J. Pharm. Sci. 72, 324–325.
58 Srivastva, D.N. and Farquhar, D. (1984) Bioorg. Chem. 12, 118–129.
59 Sastry, J.K., Nehete, P.N., Khan, S., Nowak, B.J., Plunkett, W., Arlinghaus, R.B. and Farquhar, D. (1991) Mol. Pharmacol. 41, 441–445.
60 Farquhar, D., Khan, S., Srivastva, D.N. and Saunders, P.P. (1994) J. Med. Chem. 37, 3902–3909.
61 Pompon, A., Lefebvre, I., Imbach, J.-L., Kahn, S. and Farquhar, D. (1994) Antiviral Chem. Chemother. 5, 91–98.
62 Schultz, C., Vajanaphanich, M., Harootunian, A.T., Sammak, P.J., Barrett, K.E. and Tsien, R.Y. (1993) J. Biol. Chem. 268, 6316–6322.
63 Starrett Jr., J.E., Tortolani, D.R., Russell, J., Hitchcock, M.J.M., Whiterock, V., Martin, J.C. and Mansuri, M.M. (1994) J. Med. Chem. 37, 1857–1864.
64 Starrett Jr., J.E., Tortolani, D.R., Hitchcock, M.J.M., Martin, J.C. and Mansuri, M.M. (1992) Antiviral Res., 1992, 19, 267–273.
65 Srinivas, R.V., Robbins, B.L., Connelly, M.C., Gong, Y.-F., Bischofberger, N. and Fridland, A. (1993) Antimicrob. Agents Chemother. 37, 2247–2250.
66 Srinivas, R.V., Robbins, B.L., Connelly, M.C., Gong, Y.-F., Fridland, A. and Bischofberger, N. (1994) Int. Antiviral News 2, 53–55.
67 Naesens, L., Neyts, J., Balzarini, J., Bischofberger, N. and De Clercq, E. (1995) Nucleosides Nucleotides 14, 767–770.

68 Naesens, L., Balzarini, J., Bischofberger, N. and De Clercq, E. (1996) Antimicrob. Agents Chemother. 40, 22–28.
69 Serafinowska, H.T., Ashton, R.J., Bailey, S., Harnden, M.R., Jackson, S.M. and Sutton, D. (1995) J. Med. Chem. 38, 1372–1379.
70 Iyer, R.P., Yu, D. and Agrawal, S. (1994) Bioorg. Med. Chem. Lett. 4, 2471–2476.
71 Barber, I., Rayner, B. and Imbach, J.-L. (1995) Nucleosides Nucleotides 14, 1083–1085.
72 Iyer, R.P., Phillips, L.R., Biddle, J.A., Thakker, D.R. and Egan, W. (1989) Tetrahedron Lett. 30, 7141–7144.
73 Iyer, R.P., Boal, J.H., Phillips, L.R., Thakker, D.R. and Egan, W. (1994) J. Pharm. Sci. 83, 1269–1273.
74 Thomson, W., Nicholls, D., Mitchell, A.G., Corner, J.A., Irwin, W.J. and Freeman, S. (1993) J. Chem. Soc., Perkin Trans. 1, 2303–2308.
75 Vajanaphanich, M., Schultz, C., Rudolf, M.T., Wasserman, M., Enyedi, P., Craxton, A., Shears, S.B., Tsien, R.Y., Barrett, K.E. and Traynor-Kaplan, A. (1994) Nature (London) 371, 711–714.
76 Friis, G.J. and Bungaard, H. (1996) Eur. J. Pharm. Sci. 4, 49–59.
77 Farquhar, D., Khan, S., Wilkerson, M.C. and Andersson, B.S. (1995) Tetrahedron Lett. 36, 655–658.
78 Freed, J.J., Farquhar, D. and Hampton, A. (1989) Biochem. Pharmacol. 38, 3193–3198.
79 Farquhar D., Chen, R. and Khan S. (1995) J. Med. Chem. 38, 488–495.
80 Gurney, A.M. and Lester, H.A. (1987) Physiol. Rev. 67, 583–617.
81 McCray, J.A. and Trentham, D.R. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 239–270.
82 Walker, J.W., Reid, G.P., McCray, J.A. and Trentham, D.R. (1988) J. Am. Chem. Soc. 110, 7170–7177.
83 Nerbonne, J.M., Richard, S., Nargeot, J. and Lester, H.A. (1984) Nature (London) 310, 74–76.
84 Walker, J.W., Somlyo, A.V., Goldman, Y.E., Somlyo, A.P. and Trentham, D.R. (1987) Nature (London) 327, 249–252.
85 Walker, J.W., Feeney, J. and Trentham, D.R. (1989) Biochemistry 28, 3272–3280.
86 Freeman, S., Irwin, W.J., Mitchell, A.G., Nicholls, D. and Thomson, W. (1991) J. Chem. Soc., Chem. Commun., 875–877.
87 Mitchell, A.G., Thomson, W., Nicholls, D., Irwin, W.J. and Freeman, S. (1992) J. Chem. Soc., Perkin Trans. 1, 2345–2353.
88 Thomson, W., Nicholls, D., Irwin, W.J., Al-Mushadani, J.S., Freeman, S., Karpas, A., Petrik, J., Mahmood, N. and Hay, A.J. (1993) J. Chem. Soc., Perkin Trans. 1, 1239–1245.
89 Routledge, A., Walker, I., Freeman, S., Hay, A. and Mahmood, N. (1995) Nucleosides Nucleotides 14, 1545–1558.
90 Meier, C., Habel, L.W., Balzarini, J. and De Clercq, E. (1995) Liebigs Ann. 2203–2208.
91 Glazier, A., Phosphorus Prodrugs, International Patent Publication Number WO/91/19721, Dec. 1991.
92 Glazier, A., Kwong, C., Rose, J., Buckheit, R., Korba, B., Abou-Donia, M., Smith, E. and Wright, G.E. (1992) Antiviral Res. Suppl. 1, Abstr. 66, 77.
93 Meier, C. (1996) Angew. Chem., Int. Ed. Engl. 35, 70–72.
94 Perigaud, C., Gosselin, G., Lefebvre, I., Girardet, J.-L., Benzaria, S., Barber, I. and Imbach, J.-L. (1993) Bioorg. Med. Chem. Lett. 3, 2521–2526.
95 Puech, F., Gosselin, G., Lefebvre, I., Pompon, A., Aubertin, M., Kirn, A. and Imbach, J.-L. (1993) Antiviral Res. 22, 155–174.
96 Lefebvre, I., Périgaud, C., Pompon, A., Aubertin, A.-M., Girardet, J.L., Kirn, A., Gosselin, G. and Imbach, J.-L. (1995) J. Med. Chem. 38, 3941–3950.

97 Lefebvre, I., Pompon, A., Périgaud, C., Girardet, J.L., Gosselin, G., Aubertin, A.-M., Kirn, A. and Imbach, J.-L. (1995) Nucleosides Nucleotides, 14, 763–766.
98 Benzaria, S., Gosselin, G., Pelicano, H., Maury, G., Aubertin, A.-M., Obert, G., Kirn, A. and Imbach, J.-L. (1995) Nucleosides Nucleotides 14, 563–565.
99 Périgaud, C., Aubertin, A.-M., Benzaria, S., Pelicano, H., Girardet, J.-L., Maury, G., Gosselin, G., Kirn, A. and Imbach, J.-L. (1994) Biochem. Pharmacol. 48, 11–14.
100 Périgaud, C., Gosselin, G., Benzaria, S., Girardet, J.-L., Maury, G., Pélicano, H., Aubertin, A.-M., Kirn, A. and Imbach, J.-L. (1995) Nucleosides Nucleotides 14, 789–791.
101 Girardet, J.-L., Gosselin, G., Périgaud, C., Balzarini, J., De Clercq, E. and Imbach, J.-L. (1995) Nucleosides Nucleotides, 645–647.
102 Barber, I., Rayner, B. and Imbach, J.-L. (1995) Bioorg. Med. Chem. Lett. 5, 563–568.
103 Imbach, J.-L., Gosselin, G. and Rayner, B. (1995) Nucleosides Nucleotides 14, 459.
104 Meier, C. (1993) Angew. Chem., Int. Ed. Engl. 32, 1704–1706.
105 Meier, C., Habel, L., Laux, W., De Clercq, E. and Balzarini, J. (1995) Nucleosides Nucleotides 14, 759–762.
106 Meier, C., Habel, L.W., Balzarini, J. and De Clercq, E. (1995) Liebigs Ann. 2195–2202.
107 Meier, C. and Mauritz, R. (1995) Nucleosides Nucleotides 14, 803–804.
108 Song, Y. and Kluger, R. (1994) Bioorg. Med. Chem. Lett. 4, 1225–1228.
109 Bonnaffé, D., Dupraz, B., Ughetto-Monfrin, J., Namane, A. and Huynh-Dinh, T. (1995) Nucleosides Nucleotides 14, 783–787.
110 Bonnaffé, D., Dupraz, B., Ughetto-Monfrin, J., Namane, A. and Huynh-Dinh, T. (1995) Tetrahedron Lett. 36, 531–534.
111 Gosselin, G., Périgaud, C., Lefebvre, I., Pompon, A., Aubertin, A.M., Kirn, A., Szabo, T., Stawinski, J. and Imbach, J.-L. (1993) Antiviral Res. 22, 143–153.
112 Boal, J.H., Iyer, R.P. and Egan, W. (1993) Nucleosides Nucleotides 12, 1075–1084.
113 Poos, G.I., Hirschmann, R., Bailey, G.A., Cutler, F.A., Sarett, L.H. and Chemerda, J.M. (1958) Chem. Ind., 1260–1261.
114 Girotra, N.N., Biftu, T., Ponpipom, M.M., Acton, J.J., Alberts, A.W., Bach, T.N., Ball, R.G., Bugianesi, R.L., Parsons, W.H., Chabala, J.C., Davies, P., Doebber, T.W., Doherty, J., Graham, D.W., Hwang, S.B., Kuo, C.H., Lam, M.-H., Luell, S., MacIntyre, D.E., Meurer, R., Roberts, C.D., Sahoo, S.P. and Wu, M.S. (1992) J. Med. Chem. 35, 3474–3482.
115 Ianelli, S. and Nardelli, M. (1995) Acta Cryst. C51, 1338–1341.
116 Pettit, G.R., Freeman, S., Simpson, M.J., Thompson, M.A., Boyd, M.R., Williams, M.D., Pettit III, G.R. and Doubek, D.L. (1995) Anti-Cancer Drug Design, 10, 243–250.
117 Pettit, G.R., Temple, C., Narayanan, V.L., Varma, R., Simpson, M.J., Boyd, M.R., Rener, G.A. and Bansal, N. (1995) Anti-Cancer Drug Design 10, 299–309.
118 Bedford, S.B., Quarterman, C.P., Rathbone, D.L., Slack, J.A., Griffin, R.J. and Stevens, M.F.G. (1996) Bioorg. Med. Chem. Lett. 6, 157–160.
119 Saulnier, M.G., Langley, D.R., Kadow, J.F., Senter, P.D., Knipe, J.O., Tun, M.M., Vyas, D.M. and Doyle, T.W. (1994) Bioorg. Med. Chem. Lett. 4, 2567–2572.
120 Fields, S.Z., Igwemezie, L.N., Kaul, S., Schacter, L.P., Schilder, R.J., Litam, P.P., Himpler, B.S., McAleer, C., Wright, J., Barbhaiya, R.H., Langer, C.J. and O'Dwyer, P. (1995) Clinical Cancer Res., 1, 105–111.
121 Vyas, D.M. and Kadow, J.F. (1995) Prog. Med. Chem. 32, 289–337.
122 Vyas, D.M., Wong, H., Crosswell, A.R., Casazza, A.M., Knipe, J.O., Mamber, S.W. and Doyle, T.W. (1993) Bioorg. Med. Chem. Lett. 3, 1357–1360.
123 Mamber, S.W., Mikkilineni, A.B., Pack, E.J., Rosser, M.P., Wong, H., Ueda, Y. and Forenza, S. (1995) J. Pharmacol. Exp. Ther. 274, 877–883.

124 Ueda, Y., Mikkilineni, A.B., Knipe, J.O., Rose, W.C., Casazza, A.M. and Vyas, D.M. (1993) Bioorg. Med. Chem. Lett. 3, 1761–1766.
125 Ueda, Y., Matiskella, J.D., Mikkilineni, A.B., Farina, V., Knipe, J.O., Rose, W.C., Casazza, A.M. and Vyas, D.M. (1995) Bioorg. Med. Chem. Lett. 5, 247–252.
126 Safadi, M., Oliyai, R. and Stella, V.J. (1993) Pharm. Res. 10, 1350–1355.
127 Blakey, D.C., Burke, P.J., Davies, D.H., Dowell, R.I., Melton, R.G., Springer, C.J. and Wright, A.F. (1995) Biochem. Soc. Trans. 23, 1047–1050.
128 Deonarain, M.P. and Epenetos, A.A. (1994) Br. J. Cancer 70, 786–794.

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Progress in Medicinal Chemistry – Vol. 34 edited by G.P. Ellis and D.K. Luscombe

4 Aldehydes as Biocides

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INTRODUCTION

Aldehydes have long been recognized for their biocidal properties. Disinfection and sterilization procedures with formaldehyde as the active agent in aqueous solution or as a gas have been used for many years [1]. Glutaraldehyde was first synthesized in 1908 [2] by boiling the ozonide of cyclopentene with water to give several products, including the aldehyde. Nevertheless, glutaraldehyde remained virtually unknown until research proved its suitability as a leather tanning agent [3]. The commercial availability of glutaraldehyde led to subsequent use as a fixative in electron microscopy [4] and as a cross-linking agent for proteins and enzymes [5]. Its sporicidal properties were established in 1963 [6, 7] and the compound was thus introduced into the field of sterilization and disinfection. Other aldehydes have also been evaluated for biocidal activity, but have shown varying degrees of efficacy [8].

Prior to the introduction of aldehydes, few chemical agents had the ability to kill spores within a relatively short period of time. Low temperature surface sterilization in liquid phase was largely restricted to formaldehyde, although contact times of up to 20 hours were required for sterilization [9], and had disadvantages such as irritation and toxicity. Glutaraldehyde, in contrast, was considered safer and more efficient, although its toxicity has been increasingly recognized since early reviews [5, 10]. This awareness has caused problems in practice, and an evaluation of aldehydes as biocides must necessarily take such health risks into account.*

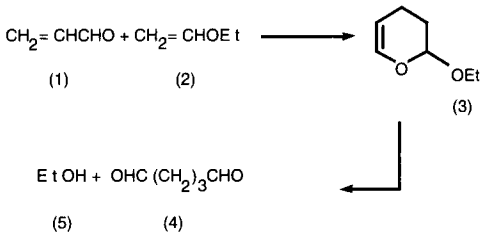
Today, aldehydes are used for purposes other than as biocides, for example, in leather tanning and electron microscopy. This review is concerned ex-

*The following abbreviations are used in this review: AIDS, acquired immune deficiency syndrome; AOAC, Association of Official Analytical Chemists; APase, alkaline phosphatase; BATH, bacterial adherence to hydrocarbons; cfu ml^{-1}, colony forming units per millilitre; EDTA, ethylenediaminetetra-acetic acid; HBV, hepatitis B virus; HIV, human immunodeficiency virus; kD, kilo Daltons; LPS, lipopolysaccharide; LTSF, low temperature steam with formaldehyde; MAI, *Mycobacterium avium-intracellulare*; mg l^{-1}, milligrams per litre; ppm, parts per million; RH, relative humidity; SDS-PAGE, sodium dodecyl sulphate polyacrylamie gel electrophoresis; w/v, weight by volume; w/w, weight by weight.

clusively with biocidal aspects and concentrates mainly on glutaraldehyde and formaldehyde since the vast majority of publications focus on these two compounds. Reference to other aldehydes is made where appropriate.

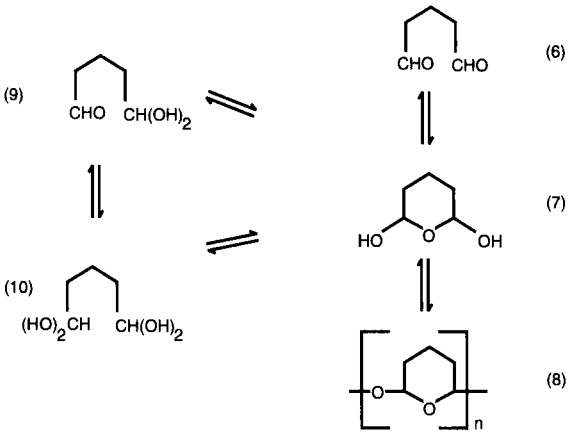
CHEMICAL PROPERTIES

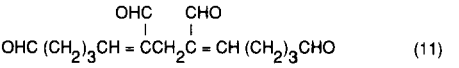
Glutaraldehyde (1,5-pentanedial) is a 5-carbon dialdehyde, empirical formula $C_5H_8O_2$. Its industrial production involves a two-step synthesis from acrolein (1) and vinyl ethyl ether (2) via an ethoxy dihydropyran (3) to yield glutaraldehyde (4) and ethanol (5) (*Scheme 4.1*). The two aldehyde groups react readily under suitable conditions, particularly with proteins, to form bisulphite complexes, oximes, cyanohydrins, acetals and hydrazones [11–13]. In the ultraviolet region, pure glutaraldehyde exhibits a single absorption maximum at 280nm, although a second peak at 235nm is often observed in commercial solutions due to impurities or polymers [14]. The appearance of polymers depends on storage temperature [15], and the rate of polymerization depends on temperature and pH [16, 17]. Analysis of the vapour evolved from aqueous glutaraldehyde solutions using gas chromatography, Fourier transform infra-red spectrometry and high-performance liquid chromatography suggests that the main component is monomeric glutaraldehyde with smaller amounts of butyraldehyde and methanol [18].



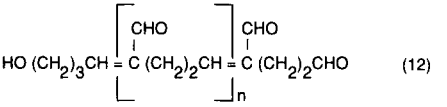
Scheme 4.1. Industrial production of glutaraldehyde.

The state of the glutaraldehyde molecule in solution remains controversial. In its simplest form, glutaraldehyde exists as a monomer (6) which is thought to exist in equilibrium with a cyclic hemiacetal (7) and an acetal-like polymer (8), with small amounts of mono- (9) and di-hydrates (10) [19, 20]. An increase in temperature produces more free aldehyde groups in acid solution, while in alkaline solution loss of reactive aldehyde groups is possible. Progression to the higher polymeric form (12), via an aldol-type polymer (11) [20] can occur with increased time and pH, since there is extensive loss





Intermediates



of aldehyde groups from polymerization in alkaline solution [3]. This may explain the rapid loss of biocidal activity of alkaline solutions on storage. It is considered that polymers in the alkaline range are unable to revert to the monomer, whereas those in the neutral and acid range revert easily [19]. Increased biocidal activity of the acid solution through heat or ultrasonics [21] may be due to displacement of equilibrium towards the monomeric form. The interrelationship between pH, time, temperature and the state of the glutaraldehyde molecule, and hence its biocidal activity, is depicted in *Figure 4.1* [20].

Glutaraldehyde is a highly reactive molecule. It reacts with various enzymes and proteins, but does not alter them sterically to inhibit all activity. The rate of reaction is pH-dependent, with an optimum over the range pH

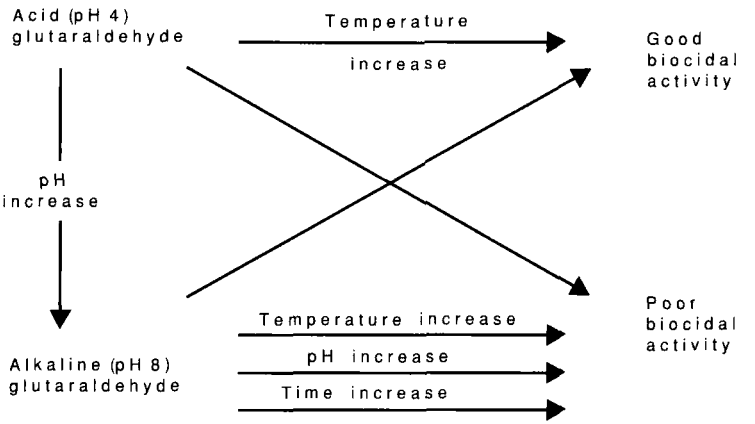
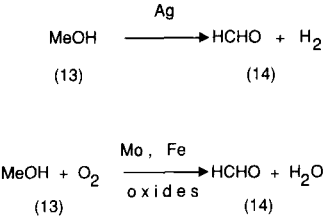


Figure 4.1. Influence of time, temperature and pH on the biocidal activity of acid and alkaline solutions (see [19]).

4–9 [22]. Glutaraldehyde prevents the dissociation of free ribosomes [5], but under the normal conditions of fixation [23], little reaction is apparent between nucleic acids and the aldehyde.

Formaldehyde (14) is a colourless gas with a typical unpleasant odour. The affinity of the gas for water is high, and at room temperature a solution of *c.* 37% w/w formaldehyde can be obtained [24]. Formaldehyde is produced industrially from methanol (13) by using a silver catalyst, or reacting it with oxygen in the presence of molybdenum and iron oxides [25]. The reactions are summarized in *Scheme 4.2.*

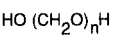


Scheme 4.2. Industrial production of formaldehyde (14) from methanol (13).

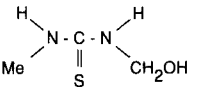
At temperatures below *c.* 80°C, formaldehyde gas polymerizes readily to various solid polymers. Trioxane (15) is the cyclic trimer of formaldehyde and is a colourless, crystalline water-soluble solid [25]. Paraformaldehyde (16, n = ~12–30) is the most common polymer and vapourizes at room tem-

perature to the monomeric form [25]. Paraformaldehyde is therefore often used as the source of formaldehyde gas [26] for disinfecting large areas. Formaldehyde in aqueous solution is normally marketed as formalin (*c.* 34–38% formaldehyde) containing 6–15% methanol as a stabilizer to prevent polymerization [25]. Formaldehyde vapour may also be released from formalin by the addition of potassium permanganate [13]. The activity of the vapour depends on aldehyde concentration, temperature and relative humidity.

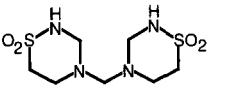
(15)



(16)



(17)



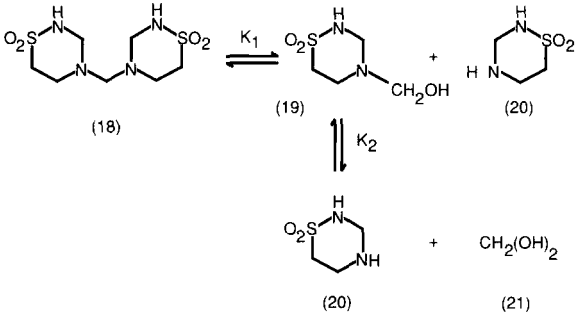
(18)

Noxythiolin (oxymethylenethiourea, 1-hydroxymethyl-3-methylthiourea, 17) is a bactericidal agent [27–29] that is believed to achieve its action by the release of formaldehyde [27, 30];

$$MeNHCSNHCH_2OH \rightarrow MeNHCSNH_2 + HCHO.$$

The amino sulphonic acid taurine has been used as the starting point for the antibacterial agent taurolin (4,4′-methylenebis(tetrahydro-1,2,4-thiadiazine-1,1,1′,1′-tetraoxide), 18) which is a condensate of two molecules of taurine and three of formaldehyde. Taurolin is water-soluble and stable in aqueous solution [31, 32]. It was considered to act as a non-toxic formaldehyde carrier, donating methylol groups to bacterial protein and lipopolysaccharide [32]. Taurolin was alleged to have lower affinity for formaldehyde than bacterial protein, but greater affinity than animal protein, resulting in selective lethal effects. However, more recent evidence suggests that such a scheme. is incorrect. When taurolin is dissolved in water, two molecules of the monomer (tetrahydro-1,2,4-thiadiazine 1,1-dioxide, GS204, 20) and its *N*-hydroxymethyl derivative (19) are released together with hydrated formaldehyde (21) [33], as illustrated in *Scheme 4.3*. The antibacterial activity of taurolin is greater than that of free formaldehyde [33, 34] and therefore

the activity of taurolin is not due entirely to the action of formaldehyde. Since GS204 has little antibacterial activity, the carbinolamine must have an important role.



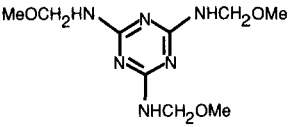
Scheme 4.3. Postulated equilibrium of taurolin in solution.

Another formaldehyde-releasing agent is hexamine (hexamethylenetetramine, methenamine). Hexamine is microbiologically inactive but breaks down by acid hydrolysis to release formaldehyde [34].

Other formaldehyde-releasing agents are the resins melamine formaldehyde (22) and urea formaldehyde (23). Melamine formaldehyde is produced from formaldehyde and melamine under alkaline conditions and urea formaldehyde is a mixture of monomethyloyl urea and dimethyloyl urea. These compounds release formaldehyde at raised temperatures, the rate of release being a function of time and temperature. These agents are, however, much less effective as sterilizing or disinfecting sources of formaldehyde than paraformaldehyde [35]. There is much greater release of formaldehyde vapour from paraformaldehyde than from the resins at various temperatures, and the biocidal activity is strictly a function of the available formaldehyde vapour.

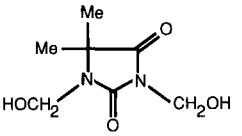
Dantoin (1,3-di(hydroxymethyl)-5,5-dimethylimidazole-2,4-dione, 24) is an imidazolone derivative. The compound is water-soluble, stable and non-corrosive with a slight odour of formaldehyde. Its mode of action is attributed to its ability to release formaldehyde. Its rate of release is greater at higher pH values, although it is more stable in the range pH 6–8 [13].

Formaldehyde interacts with protein by attaching itself to the primary amide as well as the amino group [36] and gives intermolecular cross-linkage of protein amino groups with phenolic or indole residues [37, 38]. In addition to reacting with many terminal groups in proteins, formaldehyde also reacts extensively with nucleic acids [39]. It appears to react by an initial loose binding, followed by a firm attachment after extensive reaction. For-



(22)

$HOCH_2NHCONHCH_2OH$ (23)



(24)

maldehyde reacts with the amino groups of bases, but is much less reactive with DNA than with RNA. A stable product results from the formation of methylene bridges with adenosine [39, 40]. Sublethal concentrations of formaldehyde inhibit the synthesis of cytoplasmic and nucleic acid material in bacteria [41–43] but subsequently the aldehyde is metabolized to carbon dioxide, allowing the resumption of cellular growth [44]. Formaldehyde also prevents the dissociation of ribosomes [45], although it is likely that the reaction here is with ribosomal protein, thus leading to the formation of cross-links between the protein subunits.

ANTIMICROBIAL ACTIVITY

LEVELS OF BIOCIDAL ACTIVITY

The categories of biocidal activity have been described in various publications [46–49] and are summarized in *Table 4.1*. High level biocides are active against all known microbial life forms, with the possible exception of large numbers of bacterial spores. Intermediate-level biocides are active against non-sporulating bacteria, fungi and most viruses, but not bacterial spores. Low level biocides lack activity against acid-fast bacilli (tubercle bacilli) but retain activity against most vegetative bacteria, some fungi and some viruses. The data presented in this review establish the two major biocidal al-

Table 4.1. LEVELS OF BIOCIDAL ACTIVITY

Level of biocide	*Biocidal action against*
High	Spores[1], tubercle bacilli, vegetative bacteria, fungi[2] and viruses
Intermediate	Tubercle bacilli, vegetative bacteria, fungi[2] and viruses[3]
Low	Vegetative bacteria, fungi[2] and viruses[3]

[1] Prolonged periods of time may be necessary
[2] Prolonged periods may be required for fungal spores
[3] Virucidal activity may be limited against non-lipid viruses

dehydes, glutaraldehyde and formaldehyde, as high-level biocides. Other aldehydes may be more properly regarded as intermediate-level biocides.

FACTORS INFLUENCING ACTIVITY

The activity of biocides depends on three main factors; (a) the nature of the physical environment, (b) the nature and condition of the micro-organism, and (c) the ability of the organism to render the biocide inactive. These processes have been extensively reviewed [50] and only those specific aspects relevant to the aldehydes will be discussed here.

The most significant factors influencing the activity of aldehydes include time of contact, temperature, concentration, pH and the presence of organic matter (for example, in soiled material). Biocidal efficacy increases with increasing concentration and as the period of contact with micro-organisms lengthens, although there is considerable variation in activity with different organisms. Thus, 2% alkaline glutaraldehyde kills vegetative bacteria more rapidly than bacterial spores and a concentration of 0.02% inhibits *Escherichia coli* and *Staphylococcus aureus* and 0.5% is fungicidal [51]. Suspension tests using commercial glutaraldehyde-based disinfectants have shown that formulations containing 2% of the aldehyde are effective whereas those with 1% glutaraldeyde have diminished activity [52]. Formaldehyde shows a similar range of activity although it is less effective against spores than glutaraldehyde [53].

The complex relationship between the parameters of temperature, pH and concentration for glutaraldehyde has been mentioned previously. The pH level has been proposed as the single most important factor for understanding the reaction of glutaraldehyde with the microbial cell (see [49]) and has significant effects on the stability and biocidal activity of the aldehyde [13, 51, 54–61]. Alkaline glutaraldehyde solutions become unstable and lose biocidal activity over time (*Figure 4.1*). Freshly prepared solutions, for which

chemical instability is irrelevant, are much more active at alkaline pH, suggesting that the increased biocidal activity is due to changes in the microbial cell surface rather than the dialdehyde molecule (see [49]). The number of reactive sites to which glutaraldehyde binds is increased with an increase in pH, resulting in a greater biocidal effect.

Maximum antimicrobial activity at 20°C resides at alkaline pH. A 99.99% reduction in viable count of *Bacillus anthracis* spores was obtained by 2% alkaline glutaraldehyde, whereas the acid solution caused a 50% reduction in the same time interval [62]. A similar pattern was observed by McGucken and Woodside [63], who exposed *E. coli* to 0.01% alkaline or acid glutaraldehyde. As temperature increases, this difference between alkaline and acid solutions is reduced [64, 65]. At 70°C, both solutions were found to be equally effective, producing a complete kill of *Bacillus subtilis* spores within 5 minutes [64], possibly due to a breakdown in polymeric forms to yield more free aldehyde groups at such an elevated temperature [20]. Indeed, there is little difference in activity at temperatures above 40°C, although the alkaline formulation tends to be less stable at higher temperatures [10, 51, 66].

Glutaraldehyde interacts strongly with the constituents (especially proteins, due to reactions with amino groups) of nutrient culture media at neutral to slightly alkaline pH, effectively resulting in a loss of 'free' aldehyde [5, 10, 49, 51]. It is therefore possible to obtain values for MICs in broth against particular organisms which are significantly greater than the minimum bactericidal concentration in a broth-free environment measured over a shorter time period [49]. It has therefore been suggested that MIC values of glutaraldehyde are meaningless [49]. The interaction of amino groups with glutaraldehyde has nevertheless been applied in experiments designed to assess its antimicrobial activity. Glycine, at a concentration of 2% w/v, can be used to neutralize glutaraldehyde to reduce carryover in the subsequent determination of biocidal effects [8]. Glycine itself may exert an antibacterial action against certain organisms [10, 49] and proper controls should be applied to test its lack of antimicrobial action under the appropriate conditions. Sodium bisulphite also reacts with glutaraldehyde via formation of a proposed glutaraldehyde-bisulphite complex, and, because of its lack of microbiocidal activity, has been proposed as a suitable alternative neutralizing agent [67, 68].

The interaction between glutaraldehyde and amino acids (and therefore proteins), would lead one to expect that the presence of organic soiling material would greatly reduce its effectiveness [5, 10]. However, numerous reports contradict this hypothesis, suggesting that the relationship between organic material and glutaraldehyde is complicated. Gelinas and Goulet [69] reported a high resistance of glutaraldehyde to neutralization by organic mat-

ter. The presence of 20% blood serum [70] or 1% whole blood [71] does not appear to adversely affect activity of glutaraldehyde. However, a 60% decrease in free aldehyde concentration occurs when alkaline glutaraldehyde is added to malt extract broth [72], although this occurs over 6 hours at 37°C. These authors propose that organic matter may not appear to affect activity adversely since the reaction is slow compared with the rapid rate of uptake of the aldehyde by cells. Thus, higher concentrations of glutaraldehyde may be reduced to a certain extent, but loss of activity will become more apparent when lower concentrations are used. Nevertheless, a commercial recovery medium has been described which effectively neutralizes the antimicrobial activity of glutaraldehyde [73].

In the liquid phase, time-survival curves of bacterial spores treated with formaldehyde have been reported to show initial shoulders, irrespective of whether aqueous or alcoholic solutions are used [62, 74], although this phenomenon has not been observed by other workers [75]. Various alcohols have also been shown to reduce the sporicidal activity of glutaraldehyde [62, 63].

The bactericidal activity of formaldehyde, and formaldehyde-releasing disinfectants, is influenced significantly by temperature, with extensive spore inactivation at temperatures of 40°C and above [75–77]. pH does not seem to influence the antimicrobial efficacy of formaldehyde, or any other aldehyde with the notable exception of glutaraldehyde [8].

In the vapour phase, there is a linear relationship between the concentration of formaldehyde and the killing rate, but little effect on disinfection rate from variation in temperature over the range 0–30°C [78]. However, the rate of killing of spores exposed to formaldehyde vapour has been reported to increase in the range 10–70°C [1]. The addition of formaldehyde to steam under subatmospheric pressure at temperatures below 90°C significantly enhances the sporicidal activity of steam alone [79], with deep penetration into fabrics [80]. At temperatures below 80°C, formaldehyde tends to polymerize, resulting in the condensation of paraformaldehyde, imparting an irritant smell to the products being treated, a toxological hazard to personnel and a gas concentration lower than expected [24].

Organic matter (blood, sputum or soil) reduces the rate of formaldehyde-induced bacterial inactivation [1]. As with ethylene oxide, spores can be protected from formaldehyde vapour by their inclusion within a crystal mass [81].

The activity of formaldehyde vapour is dependent on RH. Various RH levels for optimum antibacterial activity have been proposed. An optimum RH of 80–90% but with no great increase in disinfection above 58% has been reported [78, 82]. Another study found an increase in the rate of kill

up to 50% RH with little effect thereafter [1], although others suggest there is no bactericidal effect unless the RH is 70% or more [83]. A confusing report suggests that formaldehyde is effective at RH values below 50%, and that formaldehyde generated from paraformaldehyde is more active than an equivalent amount generated from formalin solution [84]. More recent studies suggest that the optimum RH for formaldehyde activity lies in the range 75–100% [24].

The biocidal activity of aldehydes tends to be inversely proportional to the length of the methylene chain separating the aldehyde groups [8, 21]. Glutaraldehyde is the exception and it is the most effective sporicide in the aldehyde series. It has been proposed that the excellent sporicidal activity of glutaraldehyde may be due to optimum spacing of the aldehyde groups [8] in terms of cross-linking reactions with spore components. Aldehydes of lower molecular weight (for example, formaldehyde, glyoxal) may penetrate the spore more readily, but may not be as efficient in cross-linking. Aldehydes of higher molecular weight (for example, butyraldehyde) may be affected by the length of the side-chain [8]. The properties of various aldehydes are summarized in *Table 4.2.*

BACTERICIDAL ACTIVITY

Vegetative bacterial cells are readily susceptible to glutaraldehyde. A 0.02% w/v aqueous alkaline solution is rapidly effective against Gram-positive and Gram-negative species, whilst a 2% w/v solution is capable of killing many species including *S. aureus, E. coli, Proteus vulgaris* and *Pseudomonas aeruginosa* within 2 minutes [7]. *E. coli* is completely killed in 10 minutes by 0.01% alkaline glutaraldehyde compared to a 45% kill by the aqueous acid solution [63]. A summary of earlier reports lists killing times of less than 1

Table 4.2. PROPERTIES OF SOME ALDEHYDES

Aldehyde	*Chemical Name*	*Chemical Formulae*	*Sporicidal Activity*
Formaldehyde	Methanal	HCHO	Good[1]
Glyoxal	Ethanedial	CHOCHO	Good[2]
Malonaldehyde	Propanedial	$CHOCH_2CHO$	Slight
Succinaldehyde	Butanedial	$CHO(CH_2)_2CHO$	Slight
Glutaraldehyde	Pentanedial	$CHO(CH_2)_3CHO$	High[3]
Adipaldehyde	Hexanedial	$HO(CH_2)_4CHO$	Slight

[1]More effective when combined with low temperature steam
[2]At 10% concentration
[3]Prolonged periods of time may be required

minute for 2% aqueous glutaraldehyde solution and inactivation factors of 10^4, $>10^6$ and 10^4 after 20 minutes against *S. aureus, E. coli* and *P. aeruginosa* respectively [51]. Glutaraldehyde is effective in suspension tests and in carrier tests against *Listeria innocua* and *L. monocytogenes* in the presence of serum but ineffective when challenged with milk (2% fat) [85], though it has been found to retain activity against pre-dried *Salmonella gallinarum* cells in animal feed [86]. At low concentrations (0.5–1.0 mM), glutaraldehyde inhibits the production of sulphide without affecting the growth of sulphate-reducing bacteria [87]. Growth is inhibited at glutaraldehyde concentrations of 25–50 mM [87].

Mycobacteria are becoming increasingly important once more as the cause of serious disease in man, particularly with the re-emergence of tuberculosis and the emergence of other mycobacterial infections in AIDS patients [88]. Mycobacterial cell walls are unusual, containing a unique peptidoglycan and a mycolate of arabinogalactan [89]. The lipid-rich, hydrophobic layers of the cell wall are responsible for acid-fastness and resistance to many biocides [90]. These layers are thought to represent a barrier to the intracellular entry of many biocides [91, 92]. Many biocides that are bactericidal lack activity towards mycobacteria [90, 93]. The mycobactericidal activity of aldehydes has been the subject of many conflicting publications. Good tuberculocidal activity was attributed to glutaraldehyde in early publications [7, 70], although subsequent reports showed it to have slow action against *Mycobacterium tuberculosis* [62], being less effective than formaldehyde [94]. More recent studies have undoubtedly demonstrated that aldehydes are indeed mycobactericidal agents. A 2% alkaline glutaraldehyde solution is effective against *M. tuberculosis, M. smegmatis, M. fortuitum* and *M. terrae* [95–99], although there is variation in resistance of strains to formaldehyde and glutaraldehyde [100]. Undoubtedly, the MAI group tend to be more resistant than *M. tuberculosis* to aldehydes [101, 102]. *M. gordonae* is more resistant than virulent tubercle bacilli to inactivation by glutaraldehyde, whereas other strains, including *M. smegmatis* and *M. fortuitum*, were highly sensitive [97]. Various formulations (2% alkaline glutaraldehyde, 3.2% alkaline glutaraldehyde and 0.5% glutaraldehyde-0.03% phenol) were effective in removing 10^8 cfu ml^{-1} of *M. gordonae* after 20 minutes at 20°C and after 10–12 minutes at 25°C [103]. *M. terrae* has a similar sensitivity as *M. tuberculosis* to glutaraldehyde [98] and has thus been proposed as an indicator organism for determining tuberculocidal activity [49]. It is worth noting that in studies with 2% alkaline glutaraldehyde, the recovery medium exerts a significant influence on the recovery of glutaraldehyde-treated mycobacteria [104]. Although 2% alkaline glutaraldehyde can be considered an effective mycobactericidal agent, a significant decline in activity occurs

at lower concentrations [105], highlighting the importance of maintaining in-use solutions at the correct concentrations. A recent report, however, highlights flaws in current protocols for the mycobactericidal testing of biocides, including a lack of proper quantitation, excessive contact times at above ambient temperatures, absence of a suitable organic load, ineffective neutralization of the biocide, unsuitable surrogates for *M. tuberculosis*, unsuitable recovery media and inappropriate types of carriers [106].

A recent study has examined the antimicrobial activity of various aldehydes against *M. tuberculosis* [107]. Pentenal, benzaldehyde and phthalaldehyde showed a kill greater than 10^5 cfu ml^{-1} in 5 minutes, though benzaldehyde was only active in the presence of 2% glutraldehyde. Substituents (nitro, chloro, methyl and methoxy) on the benzaldehyde ring reduced this synergism, but still showed increased activity over 2% glutaraldehyde alone [107].

Formaldehyde is well established as a bactericidal agent [13], although most of the research has focused on its sporicidal effects [24, 74] which are therefore considered in more detail in the following section.

SPORICIDAL ACTIVITY

Relatively few antibacterial agents are actively sporicidal [108–110]. Many agents may be inhibitory to germination or outgrowth of bacterial spores, yet not necessarily kill them [108]. Actively sporicidal agents include the aldehydes [111], although it must be noted that much higher concentrations are required than for bactericidal activity [49].

A 2% alkaline glutaraldehyde solution has long been considered to provide the minimum concentration and conditions necessary for relatively rapid sporicidal activity. This solution tends to have greater activity than 8% formaldehyde [10]. At the use-diluton of 2%, glutaraldehyde is capable of killing spores of *Bacillus globigii, B. subtilis, Clostridium tetani* and *C. perfringens* in 3 hours [7, 70]. A 99.99% kill of spores of *B. anthracis* and *C. tetani* in 15 and 30 minutes respectively has also been reported [62]. A number of authors, using time-survivor measurements and aqueous suspensions of *B. subtilis* spores, indicated that a three-hour exposure to the aldehyde resulted in approximately a 6-log drop in viable count [64, 112–115]. Experiments using the AOAC sporicidal test and vacuum-dried spores showed that 10 hours were necessary for a complete kill of *B. subtilis* and *C. sporogenes* spores [19, 116]. Dilution of the aldehyde below 2% results in loss of sporicidal activity [116]. Not all spores are equally susceptible to glutaraldehyde [117]. *B. subtilis* and *B. pumilis* spores appear to be the most resistant whereas spores of *C. difficile* are very susceptible to the aldehyde [61, 117].

Formaldehyde solution is rapidly sporicidal to *B. subtilis*, but *C. sporogenes* spores are not affected after 2 hours [118]. Borax-formalin and formaldehyde-alcohol have been found to destroy *B. anthracis*, *C. tetani* and *C. perfringens* spores within 3 hours, although some of these experiments failed to neutralize formaldehyde in the growth media [119]. Failure to control sporostasis in subculture media can lead to the survival of spores even after long periods of exposure to formaldehyde. Spores of various clostridia can survive even after 8 hours exposure to formaldehyde solution, and *B. subtilis* spores can survive a four-hour treatment with 8% formaldehyde [120, 121]. A similar lack of sporicidal activity of 8% formaldehyde has been described by other workers [6]. More recent work has shown formaldehyde to be a good sporicidal agent, although not as effective as alkaline glutaraldehyde [8]. 8% formaldehyde treatment of aqueous suspensions of *B. subtilis* resulted in a 3-log kill in 3 hours at room temperature, compared to a 5-log kill for 2% alkaline glutaraldehyde [8]. The biocidal activity of formaldehyde is not greatly modified by changes in pH [62] and the addition of 0.3% sodium bicarbonate solution does not potentiate its activity as it does to glutaraldehyde [8].

The sporicidal activity of aldehydes other than glutaraldehyde and formaldehyde has not been examined in much detail. Glyoxal has been found to possess good activity against aqueous suspensions of spores of *B. subtilis* [8, 49], producing a 2-log reduction in 3 hours at room temperature. Butyraldehyde was found not to have any activity against *B. subtilis* spores [8]. As with formaldehyde, the addition of 0.3% sodium bicarbonate solution does not potentiate the sporicidal activity of these aldehydes [8]. The potentiating effect of bicarbonate on glutaraldehyde is not due to simple changes in pH. The addition of sodium hydroxide does not increase the biocidal activity of acid glutaraldehyde to the same extent as sodium bicarbonate. Since the latter is not itself sporicidal, its action is thought to be primarily on the spore outer layers [121, 122], with subsequent alterations aiding interaction and/or penetration of glutaraldehyde with potential substrates such as proteins, enzymes and peptidoglycan [8]. The spacing of the aldehyde groups in glutaraldehyde has been proposed to be optimal for its interaction with sodium bicarbonate and sporicidal activity [8, 21].

Some commercial formulations have combined aldehydes with other compounds to enhance sporicidal effects. Gigasept (succinaldehyde plus formaldehyde) has been recommended for the disinfection of endoscopes [123]. The formulation effects a 2-log and 5-log reduction at concentrations of 5% and 10% respectively against aqueous suspensions of *B. subtilis* spores [8] and is therefore more effective than formaldehyde alone.

Sporicidin (glutaraldehyde plus phenol plus phenate) achieved a 6-log kill

in 3 hours and was more effective than glutaraldehyde alone [8]. It is not sporicidal at a recommended dilution of 1:16 for disinfection [8, 124]. Various reports suggest it is a better disinfectant than 2% alkaline glutaraldehyde alone [125–130] in terms of biocidal activity, stability and reduced toxicity (at a 1:16 dilution). However, there is no mention of an appropriate neutralizing agent for glutaraldehyde (for example, glycine) and the use of 0.5% Tween 80 is unsatisfactory for neutralization of residual phenol, since plate counts were often higher in more dilute suspensions during sporicidal tests [8]. The sporostatic activity of phenol is well documented [74, 131] and is thought to inhibit germination [130]. It is therefore evident that an overestimation of the sporicidal efficacy of Sporicidin could occur due to an inadequate enumeration procedure [8]. Mixtures of glutaraldehyde and formaldehyde are found to be ten times more effective than either aldehyde alone [132].

Germinating and outgrowing spores are much more susceptible than mature spores to the lethal effects of sporicidal aldehydes, their susceptibility being equivalent to that of vegetative cells [109, 110].

FUNGICIDAL ACTIVITY

Glutaraldehyde has been shown to have both fungistatic [133] and fungicidal activity [7, 10, 133–135]. Growth of *Trichophyton interdigitale* is inhibited by a 5 minute exposure to 2% alkaline glutaraldehyde [7]. At the time, this was more potent than the commercial formulations available [7]. A 1% solution of glutaraldehyde is also fungicidal [133], but porous surfaces contaminated with *Candida albicans* and *Microsporium gypseum* are significantly more difficult to disinfect than are smooth surfaces [136]. This observation is likely to be applicable to all liquid biocides and should not be considered an inherent property of glutaraldehyde.

Aspergillus niger is more resistant than other fungi to glutaraldehyde [62, 134]. As with other fungal species, however, mycelial growth and sporulation are inhibited by 0.5% alkaline glutaraldehyde while spore swelling is entirely halted [10, 134]. Fungicidal activity is also apparent with a 2-log kill in 60 minutes when exposed to 0.5% alkaline glutaraldehyde [134]. The acid solution is much less potent with a 1-log kill after 60 minutes and little reduction in viable count thereafter [134].

Formaldehyde is as effective in killing *C. albicans* as vegetative Gram-negative bacteria [137]. The conidia of *A. niger* are more resistant than *C. albicans*, but are equally sensitive to *S. aureus* [137]. A natural aldehyde, 3-[4-hydroxy-3-(3-methyl-2-butenyl)phenyl]-2-(*E*)-propenal has been isolated from the peel of citrus fruits and found to have antifungal activity [138].

There has been little more of note to report in recent years [49], although this is true for all types of biocides. The following general conclusions on the effects of biocides have been drawn recently [53]: (a) fungicidal concentrations are often much higher than those needed to inhibit growth [139]; (b) moulds are sometimes, but not always, less sensitive than yeasts [137]; and (c) non-sporulating bacteria (except mycobacteria) tend to be more sensitive than fungi to biocides [140].

VIRUCIDAL ACTIVITY

The virucidal activity of aldehydes has been acknowledged for many years [5, 10, 141, 142]. In recent years, there have been additional reports of their effectiveness against HIV, HBV, poliovirus, herpes viruses and other viruses.

Studies with reverse transcriptase indicated that concentrations of glutaraldehyde greater than 1% inactivated HIV [143]. It has been suggested that methods based on monitoring residual viral enzyme activity are not appropriate alternatives to tests for infectious viral particles [49, 144]. Both 1% and 2% alkaline glutaraldehyde have been demonstrated to inactivate cell-free HIV within 1 minute, and 2% to inactivate cell-free virus in serum within 2 minutes, with 1% having no effect after 15 minutes [145, 146], presumably due to the interaction of glutaraldehyde with the amino groups present in serum (see earlier). Cell-associated HIV is also inactivated within 2 minutes at the higher concentration [145]. The margin of safety for the inactivation of HIV by glutaraldehyde is less than had previously been thought [143]. Freshly prepared solutions of 2% alkaline glutaraldehyde remain effective [145, 147], but efficacy is lost when solutions become too old or diluted and would not be appropriate for inactivating HIV associated with organic matter [49].

Formalin, at a concentration of 0.1%, can inactivate HIV, although 48 hours are required to halt the production of reverse transcriptase [143]. Paraformaldehyde can inactivate the virus at concentrations below those used in routine disinfection [148].

HBV is often regarded as possessing greater resistance to biocides than other viruses, although Russell [49] suggests that this may be due, in part, to the methods used to assess residual viral activity. In a chimpanzee model system, where animals are observed for signs of infection for nine months after injection with the treated material, 2% alkaline glutaraldehyde is virucidal within 5 minutes [149, 150]. In the presence of organic matter, such as dried plasma, the time for inactivation doubles to 10 minutes [149]. Alternative test methods based on alterations in HBV antigenicity, 2% alkaline glutaraldehyde produced approximately a 60% reduction in surface antigen

(HB_sAg) and 90% reduction in core antigen (HB_cAg) activity [151]. These results were superior to those obtained with formaldehyde, and the authors proposed that glutaraldehyde should be used for disinfection of items exposed to HBV in place of formaldehyde or hypochlorite solutions. However, the authors did point out that more direct approaches to determine viral infectivity after exposure were required. The aldehyde is thought to interact with lysine residues present on the most exposed structural protein of hepatitis A virus [152] and it is feasible that this interaction occurs also with HBV.

Alkaline glutaraldehyde (2%) produces a 6-log reduction of herpes simplex virus dried onto surfaces within 1 minute [153]. Similar results are obtained with poliovirus (non-lipid enveloped), although it is generally more resistant to disinfectants than herpes simplex virus [49, 154]. The high virucidal activity of glutaraldehyde toward enteroviruses has also been demonstrated by other workers [155–158]. Glutaraldehyde (0.1%) is effective against poliovirus, producing a 3-log reduction in virus titre at pH 7.2, with greater efficacy at alkaline pH but no activity at acid pH [156].

Human rotaviruses are inactivated in suspension tests and dried on surfaces by glutaraldehyde within 1 minute, in the presence of both high and low organic load [159, 160], with morphological changes induced by the aldehyde [161]. Formaldehyde (8%) was effective in abolishing the infectivity of human rotaviruses after 5 minutes exposure [162].

A solution of 2% alkaline glutaraldehyde at pH 7.4 and 25°C reduced the titre of infectious coxsackie virus by 2-log in 1 minute. The efficacy of the aldehyde was not affected by the presence of high concentrations of organic matter [163]. Other workers have confirmed the efficacy of glutaraldehyde against coxsackie virus and also shown it to be completely virucidal for echovirus [164].

Alkaline glutaraldehyde (2%) inhibits the transduction of *P. aeruginosa* PAO by bacteriophage F116, but is less effective in inactivating the phage [165]. Lower concentrations of the aldehyde (0.5%) reduce the titer of MS2- and K-coliphage by a factor of 10^4 within 20 minutes [166].

Aldehydes have also been shown to be effective against animal viruses. Swine vesicular disease virus is completely inactivated by formaldehyde after 30 minutes at 25°C [167]. Glutaraldehyde (0.25%) inactivates the virus over a period of 11 days at 22–26°C [168].

Solutions of glutaraldehyde (2%) and formaldehyde (8%) have been shown to be effective against porcine parvovirus after 20 minutes, and pseudorabies and transmissible gastroenteritis viruses after 5 minutes' exposure [169]. Aleutian disease virus of mink was inactivated by formalin only after 30 minutes exposure, but glutaraldehyde inactivated 4-log of infectious virus within 5 minutes at 23°C [170]. Formalin (0.2%) was found to be ineffective

against infectious pancreatic necrosis virus (isolated from rainbow trout) after 60 minutes exposure [171]. Formalin and glutaraldehyde can inactivate 4-log of equine infectious anaemia virus within 5 minutes at 23°C, although their action is slower than phenolic and halogen disinfectants [172].

MECHANISM OF ACTION

Most studies on the mode of action of biocides have examined vegetative bacteria, although recent work has also provided information about the inactivation of spores. Other micro-organisms have perhaps been more neglected, although there are some data on the mechanism of action of aldehydes against fungi and viruses.

VEGETATIVE BACTERIA

Considerable cross-linking of the abundant peptidoglycan in the cell wall has been shown to occur in Gram-positive bacteria, resulting in a strengthening and sealing effect [173]. Glutaraldehyde-treated peptidoglycan is less sensitive to lysis than the untreated polymer. The dialdehyde reacts with 30–50% of the NH_2 groups in isolated peptidoglycan and it was proposed that two tripeptide side-chains could be linked when free amino groups are available [173]. Pretreatment with glutaraldehyde of peptidoglycan in *S. aureus* reduces lysis on subsequent exposure to lysostaphin [174]. Intermolecular bonding of teichoic acid chains [173] and interaction with cell wall protein could also contribute to the aforementioned effects.

Earlier work suggested that glutaraldehyde does not penetrate to the peptidoglycan layer of the Gram-negative cell [175], but exerts its effect by a partial sealing of the outer layers or lipoprotein and an inactivation of cell wall-associated or periplasmic enzymes. The extent of possible sealing was investigated by comparing the transport of a low molecular weight amino acid in glutaraldehyde-treated and untreated cells [175]. Glutaraldehyde was proposed to act mainly on the outer region of the cell because transport was reduced by 50% in treated cells. Evidence for the reaction of glutaraldehyde with the outer layers has also been provided by other workers [63, 121]. However, since 99.99% kill of *E. coli* is obtained with 0.01% glutaraldehyde in the time taken for these cellular effects, it is unlikely that sealing of the cell envelope is the only cause of biocidal action.

At concentrations having little effect on viability, glutaraldehyde inhibits dehydrogenase activity [121]. Various concentrations inhibit several periplasmic enzymes, including APase [175]. Studies on the interaction of a

mammalian enzyme, a-chymotrypsin, indicate that an initial cross-linking reaction occurs due to intra-molecular cross-links, whereas later particle formation is due to inter-molecular cross-links [176]. A similar reaction with bacterial enzymes is feasible. The inhibitory effect of the dialdehyde on RNA, DNA and protein synthesis is complete within 10 minutes of exposure [63]. These workers had proposed that this effect was not due to direct action, but because of inhibition of precursor uptake as a consequence of glutaraldehyde-protein reactions in the outer structures of the cell.

A review [51] of early studies draws the following observations: (a) strong binding of glutaraldehyde to outer cell layers, (b) the prevention of lysis by sodium lauryl sulphate (*E. coli*), lysostaphin (*S. aureus*) and EDTA-tris-lysozyme (*P. aeruginosa*), (c) the inhibition of sphaeroplast lysis in hypotonic media, (d) the inhibition of RNA, DNA and protein synthesis, and (e) the inhibition of cell enzyme activity by low concentrations [49].

The extensive interaction with outer cell layers is still considered to be a major factor in the bactericidal activity of glutaraldehyde [51]. Moderate changes in pH tend to have little effect on the chemical nature of glutaraldehyde over short periods of time, but significant effects on its antimicrobial action. Unprotonated amines on the cell surface are thought to represent the reactive sites for glutaraldehyde action [177]. Current thinking suggests that as the external pH is altered from acid to alkali, more reactive sites are formed at the cell surface leading to a more rapid bactericidal effect. The cross-links so produced mean that the cell is unable to undertake most of its essential functions [49]. It should be borne in mind, however, that the chemical used to increase pH may have some effect, as discussed previously. With prolonged contact times, the effects of increase in pH become less significant [177]. Cell walls, lipopolysaccharide, proteins, nucleic acids and lipids have all been implicated in reactions with glutaraldehyde, and it is possible that more than one reaction leads to cell death [178]. Known reactions of glutaraldehyde with ε-amino groups of lysine also implicate cell wall components such as porins and membrane transport proteins as lethal targets for the aldehyde [178]. Glutaraldehyde reacts with lysine residues in the molar ratio 4:1 [179]. One hypothesis suggests that aldol-type polymers formed in alkaline solution react with the amino groups to give an imino bond stabilized by resonance with the ethylenic double bond, and that the aldehyde reacts with proteins as an unsaturated polymer rather than in its free form [180]. However, the tanning effects of purified and unpurified solutions are virtually identical indicating that potential cross-linking reactions are not dependent on the initial presence of unsaturated species [12, 181, 182]. The products of the reaction between glutaraldehyde with 6-aminohexanoic acid and with α-*N*-acetyl-lysine are tetrasubstituted pyridinium salts and it

has been suggested that such structures are present in the cross-linked products formed in the reaction between glutaraldehyde and proteins [181, 182].

Alkaline glutaraldehyde reduces or prevents lysis of *E. coli* cells and cell walls by sodium lauryl sulphate at 35–40°C [183]. This protection is due to interaction with lipoprotein and is greater with whole cells than isolated cell walls and with alkaline than with acid glutaraldehyde. The aldehyde inhibits, but does not prevent the liberation of reducing compounds from *E. coli* cell envelopes treated with acid [63]. Glutaraldehyde reduces lysis of *S. aureus* and *P. aeruginosa* cells by lysostaphin and EDTA-lysozyme respectively [174, 184] and strengthening of the outer layers of sphaeroplasts has also been reported [185]. These observations indicate a strong binding of glutaraldehyde to outer cell surfaces.

Cell agglutination occurs on exposure of various micro-organisms to aldehydes and is thought to be due to the formation of intercellular bonds [186], confirming a strong interaction with the outer cell layers. The unusual colour development in bacteria treated with glutaraldehyde is thought to be due to a reaction with amino groups in the outer layers, and the greater colouration observed with Gram-negative organisms may correlate with the suggestion that interaction with lipoprotein is responsible [186, 187].

The extent of possible sealing of the outer cell layers has been determined by comparing the transport of the amino acid α-aminoisobutyric acid-1-^{14}C in untreated cells of *E. coli* and those treated with 0.01% glutaraldehyde [175]. The extent of cell envelope penetration was also assessed using glutaraldehyde-treated and untreated mureinoplasts of *E. coli*. These cell forms lack the outer lipoprotein layer and therefore have the peptidoglycan layer exposed to the environment [188]. Glutaraldehyde tends to act mainly on the outer regions of the cell envelope, resulting in only a 50% reduction in transport of the amino acid. Cell volume and protein content are reduced by 10–15%, suggesting that deep penetration does not occur [175].

Studies on cell enzyme activity have shown that dehydrogenase activity is inhibited at concentrations of glutaraldehyde which have minimal effects on cell viability [189], possibly because reaction at the outer layers prevents access of substrate to enzyme. Glutaraldehyde also prevents the selective release of some enzymes from the cytoplasmic (inner) membrane of *Micrococcus lysodeikticus* [190] and inactivates several periplasmic enzymes [191–193] including APase [175, 193]. Glutaraldehyde fixation causes a shift of APase from the periplasmic space to the cell surface [194]. The inactivation of cell enzymes would therefore seem to play an important role in the biocidal activity of glutaraldehyde. One presumes that similar mechanisms underly the mycobactericidal action of glutaraldehyde, although no specific studies have been completed [90].

A solution of formalin (0.012%) has been shown to induce cell lysis in *E.coli*, staphylococci and streptococci [195]. Autolytic enzymes are presumably not inhibited at such low concentrations, and this action has thus been compared with that of penicillin, a cell wall-acting antibiotic [195].

A relatively specific reaction occurs between formaldehyde and nucleotides, with the amino groups of the purine and pyrimidine rings being considered the likely reactive sites [196]. Binding of formaldehyde to DNA is irreversible at 100°C at alkaline pH [197] compared with binding to RNA which is reversible if the reaction has not proceeded for longer than 2 hours [39]. After 2 hours, the reaction is slowly reversible only after dialysis [39]. The reaction is thought to be due to the formation of methylene bridges, $NHCH_2NH$, between bases containing amino groups [178]. Reactions of this type can occur with cellular amino groups other than those in nucleic acid molecules. Sublethal concentrations of formaldehyde inhibit the synthesis of cytoplasmic and nuclear material, but subsequent recovery of the cells occurs, with the aldehyde being metabolized to carbon dioxide [41–44].

The reactivity of aldehydes with NH_2, COOH and SH groups inevitably means that there are multiple targets in the bacterial cell; this makes the elucidation of specific lethal targets an extremely complex proposition. The different efficacies of various aldehyde molecules are likely to reflect differences in chain length and composition [21, 198] which may result in altered abilities to bind to specific sites in the cell.

BACTERIAL SPORES

There are several components of bacterial spores that could be implicated as sites for sporicidal action of aldehydes. These include the spore coat, the cortex, the spore membranes and the spore core. Low concentrations of glutaraldehyde (0.1%) inhibit the germination of spores of *B. subtilis* and *B. pumilis*, while higher concentrations (2%) are sporicidal [65]. The aldehyde, at acid and alkaline pH, appears to interact considerably with the outer layers of bacterial spores. This interaction reduces the release of DPA from *B. pumilis* and of peroxide-induced lysis of spores subsequently treated with thioglycollic acid [65]. The small differences in results obtained with acid and alkaline glutaraldehyde with respect to interaction with the spore coat do not correlate with the much greater sporicidal effect of the aldehyde at alkaline pH [10]. The explanation has been advanced that acid glutaraldehyde interacts and remains largely at the spore surface, whereas alkaline glutaraldehyde penetrates into the spore [10, 74]. Acid glutaraldehyde causes marked changes in the electrophoretic mobility of spores whereas alkaline glutaral-

dehyde causes much smaller changes [5, 121], lending support to the theory that acid glutaraldehyde interacts mainly at the cell surface.

Spores of *B. cereus* become heat-sensitive in the presence of high concentrations of salts. Cations are thought to interact with the loosely cross-linked, electronegative peptidoglycan unique to spores to cause a collapse of the cortex [199]. The replacement of mobile counterions and a consequent fall in the osmotic dehydration of the core leads to a reduction in resistance. In this respect, replacement of sodium bicarbonate by divalent metal chlorides produces a rapid sporicidal effect [17]. This indicates a role for bicarbonate in facilitating penetration and interaction of glutaraldehyde with proteins, enzymes and peptidoglycan in the cortex [10]. High concentrations of glutaraldehyde (10%) at alkaline pH interact powerfully with one or more layers of the spore coat producing an extremely tough sealed structure. This interaction is much slower at acid pH [200].

Vegetative bacterial cells tend to bind more glutaraldehyde than resting *B. subtilis* spores, with the degree of aldehyde adsorbed directly proportional to the sensitivity of the micro-organism to the disinfectant [201]. Uptake is greater during germination and outgrowth but is less than that by vegetative cells [201]. The greater sporicidal activity of alkaline glutaraldehyde compared with the acid solution is not reflected by differences in uptake, which are small, although this reflects binding and not the degree of penetration into the spore [201]. At higher concentrations, uptake of the alkaline solution increases after a relatively short plateau, whereas a long plateau is observed with an acid solution [201, 202]. The second rise observed in alkaline glutaraldehyde uptake curves is probably due to the exposure of fresh binding sites due to the action of sodium bicarbonate. The long plateau observed with the acid solution indicates that a high energy barrier must be overcome before additional adsorption to new sites can occur [202]. This energy can be supplied by heat, which probably accounts for the comparable activities of acid and alkaline formulations at high temperatures [64, 65]. It has thus been proposed that the alkaline form penetrates beyond the coats in intact spores, whereas the acid formulation is confined to the outer layers [10, 51].

An alternative hypothesis concludes the exact opposite to the above [177]. Acid glutaraldehyde will kill spores, albeit at a much slower rate than the alkaline solution. The hypothesis envisages the acid form penetrating the coat and reacting with the cortex, whereas alkaline glutaraldehyde interacts with the coat, destroying the ability of the spore to function solely as a consequence of this surface interaction. There is as yet no evidence to prove or disprove this hypothesis.

Spores of *Bacillus* spp. possess hydrophobic surface characteristics as opposed to the essentially hydrophilic nature of vegetative cells [203]. Pretreat-

ment of *B. subtilis* spores with low concentrations of glutaraldehyde increases their surface hydrophobicity as determined by the BATH technique [204]. This is probably due to the extensive interaction of the aldehyde with the outer layers of bacterial spores [10]. The proteinaceous spore components [205] are potentially hydrophobic and any glutaraldehyde-induced conformational change leading to the exposure of more hydrophobic residues is likely to account for these observations [201]. The greater effect of alkaline glutaraldehyde may again be attributed to the action of sodium bicarbonate (or the equivalent activator in commercial formulations). Increased penetration into the spore is likely to result in increased exposure of hydrophobic residues and therefore an overall increase in hydrophobicity. These observations would tend to support the hypothesis that alkaline formulations penetrate more into the spore than the acid solution.

Low concentrations of both alkaline and acid solutions inhibit germination, as determined spectrophotometrically [201]. The involvement of the spore coat in germination is established [206] and therefore interaction of glutaraldehyde with the surface layers, even with little penetration is probably sufficient to achieve an inhibitory effect. At low concentrations of glutaraldehyde, binding of L-[^{14}C]-alanine to the spore is not affected [207]. L-Alanine triggers the germination of bacterial spores [208, 209]. At these low concentrations, glutaraldehyde seems to act at the level of the trigger reaction of germination [210], inhibiting the stimulatory effect of L-alanine. There is no difference between acid and alkaline glutaraldehyde, indicating that the effects at these concentrations are attributable to the glutaraldehyde molecule itself, and not to any pH effects [207]. The effects have been attributed to sealing of the spore outer layers, with cross-linking of spore components physically preventing germination reactions [207]. At higher glutaraldehyde concentrations (1%), binding of L-alanine is significantly reduced [207]. No significant differences were observed between acid and alkaline glutaraldehyde, indicating that inhibition of germination is not directly correlated to the sporicidal action of the aldehyde.

Formaldehyde interacts with protein to give intermolecular cross-links [5] and it is considered to be sporicidal because it can penetrate into the interior of the spore [211]. Formaldehyde also inhibits the germination of *B. subtilis* spores. The inhibition is concentration-dependent and reversible [75], again indicating that inhibition of germination is not related to the sporicidal action of aldehydes.

Formaldehyde is an extremely reactive chemical, reacting with protein, DNA and RNA *in vitro* [212]. Its interaction with protein results from a combination with the primary amide as well as with amino groups, although phenol groups bind little of the chemical [46, 213]. Its extensive reactions

with nucleic acids [39] have been proposed as the reason for mutagenic effects [214], and its reactions with COOH, SH and OH groups class it as an alkylating agent as with other aldehydes [215].

It has been proposed that glutaraldehyde-treated spores might, in fact, be fixed rather than inactivated so that they would be unable to function properly [216]. In this context, it is interesting to note that aldehyde-treated spores can be revived under certain conditions. Sodium hydroxide has been used to revive spores of *B. subtilis* treated with a variety of aldehydes, with an optimum range of 20–50 mM [217–219]. Sodium hydroxide increases the permeability of bacterial spores to germinants [220] and alkali is known to remove protein from spore coats [221–223]. Of the glutaraldehyde formulations examined, 2% alkaline glutaraldehyde and Cidex (a similar commercial formulation) have similar sodium hydroxide-induced revival frequencies (10^{-6}). No revival was observed at glutaraldehyde concentrations greater than 4% [218]. Sporicidin shows a decreased frequency (10^{-7}), although as mentioned previously, difficulty was experienced in neutralizing residual phenol [217]. The highest frequency of sodium hydroxide-induced revival is observed with glyoxal (10^{-4}). Minimal revival is observed with 10% Gigasept and none with 8% formaldehyde solution [217]. It is interesting that the phenomenon of alkali-induced revival seems to be linked to dialdehyde inactivation. Glutaraldehyde and glyoxal are both dialdehydes, whereas formaldehyde is a monoaldehyde. Heat-induced revival of formaldehyde-treated spores has been reported [137] but the effect is much reduced with glutaraldehyde [219, 224]. This implies a difference in the mechanism of action of mono- and di-aldehydes. The interaction of aldehydes with proteins to give molecular cross-links and their ability to penetrate into spores has been discussed earlier. It is possible that the mode of cross-linking of dialdehydes differs from that of monoaldehydes. The presence of two aldehyde groups linked by a chain of carbon atoms may result in a form of intermolecular bridging not achieved by a molecule containing a single aldehyde group, such as formaldehyde, and this may be reflected by the varying successes of different revival treatments

Differences in revival frequencies of dialdehydes may be due to the distance between the aldehyde functions [217] which is thought to be a factor in the biocidal activity of aldehydes [21]. The aldehyde groups in glutaraldehyde may be spaced at a distance which results in more effective cross-links than glyoxal. Removal of subsequent inhibition would therefore be easier with glyoxal. Revival of Gigasept-treated spores is interesting. No revival was obtained with 5% Gigasept whereas a proportion of spores were revived after exposure to a 10% solution [217]. Gigasept contains butan-1,4-dial and formaldehyde. It is possible that at the lower concentration the majority

of sporicidal activity is due to formaldehyde, and hence no sodium hydroxide-induced revival would be expected. At a concentration of 10%, a greater proportion of the sporicidal action may be due to the dialdehyde, and the observed revival may be attributed to those spores inactivated mainly by butan-1,4-dial [217].

Some proposals have been forwarded to explain the phenomenon of alkali-induced revival [219]: (a) the high pH dissolves alkali-soluble proteins from the spore coat [225], allowing easier access to germinants, (b) the alkali may act as a trigger for germination, substituting for nutritional germinants, (c) the alkali may desorb aldehydes from treated spores, and (d) a simple pH effect. Little protein is released from glutaraldehyde-treated spores on exposure to sodium hydroxide [218]. However, the proportion of revived spores is small, compared with the initial inoculum, and it may be that only a tiny proportion of aldehyde-treated spores is susceptible to alkali treatment, accounting for the small amount of protein solubilized from the spore coats.

The proposal that sodium hydroxide acts as a germination stimulant is untenable since untreated spores do not germinate in its presence [219]. Treatment with alkali may result in the desorption of aldehyde from some spores, sufficient to allow access of germinant [219]. However, the assay employed to monitor glutaraldehyde levels [226] is not sufficiently sensitive to reach a definite conclusion. A simple pH effect is unlikely since a high pH is known to inhibit germination [227].

FUNGI

Inhibition of fungal spore germination and sporulation by aldehydes has been demonstrated [10]. Little research has been conducted into the mode of action of aldehydes on these organisms, although an examination of the chemical nature of fungi indicates a possible mechanism. The major structural component of fungal cell walls is chitin which resembles bacterial peptidoglycan and is thus a potential reactive site [134]. Fungal enzymes are also located in their cell walls and other reactive sites might include the polysaccharide-protein complexes, found in yeasts, and in which cystine residues (-S-S-bonds) are abundant [10]. Glutaraldehyde has been shown to agglutinate cells of *C. lipolytica* and *Saccharomyces carlsbergensis* and to increase their settling rate, as a result of an effect on the outer cell layers [186]. These effects are probably due to the formation of intercellular bonds (see earlier) and could well be an important factor in causing cell death [10].

VIRUSES

Glutaraldehyde is a highly effective virucidal agent. Its mechanism of action has been the subject of recent publications. The dialdehyde reduces HB_SAg and, particularly, HB_CAg activity in HBV [151]. It also interacts with lysine residues on the surface of hepatitis A virus [152]. Alkaline glutaraldehyde (0.1%) is effective against purified poliovirus, whereas poliovirus RNA is highly resistant to concentrations up to 1% at pH 7.2 with a low inactivation rate at pH 8.3 [156]. Thus, intact virus particles are much more sensitive than poliovirus RNA, from which it may be deduced that capsid changes are responsible for the loss of infectivity after exposure to the aldehyde [156]. Capsid proteins of poliovirus and echovirus react with low concentrations of glutaraldehyde (0.05% and 0.005% respectively), the differences in sensitivity reflecting major structural variations in the two viruses [157].

The differential activity of glutaraldehyde against transduction by bacteriophage F116 and inactivation of the infective particle [165] implies the aldehyde has distinct cellular target sites. Electron micrograph studies of glutaraldehyde-treated F116 showed an increase in the number of phage particles with empty heads [228], suggesting the aldehyde effects inactivation by causing loss of nucleic acid. There is also evidence that glutaraldehyde alters SDS-PAGE protein patterns of F116 though a correlation with phage inactivation is unclear [229].

A recent report divides aldehydes into those with cross-linking activity and those lacking such activity [230]. Aldehydes with specific protein-DNA cross-linking ability disrupt simian virus 40 DNA replication, causing failure of the replication fork by the 40S intermediate pathway. Replicating viral genomes become inactivated and torsionally stressed [231]. In contrast, aldehydes with no cross-linking properties had no effect on viral DNA replication, from which it can be inferred that protein-DNA cross-links block either DNA polymerase or the entire replication complex [231]. Replication failure by the 40S pathway is known to initiate recombinational events in damaged viral replicons, possibly resulting in mutations. Formaldehyde and acrolein also caused accumulation of catenated (topologically linked) virus daughter chromosomes, which is characteristic of the inhibition of DNA topoisomerase II [231].

A recent interesting article examined the effects of peptide aldehydes on a hepatitis A virus enzyme [232]. Picornaviral 3C proteinases are a group of closely related thiol proteinases responsible for processing viral polyprotein into its component proteins. These proteinases adopt a chymotrypsin-like fold [233, 234] and display an active site configuration similar to those of serine proteinases. The peptide aldehydes were based on the preferred peptide

substrates for hepatitis A virus 3C proteinase and were synthesized by reduction of a thioester precursor [231]. Acetyl-Leu-Ala-Ala-(N, N′-dimethylglutaminal) binds slowly and reversibly to the hepatitis A 3C proteinase but is 50 times less active against the rhinovirus enzyme, whose substrate specificity is slightly different, suggesting a high degree of selectivity [231]. NMR spectrometry of the adduct of the radiolabelled peptide aldehyde with the enzyme indicates that a thiohemiacetal is formed between the enzyme and the aldehyde carbon as has been reported previously for peptide aldehyde inhibitors of papain [234, 235].

MECHANISMS OF RESISTANCE

The mechanisms of resistance to biocides are relatively poorly understood in comparison with antibiotics. Bacterial resistance to antibiotics is normally considered to be of two types: (a) intrinsic (innate, natural), a natural property of the organism, or (b) acquired, either by chromosomal mutation or by the acquisition of extraneous DNA, usually acquired as plasmids or transposons [53, 236, 237].

INTRINSIC RESISTANCE

Intrinsic resistance is the most common form of resistance to biocides and is considered to involve exclusion of a biocide as a consequence of impermeability. Additionally, physiological adaptations of an organism in response to changes in the growth environment can modulate sensitivity to biocides, a classical example of which is biofilm production [236].

Gram-positive bacteria

The cell wall of staphylococci is composed of peptidoglycan and teichoic acids. Compounds of high molecular weight can traverse the wall readily, which provides an explanation of the sensitivity of these organisms to aldehydes [53]. Biocides must penetrate the outer and cytoplasmic membranes of Gram-negative bacteria and it is unlikely that the Gram-positive bacterial cell wall presents as much of a barrier to the entry of antibacterial compounds as does the lipid-rich envelope of Gram-negative organisms [50]. However, most reports on aldehydes fail to distinguish significant differences between the sensitivities of Gram-positive and Gram-negative organisms [7, 51, 63]. Indeed, one report indicates that *S. aureus* is more resistant than *E. coli* to the action of glutaraldehyde [51].

Gram-negative bacteria

The outer membrane of Gram-negative bacteria plays an important role in limiting access of susceptible target sites to antimicrobial agents [237–241], which means that Gram-negative organisms tend to be less sensitive to many biocides than Gram-positive bacteria [236]. However, as discussed previously, this does not seem to present a barrier to the action of aldehydes, presumably because of the ready availability of reactive moieties (for example, proteins) in both types of cell wall. However, there are differences in susceptibility of different Gram-negative organisms to aldehydes. Thus, *P. aeruginosa* is more resistant than *E. coli* to the action of glutaraldehyde [51]. The high Mg^{2+} content in the cell envelope of *P. aeruginosa* is thought to produce strong LPS-LPS links [236] which may account for the increased resistance of these organisms.

Mycobacteria

Mycobacteria are generally considered to be of intermediate resistance to biocides, including aldehydes, between bacterial spores and other vegetative cells [90]. Formalin, aqueous formaldehyde solution and glutaraldehyde are mycobactericidal, although some mycobacterial strains show increased resistance [90, 101, 236].

The cell walls of mycobacteria are complex and contain a covalent skeleton consisting of two covalently linked polymers (peptidocglycan and an arabinogalactan mycolate), free lipids which can be removed by neutral solvents, and peptides which can be removed by proteolytic enzymes [236]. The intrinsic resistance of mycobacteria to many biocides, including aldehydes, is thought to be related to the amount of waxy material present [242]. The waxy, and therefore hydrophobic, outer layers are believed to limit the access of biocides to sensitive target sites [236] and, in the case of aldehydes, may not present as many reactive sites, although the evidence for this is limited. Furthermore, it is difficult to define a correlation between cell wall composition and biocide resistance or sensitivity [236]. Clearly, further investigation is required into these hypotheses.

Biofilms

The interaction of bacteria with surfaces is initially reversible and then proceeds to an irreversible state. The association of bacteria with solid surfaces leads to the formation of a biofilm, which has been described as a consortium of bacteria organized within an extensive mucopolysaccharide exopol-

ymer or glycocalyx [243]. Bacteria within such a biofilm reside in a specialized microenvironment that differs from cells grown in batch culture under standard laboratory conditions [237]. Changes in growth rate and nutrient limitation result in the modulation of the cell envelope of Gram-negative cells and the cell wall of Gram-positive organisms [238, 244–246]. Bacteria existing in different regions of the biofilm are exposed to different nutrient environments so their physiology, and hence their phenotype, is affected in different ways. This physiological adaptation can lead to changes in sensitivity to biocides, and can be considered as an expression of intrinsic resistance brought about by exposure to environmental conditions [236]. *P. aeruginosa* biofilms, for example, are more resistant than planktonic cell suspensions to 2% glutaraldehyde [247].

Thus, sessile bacteria within a biofilm may be significantly different physiologically from the same cells grown planktonically under standard laboratory conditions. Bacteria in different regions of the biofilm are also likely to grow at different rates, factors which are well-known to affect physiology and therefore biocide resistance [243, 244]. Additionally, the glycocalyx may prevent the biocide from reaching the bacterial cell surface and thus its target site [236]. This will depend on various factors relating to the nature of the biofilm and the properties of the biocide. *Table 4.3* summarizes the likely mechanisms involved. The measurements of biocidal action on different micropopulations within a biofilm have not been elucidated, mainly because of technological limitations, and thus many of the proposed mechanisms remain hypothetical.

Bacterial spores

Bacterial spores are invariably the most resistant forms of bacteria to bio-

Table 4.3. RESISTANCE OF BIOFILMS TO BIOCIDES

Mechanism of resistance	*Remarks*
Exclusion or reduced access of biocide	Depends on: nature of biocide binding capacity of glycocalyx rate of growth of microcolony relative to biocide diffusion rate
Modulation of microenvironment	Associated with: nutrient limitation growth rate
Increased production of degradative enzymes	Physiological adaptation?

cides [74, 248]. Mechanisms of biocide uptake into spores are poorly understood, but the spore coat and cortex appear to prevent many compounds from reaching the spore core [53].

Three techniques have proved particularly useful in studying the resistance of spores to aldehydes: (a) the removal of outer spore layers by chemical means [217], (b) the use of mutants of *B. subtilis* 168 which cannot proceed beyond defined points in spore development [248], and (c) a 'step-down' procedure in which vegetative cells growing in a rich medium are transferred into a nutritionally poor medium, when 90% synchronous sporulation is achieved during subsequent incubation [249]. Coat removal methods utilizing urea plus dithiothreitol with or without sodium lauryl sulphate at alkaline pH are known to remove a significant amount of the spore cortex too [249], with the subsequent addition of lysozyme enhancing this effect [53].

There are seven well-defined stages in the development of a bacterial spore from a vegetative cell [248]. Of these, stages VI and VII are most often associated with reduced sensitivity to biocides. On the basis of such experiments, it is clear that resistance to formaldehyde arises as an early event in sporulation (Stage III/IV) which coincides with the encystment of the forespore and the formation of the cortex [74]. The spore cortex would therefore seem to endow spores with their intrinsic resistance to this aldehyde. Resistance to glutaraldehyde, however, does not manifest itself until much later, commencing at late stage V and being fully developed by the end of stage VI [217]. These stages correspond to the synthesis of the spore coats with subsequent maturation, where the coat material becomes more dense and an increase in refractility typical of mature spores is observed [74]. Clearly, the spore coats are major factors in the intrinsic resistance of bacterial spores to glutaraldehyde. Unlike formaldehyde, the spore cortex does not seem to have an important role in resistance to glutaraldehyde. This may reflect different abilities to penetrate into the spore of different aldehydes as discussed earlier.

Fungi

The same two basic mechanisms of resistance, intrinsic and acquired, can be envisaged for fungi [250, 251]. Fungi tend to be less sensitive than vegetative bacterial cells (except mycobacteria) to aldehydes [146, 251]. Various types of polymers are found in fungal cell walls: chitin and chitosan (Zygomycetes), chitin and glucan (mycelial forms of Ascomycetes and Deuteromycetes) and glucan and mannan (yeast forms of Ascomycetes and Deuteromycetes) [251]. The exclusion of aldehydes and other biocides by such structures remains to be determined but the possibility is feasible in theory.

Glucan chains have been shown to play an important role in the exclusion of the polyenic antibiotic, amphotericin B, in *C. albicans* [252], acting as a barrier to the penetration of the antibiotic to its target site at the membrane. Studies using protoplasts of *S. cerevisiae* have shown that they are lysed rapidly by low concentrations of other biocides and that glucan might act as a barrier restricting the entry of these biocides [253]. The principle may be applied to other biocides, including aldehydes, although further studies are required to support this contention and to examine other factors such as uptake which may influence biocidal efficacy.

There is no evidence associating the presence or acquisition of plasmids with resistance to biocides in fungi, although acquired resistance (non-plasmid) of yeasts to organic acids has been demonstrated [254].

Viruses

Conflicting results are often reported about the action of biocides on different types of viruses [255]. It is suggested that the structural integrity of a virus is altered by an antimicrobial agent reacting with viral capsids to increase viral permeability, so that a 'two-stage' disinfection could offer an efficient means of viral inactivation [255]. This would overcome the possibility of multiplicity reactivation [53], which accounts for an initial reduction followed by a subsequent increase in titre of biocide-treated bacteriophage observed in some experiments.

The relative sensitivities and resistances to biocides of viruses have been postulated as relating to whether the virus contains lipid or not [256]. Viruses have been classified into three groups: A, lipid-containing; B, non-lipid picornaviruses; and, C, other non-lipid viruses (larger than the picornaviruses), and biocides into two groups, broad spectrum biocides that inactivate all viruses and lipophilic biocides that fail to inactivate picornaviruses and parvoviruses [256]. The aldehydes would tend to be classified in the former group since they are capable of inactivating all types of viruses (see earlier). Nevertheless, there are differences in susceptibility. Poliovirus (non-lipid enveloped) tends to be more resistant than herpes simplex virus (enveloped) to glutaraldehyde [49, 153, 154], presumably because of differences in the outer layers affecting the penetration of the aldehyde into the viral particles. The ability of aldehydes to cross-link protein and DNA has also been shown to be important in the activity against simian 40 virus [230]. The cross-linking abilities are likely to relate to optimal spacing of reactive groups in the virus and CHO groups in the aldehydes, and it can therefore be envisaged that different structures in different viruses will affect the

cross-linking abilities of aldehydes. Evidence is required to support this contention.

The penetration of biocides into viruses of different types has not been examined in any detail, nor has the interaction between viral protein and nucleic acid [53]. It is therefore difficult to provide cogent theories to explain the relative sensitivities or resistance of different types of virus to aldehydes.

ACQUIRED RESISTANCE

Acquired resistance to biocides arises by the acquisition of extrachromosomal genetic elements (plasmids and transposons) or as a consequence of a chromosomal gene mutation [53, 236, 237]. Acquired resistance to chromosomal mutation can arise when bacteria are sequentially exposed to increasing concentrations of a biocide.

There are few reports of acquired resistance to aldehydes in bacteria. Formaldehyde-resistant yeasts and bacteria have been found to produce extremely high formaldehyde dehydrogenase activities [257–259]. The yeast *Debaromyces vanriji* produces formaldehyde dehydrogenase constitutively and increases its production in the presence of formaldehyde [257, 259]. The bacterium *P. putida* only forms the enzyme in the presence of formaldehyde [258, 259]. The enzymes from these two organisms have virtually identical specific activities; both require reduced glutathione for their activity and yield *S*-formylglutathione as a product of the dehydrogenation reaction [259]. Formaldehyde and methylglyoxal are substrates for these enzymes [259]. Aldehyde dehydrogenases are common enzymes in many biochemical pathways [260–262], since aldehydes are often products of alcohol and sugar metabolism, and it is therefore not surprising that increased production of aldehyde dehydrogenases, presumably by mutation, leads to increased resistance to aldehydes. Nevertheless, there have been no other reports of overproduction of dehydrogenases resulting in resistance to other aldehydes.

Transferable resistance to formaldehyde and formaldehyde-releasing agents has been reported in *Serratia marcescens* [239, 263]. Although the mechanism of resistance is unclear, it is proposed that changes in cell surface components are induced in resistant cells, which carry a plasmid [263]. The changes do not involve the LPS but rather the proteins in the outer membrane. These changes in outer membrane proteins have been demonstrated in experiments with sensitive, plasmid-less cells which contain a higher proportion of 40 and 45kD proteins than resistant strains harbouring a plasmid [239, 263]. An alternative suggestion is that resistant strains take up less formaldehyde than the isogenic plasmid-less strains and, coupled with metabo-

lism of the aldehyde by native dehydrogenases, a reduction in the efficacy of formaldehyde is the end result [237].

Generally, however, there is minimal experimental evidence that plasmids are responsible for the development and spread of resistance to aldehydes, and, indeed, this is largely true for most biocides [53, 237].

BIOCIDAL USES OF ALDEHYDES

It is important to draw a distinction between the uses of biocides in liquid and gaseous forms. Liquid chemical biocides are sometimes used for sterilization purposes, but are normally employed as disinfectants. Gaseous chemical biocides are often employed as sterilants. Thus, 2% alkaline glutaraldehyde is capable of acting as a sterilizing agent but only after prolonged contact times [264]. In clinical practice where the dialdehyde is employed to 'sterilize' medical equipment, the process is actually one of disinfection [265–276].

In a clinical environment, it is essential to consider the types of medical devices and items and the classification of the infection risk when assessing whether disinfection or sterilization is necessary. Items and surfaces are classified according to the following scheme: (a) high risk (or critical) – items that are in close contact with breaks in the skin or mucous membranes or are introduced into sterile body areas or the vascular system, for example, surgical instruments, laparoscopes, catheters and syringes, (b) intermediate risk (semi-critical) – items which are in contact with intact mucous membranes and do not enter sterile body areas or the vascular system, for example, gastrointestinal endoscopes, bronchoscopes, respiratory and anaesthetic equipment and thermometers, and (c) low risk (non-critical) – items in contact with normal skin or remote from the patient, for example, handbasins, trolley tops and bedding [277, 278].

The main uses of aldehydes as biocides in a clinical environment must be considered in this context, particularly with devices coming in the intermediate risk category such as endoscopes. Autoclavable endoscopes are available [273], but there is insufficient time for a full sterilization cycle between patients and thus autoclaving tends to take place at the end of the day. Additionally, many endoscopes (or some of its components) are sensitive to heat and thus the use of aldehydes as high level disinfectants is relevant. The majority of cases of endoscope-acquired infection arise from inadequate cleaning and disinfection [265, 270, 279–281], with particular problems for immunocompromized patients [279]. Adequate disinfection is required before and after each endoscopy, since each patient might be at risk [282], although

cross-infection apparently remains uncommon, despite the increase in use of flexible fibreoptic endoscopes [283]. Nevertheless, reports confirm the occurrence of bacterial (especially mycobacterial) contamination of endoscopes and other medical equipment even after disinfection with suitable agents such as glutaraldehyde [284–289]. Precleaning of flexible fibreoptic endoscopes is essential prior to disinfection with aldehydes in order to remove as many organisms as possible plus any organic matter that could reduce the efficacy of the aldehyde [265, 270, 290]. In fact, a recent report suggests that high-level disinfection with glutaraldehyde is not associated with a higher infection rate than sterilization [291]. Numerous aldehyde formulations are on the market [8, 277, 290] containing, singly or in combination, glutaraldehyde, formaldehyde, succinaldehyde and phthalaldehyde as the most common aldehydes. Of these, glutaraldehyde is by far the main chemical aldehyde disinfectant used in hospitals [49].

A 2% alkaline solution of glutaraldehyde kills most vegetative bacteria and viruses (including HIV) within 2 minutes, although a 4-minute exposure is recommended to minimize the risk of infection by HBV [56]. Mycobacteria present more difficulties, with concern that tuberculosis could be transmitted during bronchoscopy from patients who have tubercle bacilli in their sputum [280]. Aerosols are the most common route of transmission of tuberculosis [280]. Recommended exposure times of 30 minutes for 2% alkaline glutaraldehyde are recommended, with adequate precleaning [56]. Others have suggested that an exposure time of 20 minutes should be sufficient [49, 280, 292]. Practical situations, however, often bear little relation to theoretical scenarios and two surveys in the USA found that most hospitals used glutaraldehyde-based disinfectants for longer than 20 minutes or for less than 10 minutes [278, 290]. Other reports confirm the lack of standard practices regarding glutaraldehyde use in hospitals [293]. In certain hospitals in the UK, the lack of sufficient funds has meant that endoscopes are disinfected with glutaraldehyde for a maximum of 4 minutes between patients.

The decontamination of arthroscopes and laparoscopes, or articles such as biopsy forceps, remains problematic because these items penetrate intact skin and are therefore in the high risk category [272, 294]. However, disinfected rather than sterilized laparoscopes have been in use for the past 30 years or so [269]. Nevertheless, current concern that viruses such as HIV may be transmitted due to inadequate disinfection are justified. A technique described for the disinfection of laparoscopes involves precleaning, followed by autoclaving of heat-resistant parts at 270°C for 4 minutes [269]. Heat-sensitive parts containing rubber, plastic fibreoptics and lenses are soaked in 2% alkaline glutaraldehyde for 10 minutes [269, 272], although this should be increased to at least 20 minutes if *M. tuberculosis* might be problematic

[272, 295]. These authors have suggested that glutaraldehyde- and succinaldehyde-based products are the only disinfectants suitable for the decontamination of endoscopes [272], although a recent report proposes the use of 0.5% phthalaldehyde, which achieves a 5-log reduction in bacterial load, does not require dilution and does not emit irritating vapours as with other aldehydes [296]. Alternative agents have also been proposed in experiments designed to evaluate the efficacy of disinfection of bronchoscopes contaminated with *M. tuberculosis* or MAI [297].

Glutaraldehyde-based formulations currently remain the disinfectants of choice for the chemical decontamination of endoscopes, although problems of toxicity are evident [298].These will be addressed in the following section. In the absence of suitable alternatives, stringent safety precautions are necessary for its optimal use, and include the use of automatic, enclosed, cleaning and disinfection machines and improved local ventilation [282–296]. Evidence suggests that automated disinfection of endoscopes is more efficient than manual cleaning and less likely to lead to contamination [299], but it is important to monitor solutions to ensure that adequate concentrations are maintained [300].

Glutaraldehyde is also used for high-level disinfection of biopsy forceps [301], peak flow meters [302] and, as an alternative to formaldehyde, of haemodialyzers [303–305], though there is continued criticism of the latter practice due to an increased risk of infection and chronic exposure of patients to aldehydes [303, 306]. Glutaraldehyde is the only chemical found to be effective in the disinfection of composite polishing instruments in dentistry [307].

Glutaraldehyde and formaldehyde (as formalin) have been used topically for the treatment of viral warts [308–311]. A 10% solution of glutaraldehyde in ethanol was found to be most effective, with a cure rate of 73.5% [312]. Other topical applications have included the treatment of hyperhydrosis [313] and onchomycosis, a fungal infection of the nails [314].

Aldehydes have also been the subject of a patent application for the prevention of dental calculus formation and for reducing dental caries formation [315]. Glutaraldehyde has also been described as an ingredient of a chewing gum with anti-caries activity [316]. Aldehydes, particularly formaldehyde (as formocresol) and glutaraldehyde, are used to disinfect and decontaminate the root canal during certain dental procedures [317, 318, 319] and to disinfect items such as dental stone casts, impressions and dentures [320–324].

Still in the medical field, gaseous formaldehyde, usually in the form of a formaldehyde-releasing agent such as paraformaldehyde, was used for fumigation of large areas although this practice has been discontinued because it was found to be of little value [325]. Nevertheless, its use is still recom-

mended for small areas where liquid disinfectants are inappropriate, such as for safety cabinet disinfection, or for respiratory or suction equipment not protected by filters [277].

A related use of formaldehyde (usually as formalin), and sometimes glutaraldehyde, is the detoxification of toxins, whole bacterial cells and viruses for the production of vaccines [326, 327]. The aldehyde interacts with its targets in such a way as to nullify the pathogenic properties while retaining their immunogenicity, presumably due to a combination of cross-linking and fixation. With the more recent development of vaccines that are detoxified by genetic manipulation, for example, peptide vaccines and genetically engineered strains, formaldehyde has a new role as a stabilizer of such genetically detoxified antigens [326]. Stabilization is thought to arise from the cross-linking properties of the aldehyde.

An important use of formaldehyde is in LTSF sterilization procedures, mainly used for packaged heat-sensitive rigid endoscopes and devices made from rubber, plastic and other heat-labile materials [277]. This specialized application deserves further comment. The sporicidal (and hence sterilizing) effect of adding formaldehyde to low-temperature steam, at sub-atmospheric pressure, was demonstrated over 90 years ago [328], although the process was not adapted for hospital use until the 1960s [80, 329]. The process requires specialized equipment, which originally took the form of modified autoclaves [330], but has since developed into LTSF sterilizers, where formalin vapour is introduced into the chamber with a series of air removal steam pulses [330]. This method is typical of the machines currently in use in the UK [330, 331]. A wide range of cycle parameters for LTSF procedures have been reported [330], but current guidelines recommend a temperature of 73°C for up to 3 hours [277]. The measurement of physical parameters associated with sterilization by LTSF is often impractical, and routine testing normally relies on the use of biological indicators to assess the biocidal efficacy of the process [24, 330]. When sterilization is required, biological indicators should be included with every load, but for small-scale use, where reliable disinfection rather than sterilization is required, the biological indicator should be used at regular intervals or when changes in routine procedures occur [24]. Spores of *B. stearothermophilus*, impregnated on paper strips, are usually employed as the biological indicator [304]. These spores are among the most resistant to formaldehyde, although *B. subtilis* spores are also acceptable [24]. A suitable recovery medium and conditions for the recovery of treated spores are most important to ensure the LTSF process is accurately assessed [330]. As discussed earlier, polymerization of formaldehyde, with subsequent condensation and deposition of solids on surfaces, will impede the penetration of formaldehyde vapour and therefore reduce

the efficacy of the process. A flushing procedure with water has been devised to prevent blockage of valves and delivery tubing [330, 332]. The minimum amount of formaldehyde necessary to achieve a successful spore test result should be employed to minimize condensation and reduce the hazard to the operator and the immediate use of the processed equipment [24, 330, 332]. Guidance on commissioning, recommissioning and routine testing of LTSF has been issued by the Department of Health [333].

The biocidal properties of aldehydes have been applied in fields other than those related to medicine. In the poultry industry, glutaraldehyde has been used for the sterilization of poultry processing equipment and as a disinfectant in the immersion chilling of poultry [334, 335]. In cosmetics, it has been recommended for disinfection of production equipment [336] and as a preservative [337]. Glutaraldehyde and formalin have also proved effective as fungicides in fish culture [338, 339], though glutaraldehyde was toxic at a concentration of 62 mg l^{-1} [338].

Glutaraldehyde and formaldehyde have also been assessed as disinfectants in sugar beet extraction installations [340]. Formaldehyde was more effective for microbiological control under the operating conditions concerned. Glutaraldehyde was also less resistant to invertase activity.

Aldehyde-releasing agents, particularly those which release formaldehyde, find application in a number of processes as preservatives, such as in cutting-oil emulsions and latexes [325]. Formaldehyde may be applied to natural keratin fibres in the leather and textile industry to prevent problems of anthrax contamination [341], in paints as preservatives [342] and in the construction industry as toxic washes to prevent microbial growth on large surface areas [343] or as additives in concrete itself [344]. Formaldehyde has long been used as a preservative for natural history specimens in, for example, museums, to prevent biodeterioration and maintain the structure of organs and tissues [345].

Glyoxal, glutaraldehyde and formaldehyde are all effective in increasing the decay resistance of wood, though the relative efficacies of the aldehydes depended on the type of wood treated [346–348].

TOXICITY AND SAFETY ASPECTS

The toxicity of aldehydes is well recognized [10, 349–352]. Direct contact with aldehydes can result in irritant dermatitis and the vapour may irritate mucous membranes of the nose, throat and eyes, with effects on the respiratory tract producing cough and bronchospasm [353–364]. Aldehydes can also act as allergic sensitizers, producing true allergic asthmas and allergic

dermatitis following exposure [355, 357, 365–369]. Other recorded effects of aldehydes include headache, dizziness, nausea, metallic taste and discolouration of the skin [355] and colitis or proctitis following endoscopy procedures [370–375]. Formaldehyde, in particular, has been shown to be a carcinogen in rats at levels of 10 ppm or more [376]. However, the results at lower levels of exposure are less clear, and other species of rodents seem to be less sensitive to the carcinogenic potential of formaldehyde. Epidemiological studies on humans have yielded conflicting results, and doubts as to methodologies have been expressed [357, 376]. Nevertheless, the carcinogenic effects in animal models mean that strict adherence to safety precautions is a prerequisite for handling this compound. There is concern that other aldehydes might display carcinogenic effects since they share the same reactive groups as formaldehyde, and most are mutagenic in various test systems. Butyraldehyde and crotonaldehyde induce polyploid cells in mice by a mechanism which may involve cell or nuclear fusion [377]. Acetaldehyde causes DNA damage and mutations in cultured human epithelial cells and fibroblasts [378]. It forms DNA-DNA and DNA-protein cross-links and decreases the activity of the DNA repair enzyme O6-methylguanine-DNA methyltransferase in epithelial cells and causes significant levels of 6-thioguanine resistance mutations in skin fibroblasts. This range of cytopathic effects is associated with multistep carcinogenesis [378]. Glutaraldehyde, glyoxal and formaldehyde are all mutagenic in Ames tests using *Salmonella typhimurium* TA102 [379–381] and induce chromosome loss in the yeast *S. cerevisiae* [382]. Glutaraldehyde is also mutagenic in cultured mammalian cell assays [383] and has been shown to cause damage to the upper respiratory tract of mice at levels of 1.0 ppm [384]. Furfural and methylfurfural are recognized dietary mutagens [385–388]. Furfural induces three times as many strand breaks as methylfurfural in calf thymus DNA, although only in the case of methylfurfural treatment does depurination lead to the degradation of DNA [385], possibly due to the alkylation of DNA bases and phosphates. Plasmids treated with furfural are found to have a decreased capacity to transform cells of *E. coli*, as a function of aldehyde concentration and time of exposure [386, 388]. The plasmids were found to have undergone DNA alterations including deletions [388], caused by single strand breaks in duplex DNA predominantly in AT base pairs. Furfural has also been shown to inactivate bacteriophage lambda, by inducing strand scissions in bacteriophage DNA [387]. Acrolein acts as a direct mutagen in *Drosophila melanogaster* and can also be metabolized by the oxidative activities of cytochrome P450 into an active metabolite, thought to be glycidaldehyde [389]. Interestingly, acrolein has also been shown to possess antimutagenic activity in *E. coli* cells exposed to UV radiation [390]. Antimuta-

genic activity occurs at 30°C in wild-type cells but only at higher temperatures in excision repair-deficient strains [390].

The health risks posed by aldehydes have caused significant concern. However, in the medical field, for example, there is no viable alternative to glutaraldehyde for the high level disinfection of medical devices in the intermediate risk category [255]. Numerous recommendations have been proposed to minimize the risks associated with the use of aldehydes. In the UK, COSHH regulations [391] require employers to assess the risks to the health of staff of exposure to chemicals such as aldehydes, to avoid such exposure where reasonably practicable and otherwise to ensure adequate control. Engineering means of control must be used in preference to personal protective equipment [255, 391]. A working party of the British Society of Gastroenterology Endoscopy Committee has made various recommendations on the use of aldehydes in hospitals [355]. Most mention glutaraldehyde specifically, but are applicable to all aldehydes. The main recommendations are that: (a) heat-stable items should be autoclaved where possible, (b) heat-labile items should be disinfected within automated washer/disinfectors, (c) local exhaust ventilation must be used to control glutaraldehyde vapour, (d) endoscope disinfection should be performed in a dedicated room equipped with control measures to maintain the concentration of glutaraldehyde vapour below current occupational exposure standards (0.2 ppm for glutaraldehyde, 0.75 ppm for formaldehyde), and (e) monitoring of atmospheric levels should be performed by trained personnel. The full list of recommendations, and the reasoning behind them, can be found in the working party report [355]. Additionally, many institutions now implement health surveillance for employees exposed to chemicals such as aldehydes [392].

Monitoring of aldehydes would thus seem particularly important to confirm that control measures are adequate and effective. An accepted technique for the atmospheric sampling of glutaraldehyde is the OSHA64 method [393] in which air is drawn through two filters impregnated with 2,4-dinitrophenylhydrazine and phosphoric acid. The stable glutaraldehyde derivative is trapped on the filter and eluted to be analyzed by high performance liquid chromatography. Diffusive samplers based on this technique have been developed [394, 395]. Commercially available glutaraldehyde meters are less reliable and subject to interference from other vapours in the atmosphere such as alcohol, perfume and aftershave [355].

A number of methods for monitoring formaldehyde levels in air have also been assessed [396]. These include commercial monitors based on 'passive' methods and established 'active' methods based on three different chemicals; chromotropic acid, 2-(hydroxymethyl)piperidine and 2,4-dinitrophenylhy-

drazine, with subsequent analysis by high-performance liquid chromatography. The monitors were tested for short-term and time-weighted average monitoring for formaldehyde at concentrations of 0.3, 0.5 and 1 ppm [396]. Only the monitor based on 2,4-dinitrophenylhydrazine met the Occupational Safety and Health Administration's criteria for monitoring at a 0.3 ppm ceiling [393, 396].

A recent article describes the use of various aldehyde derivatives to determine the amount of volatile aldehyde in air [397]. Aldehyde pentafluorobenzyloximes were superior to the recommended 2,4-dintrophenylhydrazones in terms of volatility and sensitivity to detection by gas chromatography and may be useful to monitor aldehyde levels in terms of their contribution to air pollution [397].

Methods of monitoring aldehydes in food and pharmaceutical samples have also been reported. A semiautomatic method by a stopped-flow flow injection analysis technique was successful in identifying the presence of furfural and 5-hydroxymethyl-2-furfuraldehyde in several commercial pharmaceutical preparations and food samples [398]. The analysis is based on the reaction of the aldehydes with 2-thiobarbituric acid, with determination of the derivatives.

Finally, levels of furfural and 5-hydroxymethyl-2-furfuraldehyde have also been monitored in processed fruit juices by a high performance liquid chromatography method using a reversed-phase macroreticular column and phosphate buffer as eluent [399]. These aldehydes are degradation products of L-ascorbic acid. The detection limit of this method was reported as 0.05 mg l^{-1} which was insufficiently sensitive to detect the presence of furfural.

CONCLUSIONS

The extensive use of aldehydes as biocides has perhaps diminished in recent years with increasing knowledge and awareness of the health risks associated with their application. Increasing targeting of their biocidal action has become evident, for example, for heat-labile medical devices such as endoscopes, where there are no viable and less toxic alternatives retaining the range and potency of the aldhydes. Additionally, the safety measures necessarily imposed on their use adds significant cost implications for hospitals whose budgets might already be limited. Nevertheless, there remains a niche for aldehydes as biocides which is currently unoccupied by potential substitutes. The exciting development of new compounds such as the peptide aldehydes which are targeted at specific microbial enzymes offers the potential of a new class of antimicrobial agent. The advent of untreatable diseases

such as AIDS and the emergence of multiple antibiotic resistance in bacteria makes the development of novel biocidal agents all the more imperative. An increased understanding of the biocidal mechanisms of aldehyde action and resistance can only serve to increase those options.

ACKNOWLEDGEMENTS

I must express my heartfelt thanks to Professor Denver Russell (Welsh School of Pharmacy) for sparking my enthusiasm in the field of antimicrobial agents, and for his ready help and advice.

REFERENCES

1 Nordgren, G. (1939) Acta Pathol. Microbiol. Scand. Suppl., 38–41.
2 Harries, C. and Frank, L. (1908) Ber. Dtsch. Chem. Ges. 41, 1701–1711.
3 Fein, M.L., Harris, E.H., Naghski, J. and Filachione, J. (1959) J. Am. Leather Chem. Assoc. 54, 488–496.
4 Sabatini, D.D., Bensch, K. and Barnett, R.J. (1963) J. Cell Biol. 17, 19–58.
5 Russell, A.D. and Hopwood, D. (1976) Prog. Med. Chem. 13, 271–301.
6 Pepper, R.E. and Chandler, V.L. (1963) Appl. Microbiol. 11, 384–388.
7 Stonehill, A.A., Krop, S. and Borick, P.M. (1963) Am. J. Hosp. Pharm. 20, 458–465.
8 Power, E.G.M. and Russell, A.D. (1990) J. Appl. Bacteriol. 69, 261–268.
9 Rahn, O. (1945) Biodynamica 3, 90.
10 Gorman, S.P., Scott, E.M. and Russell, A.D. (1980) J. Appl. Bacteriol. 48, 161–190.
11 Bowes, J.H. and Cater, C.W. (1966) J. R. Microsc. Soc. 85, 193–200.
12 Hardy, P.M., Nicholls, A.C. and Rydon, H.N. (1976) J. Chem. Soc., Perkin Trans. 1, 958–962.
13 Hugo, W.B and Russell, A.D. (1992) in Principles and Practice of Preservation, Disinfection and Sterilization (Russell, A.D., Hugo, W.B. and Ayliffe, G.A.J., eds.). pp. 89–113, Blackwell Scientific Publications, Oxford.
14 Anderson, P.J. (1967) J. Histochem. Cytochem. 15, 652–661.
15 Gillett, R. and Gull, K. (1972) Histochemistry 30, 162–167.
16 Rasmussen, K.E. and Albrechtsen, J. (1974) Histochemistry 38, 19–26.
17 Gorman, S.P. and Scott, E.M. (1979) Int. J. Pharm. 4, 57–65.
18 Scobbie, E. and Groves, J.A. (1995) Ann. Occup. Hyg. 39, 63–78.
19 Boucher, R.M.G. (1974) Am. J. Hosp. Pharm. 31, 546–557.
20 Gorman, S.P. and Scott, E.M. (1980) J. Pharm. Pharmacol. 32, 131.
21 Boucher, R.M.G. (1975) Can. J. Pharm. Sci. 10, 1–7.
22 Hopwood, D., Allen, C.R. and McCabe, M. (1970) Histochem. J. 2, 137–150.
23 Hopwood, D. (1975) Histochem. J. 7, 267–276.
24 Christensen, E.A. and Kristensen, H. in Ref. 13, pp. 557–572.
25 Committee on Aldehydes (1981) Formaldehyde and Other Aldehydes (National Research Council, USA, eds.), National Academy Press, Washington.
26 Tulis, J.J. (1973) in Industrial Sterilization: International Symposium, Amsterdam 1972

(Phillips, G.B. and Miller, W.S., eds.), pp. 209–238, Duke University Press, Durham, North Carolina.
27 Kingston, D. (1965) J. Clin. Pathol. 18, 666–667.
28 Wright, C.J. and McAllister, T.A. (1967) Clin. Trials J. 4, 680–681.
29 Browne, M.K. and Stoller, J.L. (1970) Br. J. Surg. 57, 525–529.
30 Pickard, R.G. (1972) Br. J. Surg. 59, 642–648.
31 Reeves, D.S. and Schweitzer, F.A.W. (1973) in Proceedings of the 8th International Congress of Chemotherapy, p. 583–586, Hellenic Press, Greece.
32 Browne, M.K., Leslie, G.B. and Pfirrman, R.W. (1976) J. Appl. Bacteriol. 41, 363–368.
33 Myers, J.A., Allwood, M.C., Gidley, M.J. and Sanders, J.K.M. (1980) J. Appl. Bacteriol. 48, 89–96.
34 Allwood, M.C. and Myers, E.R. (1981) in Society for Applied Bacteriology Technical Series 16, pp. 69–76, Academic Press, London.
35 Rossmore, H.W. and Sondossi, M. (1988) Adv. Appl. Microbiol. 33, 223–277.
36 Fraenkel-Conrat, H., Cooper, M. and Olcott, H.S. (1945) J. Am. Chem. Soc. 67, 950–954.
37 Fraenkel-Conrat, H. and Olcott, H.S. (1946) J. Am. Chem. Soc. 68, 34–37.
38 Fraenkel-Conrat, H. and Olcott, H.S. (1948) J. Biol. Chem. 174, 827–843.
39 Staehelin, M. (1958) Biochim. Biophys. Acta 29, 410–417.
40 Alderson, T. (1960) Nature (London) 187, 485–489.
41 Neely, W.B. (1963) J. Bacteriol. 85, 1028–1036.
42 Neely, W.B. (1963) J. Bacteriol. 85, 1420–1430.
43 Neely, W.B. (1963) J. Bacteriol. 86, 445–456.
44 Neely, W.B. (1966) J. Gen. Microbiol. 45, 187–194.
45 Tal, M. (1970) Biochim. Biophys. Acta 224, 470–476.
46 Favero, M.S. and Bond, W.W. (1991) in Sterilization of Medical Products, Vol. 5 (Morrissey, R.F. and Prokopenko, Y.I., eds.), pp. 397–405, Polyscience Publications Inc., Morin Heights, Canada.
47 Favero, M.S. and Bond, W.W. (1991) in Disinfection, Sterilization and Preservation (Block, S.S., ed.), pp. 617–641, Lea and Febiger, Philadelphia.
48 Favero, M.S. and Bond, W.W. (1993) in Sterilization Technology: A Practical Guide for Manufacturers and Users of Health Care Products (Morrissey, R.F. and Phillips, G.B., eds.), pp. 309–334, Van Nostrand Reinhold, New York.
49 Russell, A.D. (1994) Infect. Control Hosp. Epidemiol. 15, 724–733.
50 Russell, A.D. in Ref. 13, pp. 89–113.
51 Scott, E.M. and Gorman, S.P. in Ref. 47, pp. 596–614.
52 Jette, L.P., Ringuette, L., Ishak, M., Millar, M. and Saintantoine, P. (1995) J. Hosp. Infect. 30, 295–303.
53 Russell, A.D. and Russell, N.J. (1995) in Fifty Years of Antimicrobials: Past Perspectives and Future Trends (Hunter, P.A., Darby, G.K. and Russell, N.J., eds.), pp. 327–365, 53rd Symposium of the Society for General Microbiology, Cambridge University Press, Cambridge.
54 Power, E.G.M. and Russell, A.D. (1988) J. Hosp. Infect. 11, 376–380.
55 Russell, A.D. (1990) in Topley and Wilson's Principles of Bacteriology, Virology and Immunity, 8th edn., Vol. 1 (Dick, M.H. and Linton A.H., eds.), pp. 71–103, Edward Arnold, London.
56 Ayliffe, G.A.J., Collins, B.J. and Taylor, L.J. (1990) Hospital-Acquired Infection, John Wright, London.
57 Gardner, J.F. and Peel, M.M. (1991) Introduction to Sterilization, Disinfection and Infection Control, Churchill Livingstone, London.
58 Russell, A.D. in Ref. 47, pp. 29–88.

59 Russell, A.D. in Ref. 48, pp. 3–16.
60 Rutala, W.A., Cole, E.C. Wannamaker, M.S. and Weber, D.J. (1991) Am. J. Med. 91 (Suppl. B), 267S-271S.
61 Rutala, W.A., Gergen, M.F. and Weber, D.J. (1993) Infect. Control Hosp. Epidemiol. 14, 36–40.
62 Rubbo, S.D., Gardner, J.F. and Webb, R.L. (1967) J. Appl. Bacteriol. 30, 78–87.
63 McGucken, P.V. and Woodside, W. (1973) J. Appl. Bacteriol. 36, 419–426.
64 Sierra, G. and Boucher, R.M.G. (1971) Appl. Microbiol. 22, 160–164.
65 Thomas, S. and Russell, A.D. (1974) Appl. Microbiol. 28, 331–335.
66 Kawahara, J.I., Ohmori, T., Ohkubo, T., Hattori, S. and Kawamura, M. (1992) Anal. Biochem. 201, 94–98.
67 Jordan, S.L.P., Russo, M.R., Blessing, R.L. and Theis, A.B. (1996) J. Toxicol. Environ. Health 47, 299–309.
68 Kuznetsova, N.P., Mishaeva, R.N., Koltsova, S.V., Gudkin, L.R., Stragovich, L.M. and Bolshakova, E.G. (1993) Bioorganich. Khim. 19, 871–879.
69 Gelinas, P. and Goulet, J. (1983) J. Appl. Bacteriol. 54, 243–247.
70 Borick, P.M., Dondershine, F.H. and Chandler, J.L. (1964) J. Pharm. Sci. 53, 1273–1275.
71 Snyder, R.W. and Cheatle, E.L. (1965) Am. J. Hosp. Pharm. 22, 321–327.
72 Gorman, S.P. and Scott, E.M. (1978) Microbios Lett. 6, 39–44.
73 Dey, B.P. and Engley, F.B. (1994) J. Microbiol. Methods 19, 51–58.
74 Russell, A.D. (1982) The Destruction of the Bacterial Spore, Academic Press, London.
75 Trujillo, R. and David, T.J. (1972) Appl. Microbiol. 23, 618–622.
76 Willard, M. and Alexander, A. (1964) Appl. Microbiol. 12, 229–233.
77 Trujillo, R. and Lindell, K.F. (1973) Appl. Microbiol. 26, 106–110.
78 Committee on Formaldehyde Disinfection (1958) J. Hyg. 56, 488–515.
79 Deverill, C.E.A. and Cripps, N.F. (1981) J. Hosp. Infect. 2, 175–180.
80 Alder, V.G., Brown, A.M. and Gillespie, W.A. (1966) J. Clin. Pathol. 19, 83–89.
81 Abbott, C.F., Cockton, J. and Jones, W. (1956) J. Pharm. Pharmacol. 8, 709–719.
82 Baird-Parker, A.C. and Holbrook, R. (1971) in Inhibition and Destruction of the Microbial Cell (Hugo, W.B., ed.), pp. 369–397, Academic Press, London.
83 Spaulding, E.H., Grundy, K.R. and Turner, F.J. (1977) in Disinfection, Sterilization and Preservation (Block, S.S., ed.), pp. 654–684, Lea and Febiger, Philadelphia.
84 Hoffman, R.K. (1971) in Ref. 82, pp. 225–228.
85 Best, M., Kennedy, F.E. and Coates, F. (1990) Appl. Environ. Microbiol. 56, 377–380.
86 Berchieri, A. and Barrow, P. (1995) Rev. Microbiol. 26, 246–252.
87 Vatsala, T.M. and Mah, R.A. (1995) Curr. Sci. 68, 1131–1133.
88 Collins, F.M. (1989) Clin. Microbiol. Rev. 2, 360–377.
89 Draper, P. (1988) Int. J. Leprosy 52, 527–532.
90 Russell, A.D. in Ref.13, pp. 246–253.
91 David, H.L., Rastogi, N., Seres, C.L. and Clement, F. (1988) Curr. Microbiol. 17, 61–68.
92 Sareen, M. and Khuller, G.K. (1990) Lett. Appl. Bacteriol. 11, 7–10.
93 Rutala, W.A. (1990) Am. J. Infect. Control 18, 99–117.
94 Bergan, T. and Lystad, A. (1971) J. Appl. Bacteriol. 34, 751–756.
95 Collins, F.M. and Montalbine, V. (1976) J. Clin. Microbiol. 4, 408–412.
96 Collins, F.M. (1986) J. Appl. Bacteriol. 61, 87–93.
97 Collins, F.M. (1986) J. Appl. Bacteriol. 61, 247–251.
98 van Klingeren, B. and Pullen, W. (1987) J. Hosp. Infect. 10, 292–298.
99 Holton, J., Nye, P. and McDonald, V. (1994) J. Hosp. Infect. 27, 105–115.
100 Carson, L.A., Petersen, J., Favero, M.S. and Aguero, S.M. (1978) Appl. Environ. Microbiol. 36, 839–846.

101 Hanson, P.J.V. (1988) Br. J. Hosp. Med. 40, 149.
102 Hanson, P.J.V., Chadwick, M., Nicholson, G., Gaya, H. and Collins, J.V. (1988) Thorax 43, 850P.
103 Jackson, J., Leggett, J.E., Wilson, D. and Gilbert, D.N. (1996) Am. J. Infect. Control 24, 19–23.
104 Merkal, R.S. and Whipple, D.L. (1980) Appl. Environ. Microbiol. 40, 282–284.
105 Cole, E.C., Rutala, W.A., Nessen, L., Wannamaker, N.S. and Weber, D.J. (1990) Appl. Environ. Microbiol. 56, 1813–1817.
106 Sattar, S.A., Best, M., Springthorpe, V.S. and Sanani, G. (1995) J. Hosp. Infect. 30, 372–382.
107 Gordon, M.D., Ezzell, R.J., Bruckner, N.I. and Ascenzi, J.M. (1994) J. Ind. Microbiol. 13, 77–82.
108 Russell, A.D. (1990) Clin. Microbiol. Rev. 3, 99–119.
109 Russell, A.D., Dancer, B.N. and Power, E.G.M. (1991) in Mechanisms of Action of Chemical Biocides (Denyer, S.P. and Hugo, W.B., eds.), pp. 23–44, Society for Applied Bacteriology Technical Series No. 27, Blackwell Scientific Publications, Oxford.
110 Bloomfield, S.F. in Ref. 13, pp. 230–245.
111 Sagripanti, J.L. and Bonifacio, A. (1996) Appl. Environ. Microbiol. 62, 545–551.
112 Kelsey, J.C., MacKinnon, I.H. and Maurer, I.M. (1974) J. Clin. Pathol. 27, 632–638.
113 Forsyth, M.P. (1975) Develop. Ind. Microbiol. 16, 37–47.
114 Miner, N.A., McDowell, J.W., Willcockson, G.W., Bruckner, N.I., Stark, R.L. and Whitmore, E.J. (1977) Am. J. Hosp. Pharm. 34, 376–382.
115 Babb, J.R., Bradley, C.R. and Ayliffe, G.A.J. (1980) J. Hosp. Infect. 1, 63–75.
116 Rutala, W.A., Gergen, M.F. and Weber, D.J. (1993) Infect. Control Hosp. Epidemiol. 14, 713–718.
117 Dyas, A. and Das, B.C. (1985) J. Hosp. Infect. 6, 41–45.
118 Ortenzio, L.F., Stuart, L.S. and Friedl, J.L. (1953) J. Assoc. Off. Agric. Chem. 36, 480–484.
119 Klarmann, E.G. (1956) Am. J. Pharm. 128, 4–18.
120 Klarmann, E.G. (1959) Am. J. Pharm. 131, 86–90.
121 Munton, T.J. and Russell, A.D. (1970) J. Appl. Bacteriol. 33, 410–419.
122 King, J.A., Woodside, W. and McGucken, P. (1974) J. Pharm. Sci. 63, 804–805.
123 Ayliffe, G.A.J., Coates, D. and Hoffman, P.M. (1984) Chemical Disinfection in Hospitals, Public Health Laboratory Service, London.
124 Danielson, J.W., Peeler, J.T. and Oxborrow, G.S. (1994) J. Assoc. Off. Anal. Chem. Int. 77, 53–57.
125 D'Alessandro, S.M., Derevjanik, M.A. and Howard, M. (1981) J. Dent. Res. 60, Abstr. 54.
126 Leach, E.D. (1981) Infect. Control 2, 26–30.
127 Pepper, R.E. (1980) Infect. Control 1, 90–92.
128 Townsend, T.R., Wee, S-B. and Koblin, B. (1982) Infect. Control 3, 240–243.
129 Derby, B. and McGucken, E. (1984) Ortho. Nursing 3, 5.
130 Isenberg, H.D. (1985) J. Clin. Microbiol. 22, 735–739.
131 Russell, A.D. (1965) Manuf. Chem. Aerosol News 36, 38.
132 Waites, W.M. and Bayliss, C.E. (1984) in The Revival of Injured Microbes (Andrew, M.H.E. and Russell, A.D., eds.), pp. 159–172, Society for Applied Bacteriology Technical Series No. 12, Academic Press, London.
133 Dabrowa, N., Landau, J.W. and Newcomer, V.D. (1972) Arch. Dermatol. 105, 555–557.
134 Gorman, S.P. and Scott, E.M. (1977) J. Appl. Bacteriol. 43, 83–89.
135 Gray, K.G. (1980) Aust. J. Hosp. Pharm. 10, 139–141.

136 Tadeusiak, B. (1976) Rocz. Panstw. Zakl. Hig. 27, 689–695.
137 Spicher, G. and Peters, J. (1976) Zentralb. Bakteriol. Parasitenkd. Infektionskr. Hyg. 163, 468–508.
138 Strange, Jr., R.R., Midland, S.L., Eckert, J.W. and Sims, J.J. (1993) J. Nat. Prod. 56, 1627–1629.
139 Wallhauser, K.H. (1984) in Cosmetic Drug Preservation. Principles and Practice (Kabara, J.J., ed.), pp. 605–745, Marcel Dekker, New York.
140 Karabit, M.S., Juneskans, O.T. and Lundgren, P. (1989) Int. J. Pharm. 56, 169–174.
141 Springthorpe, V.S. and Sattar, S.A. (1990) Crit. Rev. Environ. Control 20, 169–229.
142 Quinn, P.J. in Ref. 13, pp. 150–170.
143 Spire, B., Barre-Sinoussi, F., Montagnier, L. and Chermann, J.C. (1984) Lancet 1, 899–901.
144 Resnick, L., Veren, K., Salahuddin, S.Z., Tondreau, S. and Markham, P.D. (1986) J Am. Med. Assoc. 255, 1887–1891.
145 Hanson, P.J.V., Gor, D., Jeffries, D.J. and Collins, J.V. (1989) Br. Med. J. 298, 862–864.
146 Druce, J.D., Jardine, D., Locarnini, S.A. and Birch, C.J. (1995) J. Hosp. Infect. 30, 167–180.
147 Hospital Infection Society (1990) J. Hosp. Infect. 15, 7–34.
148 Martin, L.S., McDougal, J.S. and Loskoski, S.L. (1985) J. Infect. Dis. 152, 400–403.
149 Bond, W.W., Favero, M.S., Peterson, N.J., Ebert, J.W. (1983) J. Clin. Microbiol. 18, 533–538.
150 Kobayashi, H. and Tsuzuki, M. (1984) J. Hosp. Infect. 5 (suppl. A), 93–94.
151 Adler-Storthz, K., Schulster, L.M., Dreesman, G.R., Hollinger, F.B. and Melnick, J.L. (1983) Eur. J. Clin. Microbiol. 2, 316–320.
152 Passagot, J., Crance, J.M., Biziagos, E., Laveran, H., Agbalika, F. and Deloince, R. (1987) J. Virol. Methods 16, 21–28.
153 Tyler, R. and Ayliffe, G.A.J. (1987) J. Hosp. Infect. 19, 22–29.
154 Tyler, R., Ayliffe, G.A.J. and Bradley, C.R. (1990) J. Hosp. Infect. 15, 339–345.
155 Chambon, M., Bailly, J.-L. and Peigue-Lafeuille, H. (1994) Appl. Environ. Microbiol. 60, 387–392.
156 Bailly, J.-L., Chambon, M., Peigue-Lafeuille, H., Laveran, H., de Champs, C. and Beytout, D. (1991) Appl. Environ. Microbiol. 57, 1156–1160.
157 Chambon, M., Bailly, J.-L. and Peigue-Lafeuille, H. (1992) Appl. Environ. Microbiol. 58, 3517–3521.
158 Sattar, S.A., Springthorpe, V.S., Karim, Y. and Loro, P. (1989) Epidemiol. Infect. 102, 493–505.
159 Springthorpe, V.S., Grenier, J.L., Lloyd-Evans, N. and Sattar, S.A. (1986) J. Hyg. 97, 139–161.
160 Lloyd-Evans, N., Springthorpe, V.S. and Sattar, S.A. (1986) J. Hyg. 97, 163–173.
161 Rodgers, F.G., Hufton, P., Kurzawska, E., Molloy, C. and Morgan, S. (1985) J. Med. Microbiol. 20, 123–130.
162 Meng, Z.-D., Birch, C., Heath, R. and Gust, I. (1987) Appl. Environ. Microbiol. 53, 727–730.
163 Saitanu, K. and Lund, E. (1975) Appl. Microbiol. 29, 571–574.
164 Narang, H.K. and Codd, A.A. (1983) J. Hosp. Infect. 4, 209–212.
165 Maillard, J.Y., Beggs, T.S, Day, M.J., Hudson, R.A. and Russell, A.D. (1995) Lett. Appl. Microbiol. 21, 215–218.
166 Maillard, J.Y., Beggs, T.S., Day, M.J., Hudson, R.A. and Russell, A.D. (1994) Appl. Environ. Microbiol. 60, 2205–2206.
167 Blackwell, J.H., Graves, J.H. and McKercher, P.D. (1975) Br. Vet. J. 131, 317–322.

168 Cunliffe, H.R., Blackwell, J.H. and Walker, J.S. (1979) Appl. Environ. Microbiol. 37, 1044–1046.
169 Brown, T.T. (1981) Am. J. Vet. Res. 42, 1033–1036.
170 Shen, D.T., Leendertsen, L.W. and Gorham, J.R. (1981) Am. J. Vet. Res. 42, 838–840.
171 Elliot, D.G. and Amend, D.F. (1978) J. Fish Biol. 12, 277–286.
172 Shen, D.T., Crawford, T.B., Gorham, J.R. and McGuire, T.C. (1977) Am. J. Vet. Res. 38, 1217–1219.
173 Hughes, R.C. and Thurman, P.F. (1970) Biochem. J. 119, 925–926.
174 Russell, A.D. and Vernon, G.N. (1975) Microbios 13, 147–149.
175 Gorman, S.P. and Scott, E.M. (1977) Microbios 19, 205–212.
176 Mohapatra, S.C. and Hsu, J.T. (1994) Biotechnol. Tech. 8, 13–16.
177 Bruch, C.W. in Ref. 46, pp. 377–396
178 Hugo, W.B. in Ref. 13, pp. 187–210.
179 Korn, A.H., Feairheller, S.H. and Filachione, E.M. (1972) J. Mol. Biol. 65, 525–529.
180 Monsan, P., Puzo, G. and Mazarguil, H. (1975) Biochimie 57, 1281–1292.
181 Hardy, P.M., Hughes, G.J. and Rydon, H.N. (1976) J. Chem. Soc., Chem. Commun. 5, 157–158.
182 Hardy, P.M., Hughes, G.J. and Rydon, H.N. (1977) J. Chem. Soc., Chem. Commun. 21, 759–760.
183 Munton, T.J. and Russell, A.D. (1972) J. Appl. Bacteriol. 35, 193–199.
184 Russell, A.D. and Haque, H. (1975) Microbios 13, 151–153.
185 Munton, T.J. and Russell, A.D. (1973) J. Appl. Bacteriol. 36, 211–217.
186 Navarro, J.M. and Monsan, P. (1976) Ann. Inst. Pasteur Paris 127B, 295–307.
187 Munton, T.J. and Russell, A.D. (1971) Experientia 27, 109–110.
188 Gorman, S.P. and Scott, E.M. (1977) Microbios 18, 123–130
189 Munton, T.J. and Russell, A.D. (1973) Appl. Microbiol. 26, 508–511.
190 Ellar, D.J., Munoz, E. and Salton, M.R.J. (1971) Biochim. Biophys. Acta 225, 140–150.
191 Done, J., Shorey, C.D., Loke, J.P. and Pollak, J.K. (1965) Biochem. J. 96, 27c–28c.
192 Wang, J.H.C. and Tu, J. (1969) Biochemistry 8, 4403–4410.
193 Wetzel, B.K., Spicer, S.S., Dvorak, H.F. and Heppel, L.A. (1970) J. Bacteriol. 104, 529–542.
194 Cheng, K.J., Ingram, J.M. and Costerton, J.W. (1970) Can. J. Microbiol. 16, 1319–1324.
195 Pulvertaft, R.J.V. and Lumb, G.D. (1948) J. Hyg. 46, 62–64.
196 Grossman, L., Levine, S.S. and Allison, W.S. (1961) J. Mol. Biol. 3, 47–60.
197 Collins, C.A. and Guild, W.R. (1968) Biochim. Biophys. Acta 157, 107–113.
198 Monsan, P., Puzo, G. and Marzaguil, H. (1976) in Analysis and Control of Immobilized Enzyme Systems (Thomas, D. and Kernevez, J.P., eds.), pp. 275–282, North-Holland Press, Amsterdam.
199 Gould, G.W. and Dring, G.J. (1975) Nature (London) 258, 402–405.
200 Thomas, S. (1977) Microbios Lett. 4, 199–204.
201 Power, E.G.M. and Russell, A.D. (1989) J. Appl. Bacteriol. 67, 329–342.
202 Gorman, S.P. and Scott, E.M. (1978) Microbios Lett. 5, 163–169.
203 Doyle, R.J., Nedjat-Haiem, F. and Singh, J.S. (1984) Curr. Microbiol. 10, 329–332.
204 Rosenberg, E., Brown, D.R. and Demain, A.L. (1985) Arch. Microbiol. 142, 51–54.
205 Warth, A.D. (1978) Adv. Microb. Physiol. 17, 1–45.
206 Kutima, P.M. and Foegeding, P.M. (1987) Appl. Environ. Microbiol. 53, 47–52.
207 Power, E.G.M. and Russell, A.D. (1990) FEMS Microbiol. Lett. 54, 271–276.
208 Powell, J.F. (1957) J. Appl. Bacteriol. 20, 349–358.
209 Hyatt, M.T. and Levinson, H.S. (1961) J. Bacteriol. 81, 204–211.

210 Stewart, G.S.A.B., Johnstone, K., Hagelberg, E. and Ellar, D.J. (1981) Biochem. J. 198, 101–106.
211 Sykes, G. (1970) J. Appl. Bacteriol. 33, 147–156.
212 Walker, H.W. (1964) J. Food Sci. 29, 360–365.
213 Fraenkel-Conrat, H. (1961) Biochim. Biophys. Acta 49, 169–180.
214 Loveless, A. (1951) Nature (London)167, 338–342.
215 Phillips, C.R. (1952) Bacteriol. Rev. 16, 135–138.
216 Power, E.G.M. and Russell, A.D. (1991) Lett. Appl. Microbiol. 11, 231–232.
217 Power, E.G.M., Dancer, B.N. and Russell, A.D. (1988) FEMS Microbiol. Lett. 50, 223–226.
218 Dancer, B.N., Power, E.G.M. and Russell, A.D. (1989) FEMS Microbiol. Lett. 57, 345–348.
219 Power, E.G.M., Dancer, B.N. and Russell, A.D. (1989) J. Appl. Bacteriol. 67, 91–98.
220 Wyatt, L.R. and Waites, W.M. (1974) J. Gen. Microbiol. 84, 391–394.
221 Gould, G.W., Stubbs, J.M. and King, W.L. (1970) J. Gen. Microbiol. 60, 347–355.
222 Duncan, C.L., Labbe, R.G. and Reich, R.R. (1972) J. Bacteriol. 109, 550–559.
223 Kulikovsky, A., Pankrantz, H.S. and Sadoff, H.L. (1975) J. Appl. Bacteriol. 38, 39–46.
224 Gorman, S.P., Hutchinson, E.P., Scott, E.M. and McDermott, L.M. (1983) J. Appl. Bacteriol. 54, 91–99.
225 Kondo, M. and Foster, J.W. (1967) J. Gen. Microbiol. 47, 257–271.
226 Hajdu, J. and Friedrich, P. (1975) Anal. Biochem. 65, 273–280.
227 Gould, G.W. in Ref. 132, pp. 199–220.
228 Maillard, J.Y., Hann, A.C., Beggs, T.S., Day, M.J., Hudson, R.A. and Russell, A.D. (1995) J. Med. Microbiol. 42, 415–420.
229 Maillard, J.Y., Beggs, T.S., Day, M.J., Hudson, R.A. and Russell, A.D. (1996) J. Appl. Bacteriol. 80, 291–295.
230 Permana, P.A. and Snapka, R.M (1994) Carcinogenesis 15, 1031–1036.
231 Malcolm, B.A., Lowe, C., Shechosky, S., McKay, R.T, Yang, C.C., Shah, V.J., Simon, R.J., Vederas, J.C. and Santi, D.V. (1995) Biochemistry 34, 8172–8179.
232 Allaire, M., Chernaia, M.M., Malcolm, B.A. and James, M.N. (1994) Nature (London) 369, 72–77.
233 Matthews, D.A., Smith, W.W., Ferre, R.A., Condon, B., Budahazi, G., Sisson, W., Villafranca, J.E., Janson, C.A., McElvoy, H.E., Gribskov, C.L. and Worland, S. (1994) Cell 77, 761–771.
234 Lewis, C.A. and Wolfenden, R. (1977) Biochemistry 16, 4890–4894.
235 Gamcsik, M.P., Malthouse, J.P.G., Primrose, W.U., MacKenzie, N.E., Boyd, A.S.F., Russell, R.A. and Scott, A.I. (1983) J. Am. Chem. Soc. 105, 6324–6325.
236 Russell, A.D. (1994) in Chemical Germicides in Health Care (Rutala, W.A., ed.), pp. 255–269, Polyscience Publications, Morin Heights, Canada.
237 Russell, A.D. and Chopra, I. (1990) Understanding Antibacterial Action and Resistance, pp. 182–210, Ellis Horwood, London.
238 Brown, M.R.W. and Williams, P. (1985) Annu. Rev. Microbiol. 39, 527–556.
239 Heinzel, M. (1988) in Industrial Biocides (Heinzel, M., ed.), pp. 52–67, John Wiley and Sons, Chichester.
240 Nikaido, H. and Vaara, M. (1985) Microbiol. Rev. 49, 1–32.
241 Vaara, M. (1992) Microbiol. Rev. 56, 395–411.
242 Rubin, J. in Ref. 46, pp. 414–421.
243 Costerton, J.W., Cheng, K.-J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M. and Marrie, T.J. (1987) Annu. Rev. Microbiol. 41, 435–464.
244 Brown, M.R.W. and Gilbert, P. (1993) J. Appl. Bacteriol. Symp. Suppl. 74, 87S–94S.

245 Gilbert, P. (1988) in Microbial Quality Assurance in Pharmaceuticals, Cosmetics and Toiletries (Bloomfield, S.F., Baird, R., Leak, R.E. and Leech, R., eds.), pp. 171–194, Ellis Horwood, London.
246 Prince, N.H., Nonemaker, W.S., Norgard, R.C. and Prince, D.L. (1978) J. Pharm. Sci. 67, 1629–1631.
247 Takeo, Y., Oie, S., Kamiya, A., Konishi, H. and Nazakawa, T. (1994) Microbios 79, 19–26.
248 Roberts, T.A. and Hitchins, A.D. in Ref. 82, pp. 451–612.
249 Bloomfield, S.F. and Arthur, M. (1994) J. Appl. Bacteriol. Symp. Suppl. 76, 91S–104S.
250 Dekker, J. (1987) In Modern Selective Fungicides (Lyr, H., ed.), p. 39, Longman, Harlow.
251 Russell, A.D. in Ref. 13, pp. 134–149.
252 Gale, E.F. (1986) Adv. Microb. Physiol. 27, 227–230.
253 Hiom, S.J., Furr, J.R., Russell, A.D. and Dickinson, J.R. (1993) Microbios 74, 111–120.
254 Warth, A.D. (1989) Int. J. Food Microbiol. 8, 343–349.
255 Thurman, R.B. and Gerba, C.P. (1988) Adv. Appl. Microbiol. 33, 75–105.
256 Klein, M. and Deforest, A. (1983) in Disinfection, Sterilization and Preservation (Block, S.S., ed.), pp. 422–434, Lea and Febiger, Philadelphia.
257 Kato, N., Miyawaki, N. and Sakazawa, C. (1982) Agric. Biol. Chem. 46, 655–657.
258 Kato, N., Shirakawa, K., Kobayashi, H. and Sakazawa, C. (1983) Agric. Biol. Chem. 47, 39–40.
259 Kato, N., Miyawaki, N. and Sakazawa, C. (1983) Agric. Biol. Chem. 47, 415–416.
260 Holmes, R.S. (1978) Comp. Biochem. 61, 339–346.
261 Messiha, F.S. (1978) Brain Res. Bull. 3, 595–599.
262 Cascales, C., Cascales, M. and Santos-Ruiz, A. (1985) Rev. Esp. Fisiol. 41, 19–27.
263 Kaulfers, P.-M., Karch, H. and Laufs, R. (1987) Zentralb. Bakteriol. Hyg. A226, 239–248.
264 Favero, M.S. and Bond, W.W. (1991) in Manual of Clinical Microbiology, 5th edn. (Balows, A., Hausler, Jr., W.J., Herrmann, K.L., Isenberg, H.D. and Shadomy, H.J., eds.), pp. 183–200, American Society of Microbiology, Washington D.C.
265 Ridgway, G.L. (1985) J. Hosp. Infect. 6, 363–368.
266 George, R.H. (1988) J. Hosp. Infect. 11(Suppl. A), 386–392.
267 Frank, U. and Daschner, F. (1989) Endoscopy 21, 276–278.
268 Hanson, P.J.V. and Collins, J.V. (1989) Thorax 44, 778–783.
269 Loffer, F.D. (1990) J. Reprod. Med. 25, 263–266.
270 Axon, A.T.R. (1991) Baillere's Clin. Gastroenterol. 5, 61–77.
271 Babb, J.R. and Bradley, C.R. (1991) J. Hosp. Infect. 18(Suppl. A), 130–135.
272 Ayliffe, G.A.J., Babb, J.R. and Bradley, C.R. (1992) J. Hosp. Infect. 22, 265–269.
273 Noone, M. and Mayes, R.W. (1993) J. Hosp. Infect. 24, 80.
274 Fraise, A.P. (1995) Lancet 346, 787–788.
275 Rey, J.F., Halfon, P., Feryn, J.M., Khiri, H., Masseyeff, M.F. and Ouzan, D. (1995) Gastroenterol. Clin. Biol. 19, 346–349.
276 Hanson, P.J.V., Bennett, J., Jeffries, D.J. and Collins, J.V. (1994) J. Hosp. Infect. 27, 61–67.
277 Ayliffe, G.A.J., Coates, D. and Hoffman, P.N. (1993) Chemical Disinfection in Hospitals, 2E. Public Health Laboratory Service, London.
278 Rutala, W.A., Clontz, E.P., Weber, D.J. and Hoffman, K.K. (1991) Infect. Control Hosp. Epidemiol. 12, 282–288.
279 Cowen, A.E. (1991) J. Gastroenterol. Hepatol. 6, 25–30.
280 Hanson, P.J.V., Chadwick, M.V., Gaya, H. and Collins, J.V. (1992) J. Hosp. Infect. 22, 137–142.
281 Bennett, S.N., Peterson, D.E., Johnson, D.R., Hall, W.N., Robinson-Dunn, B. and Dietrich, S. (1994) Am. J. Respir. Crit. Care Med. 150, 245–250.

282 British Society of Gastroenterology (1988) Gut 29, 1134–1151.
283 Ayliffe, G.A.J. and Collins, B.J. in Ref. 13, p. 292–309.
284 Wang, H.C., Liaw, Y.S., Yang, P.C., Kuo, S.H. and Luh, K.T. (1995) Eur. Resp. J. 8, 1259–1262.
285 Lewis, D.L. and Arens, M. (1995) Nature Med. 1, 956–958.
286 Reeves, D.S. and Brown, N.M. (1995) J. Hosp. Infect. 30, 531–536.
287 Takigawa, K., Fujita, J., Negayama, K., Terada, S., Yahaji, Y., Kawanishi, K. and Takahara, J. (1995) Respir. Med. 89, 423–427.
288 Kolmos, H.J., Lerche, A., Kristoffersen, K. and Rosdahl, V.T. (1994) Scand. J. Infect. Dis. 26, 653–657.
289 Campagnaro, R.L., Teichtahl, H. and Dwyer, B. (1994) Aust. N. Z. J. Med. 24, 693–695.
290 Gorse, G.J. and Messner, R.L. (1991) Infect. Control Hosp. Epidemiol. 12, 289–296.
291 Muscarella, L.F. (1996) Infect. Control Hosp. Epidemiol. 17, 183–187.
292 Urayman, S., Kozarek, R.A., Sumida, S., Raltz, S., Merriam, L. and Pethigal, P. (1995) Gastrointest. Endoscopy 41, 331.
293 Nakahara, S.A., Becker, L., Biggerstaff-Pearson, A. and Hardy, M.J. (1994) Am. J. Infect. Control 22, 50–56.
294 Kozarek, R.A., Raltz, S.L., Merriam, L.D. and Sumida, S.E. (1996) Gastrointest. Endoscopy 43, 10–13.
295 Rutala, W.A. and Weber, D.J. (1995) Infect. Control Hosp. Epidemiol. 16, 231–235.
296 Alfa, M.J. and Sitter, D.L. (1994) J. Hosp. Infect. 26, 15–26.
297 Nicholson, G., Hudson, R.A., Chadwick, M.V. and Gaya, H. (1995) J. Hosp. Infect. 29, 257–264.
298 Stingeni, L., Lapomarda, V. and Lisi, P. (1995) Contact Dermatitis 33, 172–176.
299 Duc, D.L., Sing, J.S.C., Mallaret, M.R., Soule, H., Arnaud, C., Croize, J. and Calop, J. (1996) Med. Malad. Infect. 26, 99–104.
300 Danielson, J.W. and Thompson, R.D. (1996) J. Chromatogr. 724, 398–402.
301 Reynolds, C.D., Rhinehart, E., Dreyer, P. and Goldmann, D.A. (1992) Am. J. Infect. Control 20, 283–290.
302 Ahmed, J., Brutus, A., Damato, R.F. and Glatt, A.E. (1994) Am. J. Infect. Control 22, 319–321.
303 Maidment, H.J. and Petersen, J.E. (1996) Am. J. Nephrol. 16, 52–59.
304 Sherman, R.A., Cody, R.P., Rogers, M.E. and Solanchick, J.C. (1994) Am. J. Kidney Dis. 24, 924–9226.
305 Held, P.J., Wolfe, R.A., Gaylin, D.S., Port, F.K., Levin, N.W. and Turenne, M.N. (1994) Am. J. Kidney Dis. 23, 692–708.
306 Luehmann, D.A. and Cosentino, L.C. (1994) Dialysis Transplant. 23, 248.
307 Tate, W.H., Goldschmidt, M.C., Ward, M.T. and Grant, R.L. (1995) Am. J. Dent. 8, 270–272.
308 London, I.D. (1971) Arch. Dermatol. 104, 96–97.
309 London, I.D. (1971) Arch. Dermatol. 104, 440.
310 Bunney, M.H. (1977) Drugs 13, 445–451.
311 Bunney, M.H., Nolan, M.W. and Williams, D.A. (1976) Br. J. Dermatol. 94, 667–679.
312 Allenby, C.F. (1977) Br. J. Clin. Pract. 31, 12–13.
313 Juhlin, H. and Hansson, H. (1968) Arch. Dermatol. 97, 327–330.
314 Suringa, D.W.R. (1970) Arch. Dermatol. 102, 163–167.
315 Eigen, E. (1970) U.S. Patent 3,497,590; (1970) Chem. Abstr. 72, 14887m
316 Litchfield, J.H. and Vely, V.G. (1972) U.S. Patent 3,679,792; (1972) Chem. Abstr. 77, 105617x
317 Wolff, G.K. (1994) Gen. Dent. 42, 260–263.

318 Martin, H. (1991) Curr. Opinion Dent. 1, 734–736.
319 Waterhouse, P.J. (1995) Endodont. Dent. Traumatol. 11, 157–162.
320 Ivanovski, S., Savage, N.W., Brockhurst, P.J. and Bird, P.S. (1995) Dent. Mater. 11, 19–23.
321 Jagger, D.C., Huggett, R. and Harrison, A. (1995) Br. Dent. J. 179, 93–96.
322 Christensen, W.G., Amidan, B.G. and Christensen, R.P. (1995) J. Dent. Res. 74, 236.
323 Olin, P.S., Holtan, J.R., Breitbach, R.S. and Rudney, J.D. (1994) J. Prosthet. Dent. 71, 625–630.
324 Kaplan, B.A., Goldstein, G.R. and Boylan, R. (1994) J. Prosthet. Dent. 71, 603–606.
325 Grayson, M. (1982) Antibiotics, Chemotherapeutics and Antibacterial Agents for Disease Control (Gump, W., ed.), pp. 435–474, John Wiley and Sons, Chichester.
326 Rappuoli, R. (1995) Vaccine 12, 579–581.
327 Kazhina, M.V. and Poznyak, S.B. (1993) Z. Mikrobiol. Epidemiol. Immunobiol. 5, 61–65.
328 Esmarch, E. (1902) Hyg. Rundsch. 12, 961–970.
329 Gibson, G.L., Johnston, H.P. and Turkington, V.E. (1968) J. Clin. Pathol. 21, 771–775.
330 Alder, V.G. and Simpson, R.A. in Ref. 13, pp. 483–498.
331 Gibson, G.L. (1980) J. Hosp. Infect. 1, 95–101.
332 Cripps, N., Deverill, C.E.A. and Ayliffe, G.A.J. (1976) Hosp. Eng. 19, 30–31.
333 HEI 95/80 (1980) Health Equip. Inf. 88, 26–33.
334 Mast, M.G. and MacNeil, J.H. (1978) Poult. Sci. 57, 676–680.
335 Mast, M.G. and MacNeil, J.H. (1978) Poult. Sci. 57, 681–684.
336 Janik, D., Hall, C.S. and DeNavarre, M.G. (1977) Cosmet. Toiletries 92, 99–100.
337 Meltzer, N. and Henkin, H. (1977) Cosmet. Toiletries 92, 95–98.
338 Fitzpatrick, M.S., Schreck, C.B., Chitwood, R.L. and Marking, L.L. (1995) Prog. Fish Cult. 57, 153–155.
339 Marking, L.L., Rach, J.J. and Schreier, T.M. (1994) Prog. Fish Culturist 56, 225–231.
340 Accorsi, C.A. (1994) Zuckerindustrie 119, 124–128.
341 McCarthy, B.J. in Ref. 13, pp. 418–430.
342 Springle, W.R. and Briggs, M.A. in Ref. 13, pp. 431–436.
343 Rechenberg, W. (1972) Betontech. Ber. 22, 249–251.
344 Bravery, A.F. in Ref. 13, pp. 437–458.
345 Child, R.E. in Ref. 13, pp. 474–479.
346 Preller, L., Heederik, D., Boleij, J.S.M., Vogelzang, P.F.J. and Tielen, M.J.M. (1995) Occup. Environ. Med. 52, 654–660.
347 Yusuf, S., Imamura, Y., Takahashi, M. and Minato, K. (1995) Mokuzai Gakkaishi 41, 163–169.
348 Yasuda, R., Minato, K. and Norimoto, M. (1994) Wood Sci. Technol. 28, 209–218.
349 Anon. (1991) Pharm. J. 247, 269.
350 Moutschen, J. (1985) Introduction to Genetic Toxicology, John Wiley and Sons, Chichester.
351 Bousquet, J. and Michel, F.B. (1991) Clin. Rev. Allergy 9, 357–370.
352 Andrion, A. and Pira, E. (1994) Pathol. Res. Pract. 190, 1214–1223.
353 Lyon, B. (1971) Oral Surg. 32, 895–898.
354 Sanderson, K.V. and Cronin, E. (1968) Br. Med. J. 3, 802–805.
355 British Society of Gastroenterology (1993) Gut 34, 1641–1645.
356 Beauchamp, R.O., Jr., St. Clair, M.B., Fennell, T.R., Clarke, D.O. and Morgan, K.T. (1992) Crit. Rev. Toxicol. 22, 143–174.
357 Chang, C.C. and Gershwin, M.E. (1992) Reg. Toxicol. Pharmacol. 16, 150–160.
358 French, H.M. (1990) Gastroenterol. Today 2, 48.
359 Werley, M.S., Burleighflayer, H.D. and Ballantyne, B. (1995) Toxicol. Ind. Health 11, 489–501.

360 Chan-Yeung, M., McMurren, T., Catonio-Begley, F. and Lam, S. (1993) J. Allergy Clin. Immunol. 91, 974–978.
361 Geier, J. and Scnuch, A. (1995) Dermatosen Beruf Umwelt 43, 30–31.
362 Taylor, E.W., Mehtar, S., Cowan, R.E. and Feneley, R.I. (1994) J. Hosp. Infect. 28, 5–14.
363 Siegel, J.H., Kasmin,F.E. and Cohen, S.A. (1994) Endoscopy 26, 545–548.
364 Campbell, M. and Beach, J.R. (1994) Occup. Med. 44, 165–166.
365 Gannon, P.F.G., Bright, P., Campbell, M., Ohickey, S.P. and Burge, P.S. (1995) Thorax 50, 156–159.
366 Stenton, S.C., Beach, J.R., Dennis, J.H., Keaney. N.P. and Hendrick, D.J. (1994) Occup. Med. 44, 95–98.
367 Lehman, P.A., Franz, T.J. and Guin, J.D. (1994) Contact Dermatitis 30, 176–177
368 Hayes, J.P. and Fitzgerald, M.X. (1994) Thorax 49, 198–200.
369 Legoaster, A., Deschamps, F. and Kalis, B. (1994) Semaine Hosp. 70, 88–90.
370 Babb, R.R. and Paaso, B.T. (1995) West. J. Med. 163, 477–478.
371 Ryan, C.K. and Potter, G.D. (1995) J. Clin. Gastroenterol. 21, 6–9.
372 West, A.B., Kuan, S.F., Bennick, M. and Lagarde, S. (1995) Gastroenterology 108, 1250–1255.
373 Birnbaum, B.A., Gordon, R.B. and Jacons, J.E. (1995) Radiology 195, 131–134.
374 Dolce, P., Gourdeau, M., April, N. and Bernard, P.M. (1995) Am. J. Infect. Control 23, 34–39.
375 Rozen, P., Somjen, G.J., Baratz, M., Kimel, R., Arber, N. and Gilat, T. (1994) Gastrointest. Endsocopy 40, 547–553.
376 McLaughlin, J.K. (1994) Int. Arch. Occup. Environ. Health 66, 295–301.
377 Auerbach, C., Moutschen-Dahmen, M. and Moutschen, J. (1979) Mutat. Res. 39, 317–361.
378 Grafstrom, R.C., Dypbukt, J.M., Sundqvist, K., Atzori, L., Nielsen, I., Curren, R.D. and Harris, C.C. (1994) Carcinogenesis 15, 985–990.
379 Muller, W., Engelhart, G., Herbold, B., Jackh, R. and Jung, R. (1993) Environ. Health Perspect. 101(Suppl. 3), 33–36.
380 Jung, R., Engelhart, G., Herbolt, B., Jackh, R. and Muller, W. (1992) Mutat. Res. 278, 265–270.
381 Schweikl, H., Schmalz, G. and Bey, B. (1994) J. Biomed. Mater. Res. 28, 1061–1067.
382 Zimmerman, F.K. and Mohr, A. (1992) Mutat. Res. 270, 151–166.
383 Schweikl, H. and Schmalz, G. (1996) J. Dent. Res. 75, 2524.
384 Zissu, D., Gagnaire, F. and Bonnet, P. (1994) Toxicol. Lett. 71, 53–62.
385 Uddin, S. and Hadi, S.M. (1995) Biochem. Mol. Biol. Int. 35, 185–195.
386 Khan, Q.A., Shamsi, F.A. and Hadi, S.M. (1995) Cancer Lett. 89, 95–99.
387 Khan, Q.A. and Hadi, S.M. (1994) Biochem. Mol. Biol. Int. 32, 379–385.
388 Khan, Q.A. and Hadi, S.M. (1993) Biochem. Mol. Biol. Int. 29, 1153–1160.
389 Barros, A.R., Sierra, L.M. and Comendador, M.A. (1994) Mutat. Res. 321, 119–126.
390 Aikawa, K. and Miwa, M. (1993) Mutat. Res. 301, 93–97.
391 Control of Substances Hazardous to Health Regulations (1988) Approved code of practice control of substances hazardous to health and approved code of practice control of carcinogenic substances. HM Stationery Office, London.
392 Smedley, J. and Coggon, D. (1996) Occup. Med. 46, 33–36.
393 US Department of Labor, Occupational Safety and Health Administration (1987) Anal. Methods Manual, Method 64.
394 Lindahl, R. and Levin, J.O. (1995) J. Chromatogr. 710, 175–180.
395 Cuthbert, J. and Groves, J. (1995) Ann. Occup. Hyg. 39, 223–233.
396 Noble, J.S., Strang, C.R. and Michael, P.R. (1993) Am. Ind. Hyg. Assoc. J. 54, 723–732.

397 Nishikawa, H. and Sakai, T. (1995) J. Chromatogr. 710, 159–165.
398 Espinosa-Mansilla, A., Munoz de la Pena, A. and Salinas, F. (1993) J. Assoc. Off. Anal. Chem. Int. 76, 1255–1261.
399 Roig, M.G., Bello, J.F., Kennedy, J.F., Rivera, Z.S. and Lloyd, L.L. (1992) Bioseparation 3, 177–184.

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Progress in Medicinal Chemistry – Vol. 34 edited by G.P. Ellis and D.K. Luscombe

5 Synthetic Methods for Enantiomers of Drugs

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GENERAL PRINCIPLES AND RANGE OF AVAILABLE CHIRAL METHODOLOGIES

NEED FOR SINGLE ISOMER DRUGS

Almost every pharmaceutical R&D programme nowadays involves chiral compounds. While initial screens for biological potency of new chemical entities can rationally use racemic mixtures provided one enantiomer does not antagonize the other, as soon as selectivity for one desired target against another becomes an issue, then it can become essential to work with pure enantiomers. Indeed, it has been asserted by Ariens that much *in vivo* work on racemates has generated 'sophisticated nonsense' [1]. For instance, pharmacokinetic data of a racemate may be dominated by a longer lasting or more bioavailable inactive component suppressing the information sought on the active component. Work with pure enantiomers is also necessary to gain a fuller understanding of molecular modelling against an enzyme or receptor. Another factor to be taken into account is regulatory policy; the US Food and Drug Administration (FDA) requires that clinical studies on a mixture of isomers must include pharmacokinetic and pharmacodynamic studies on each of the isomeric components [2,3]; thus, while the FDA does not actually prevent racemates being developed as drugs, it makes it expensive to register a new racemate.

Analysis of trends in drug development shows an increasing tendency to move towards synthetic drugs in single enantiomer form as shown in *Figure 5.1* [4]. Thus, from 1982 to 1991 the proportion of drugs sold as a synthetic single enantiomer is reported to have increased from 3.4% to 16.6%. This increase is partly the result of the quest for drugs of greater target specificity, and so fewer side-effects and increased safety. This inevitably leads to a greater structural complexity and a greater likelihood that chirality will be a feature of the drug entities. Another way of viewing this is to consider Pfeiffer's rule which states that the lower the effective dose of a drug, the

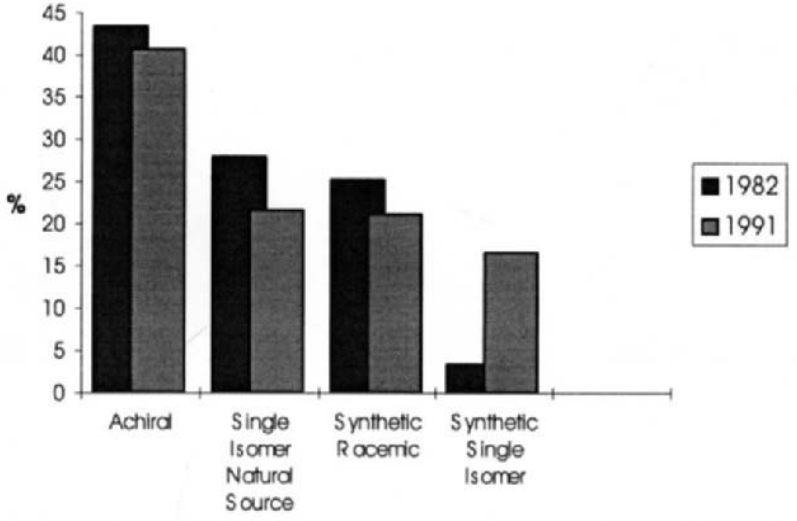


Figure 5.1. Trends in the ratio of achiral, racemic and single-enantiomer drugs.

greater the difference between the pharmacological effect of its optical isomers is likely to be [5]. Essentially this indicates that as a drug becomes a better fit for its target, its enantiomer is likely to be a worse fit; similarly, an achiral mimic can not be expected to fit as well, and thus it is inevitable that the number of chiral drugs will increase.

A further point is that the synthetic accessibility of single isomers has improved markedly over the last decade. This chapter aims to provide an overview of methodologies used to make single enantiomers to assist medicinal chemists in preparing the desired single enantiomer. In addition, we review methods used to manufacture single enantiomers of pharmaceutical agents and emphasize the different requirements of a synthetic procedure to make the first amounts of material and to manufacture a drug in bulk.*

*The following abbreviations are used: ACE, angiotensin-converting enzyme; AIBN, α,α′-azoisobutyronitrile; BINAP, 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl; Boc, tert-butoxycarbonyl; BOP reagent, benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate; COD, 1,5-cyclooctadiene; DABCO, 1,4-diazabicyclo[2.2.2]octane; DEAD, diethyl azodicarboxylate; DIOP, 1,4-bis(diphenylphosphino)-1,4-dideoxy-2,3-*O*-isopropylidenethreitol; DiPAMP, 1,2-di[phenyl(2-methoxyphenyl)phosphinyl]ethane ; DMAP, 4-(dimethylamino)-pyridine; EDAC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide; HMDS, hexamethylsilazide; Ipc, isopinocampheyl; LDA, lithium di-isopropylamide; MOM, methoxymethyl; NCE, new chemical entity; Ra-Ni, Raney nickel; SAMP, (S)-amino-2-(methoxymethyl)pyrrolidine; TBDPS, tert-butyldiphenylsilyl; TFA, trifluoroacetic acid; TMS, trimethylsilyl; Z, benzyloxycarbonyl.

RANGE OF METHODOLOGIES FOR SINGLE ENANTIOMERS

Methodologies for single enantiomers can be divided into three basic types:

(i) *chirality pool:* the product is made by synthetic modification of an available single-enantiomer raw material with maintenance or transfer of stereochemical integrity throughout the synthesis.

(ii) *resolution:* a synthetic racemic mixture is separated into its enantiomers.

(iii) *asymmetric synthesis:* control is at the point where the chirality is created by asymmetric modification of a prochiral substrate.

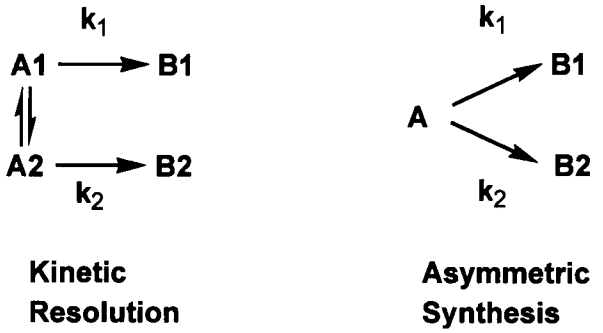
All methods for providing a single enantiomer, whether chemical or biochemical, can be considered as being comprised of one or more of the above. For instance, a fermentation may involve a microbial strain effecting an asymmetric synthesis, etc.

USE OF THE CHIRALITY POOL

The most straightforward way to obtain a single enantiomer of a pharmaceutical agent may be by chemical modification of a chirality pool material. The most important of these are the natural L-amino acids and sugars, although in effect any starting material can be considered as being of the chirality pool if enough is available to produce the desired single enantiomer in the amount required. An example of a chirality pool-based synthesis would be of an oligopeptide pharmaceutical containing L-cyclohexylalanine, where this amino acid is produced by hydrogenation of the aromatic ring of the natural amino acid L-phenylalanine [6]. A common disadvantage of a chirality pool approach is that many synthetic steps and awkward functional group protection may be required. Also, in each of the steps it may be necessary to carry out detailed experiments to verify the maintenance of necessary centres of chirality or the configuration of any new chiral centres created in the molecule. In such cases, the benefit of using the chirality pool has to be weighed against that of a synthesis based on resolution or asymmetric synthesis as discussed below.

RESOLUTION VS ASYMMETRIC SYNTHESIS METHODS

It is worth comparing the kinetic consequences of operating either a kinetic resolution or an asymmetric process. These may be represented by the generalized conversions shown in *Scheme 5.1* where in a kinetic resolution two substrates A1 and A2 which are enantiomers are converted into corresponding products B1 and B2 respectively at different rates, while in an asym-



Scheme 5.1. Generalized resolution and asymmetric synthesis.

metric synthesis one prochiral substrate A is converted into the enantiomeric products. For an asymmetric synthesis, the enantioselectivity (q) may be represented by the ratio of the rate constants (k_1, k_2) of the two paths as $q = k_1/k_2$ [7] and the enantiomeric excess (ee) of the product formed is theoretically constant throughout the conversion and given by the equation: $q = (1+ee)/(1-ee)$ or $ee = (q-1)/(q+1)$. Selke *et al.* have shown that in comparing different catalysts in an asymmetric process, it is more informative to compare the ratios of the q values rather than the product ee values [7]. The equation $\Delta(\Delta G^{\ddagger})=RT\ln(q)$ then gives the free energy of activation difference between the transition states of the two enantiomeric reaction paths needed to provide a given selectivity. Thus, a free energy of activation difference of 3 kcal/mol gives a q value of 158 at 25°C (250 at 0°C) and a product of 98.8% ee. The situation with kinetic resolutions is more complicated since the enantiomeric composition of the reactants change as the resolution progresses. The ratio of the rate constants k_1/k_2, which is the enantiospecificity, has been referred to as an E value [8,9] and is theoretically given by the equations: $E = \ln[(1-c)(1-ee_S)]/\ln[(1-c)/(1+ee_S)]$ or $E = \ln[1-c(1+ee_P)]/\ln[(1-c(1-ee_P)]$ where c is the extent of conversion (0 to 1) and ee_S and ee_P are the fractional enantiomeric excesses (0 to 1) of the substrate and product, respectively. These equations, which are used very frequently for biocatalytic resolutions, are expressed graphically for given different values of E in *Figure 5.2*, and the following points can be made:

(i) The enantiomeric excess of the product declines as the resolution progresses and for acceptable enantiomeric purity a resolution with $E > 100$ is generally sought. Only initially is the enantiomeric excess of the product the same as that given by an asymmetric synthesis having the same ratio of rate constants k_1/k_2.

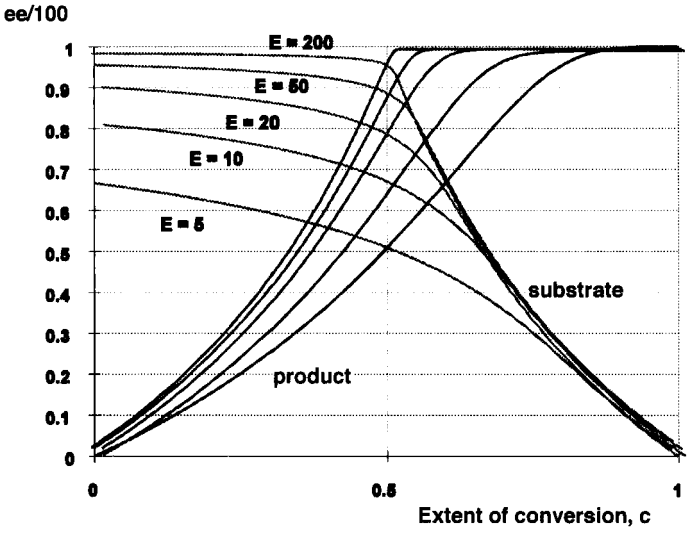


Figure 5.2. Profile of kinetic resolutions with varied enantiospecificity, E.

(ii) The enantiomeric excesses of the product and residual reactant is the same at 50% conversion.

(iii) The enantiomeric excess of the residual reactant rises to approach 100% ee for any value of E as conversion extends to over 50%. This is one of the benefits of the resolution method when a very high enantiomeric excess of material is needed.

There are various points to be considered when choosing an asymmetric or resolution method leading to a given product. A resolution method has the advantage that it will generally give both enantiomers of the material required, as is often needed in early pharmaceutical evaluation, and resolution methods can often be accomplished with less effort than an asymmetric synthesis. Upon scale-up, however, the problem with resolution is utilization of the incorrect enantiomer. If it can be recycled back into the process, then resolution can be a preferred method of manufacture. For asymmetric synthesis, a high degree of enantioselectivity is essential. For initial evaluation work, frequently a method such as recrystallization can be found to raise the enantiomeric purity of the product. However, on scale-up, any unwanted isomer which is produced cannot generally be reused. A further development is the concept of dynamic resolution (known also as a second order asymmetric transformation), in which a kinetic resolution is effected under conditions where the substrate racemizes rapidly. Thus, by reference

to the diagrams of *Scheme 5.1*, the substrates A1 and A2 become one and the process obeys the kinetics of an asymmetric synthesis.

ANALYTICAL METHODS FOR SEPARATING ENANTIOMERS

In the synthesis of single enantiomers of pharmaceutical agents, the availability of methods to determine the optical purity of the intermediates is paramount. It is therefore comforting that analytical methods for separating enantiomers have developed greatly over the past decade [10]. Analytical methods that might be used in the determination of enantiomeric composition are: optical rotation, nuclear magnetic resonance (NMR) spectrometry, high performance liquid chromatography (HPLC), gas chromatography (GC) and capillary zone electrophoresis (CZE). Optical rotation measurement by polarimetry is not recommended as an accurate measure of enantiomeric purity since it cannot measure small levels of the minor enantiomeric component and is too sensitive to the chemical purity and, in particular, contamination by other optically active components. NMR spectroscopy is a versatile method, especially using chiral lanthanide shift reagents, solvating agents, or derivatization with a chiral auxiliary, but it can be difficult to get good resolution in relation to the broadness of peaks and the minor enantiomeric component can easily be lost within other peaks on the baseline. Nowadays, chromatographic methods based on chiral stationary phases in the case of HPLC (and its recent development of supercritical fluid chromatography), GC, or chirally modified mobile phase in the case of CZE are becoming the methods of choice; they readily allow detection of 0.5% or less of an undesired enantiomer, and appropriate equipment and expertise are becoming increasingly available commercially.

CRYSTALLIZATION TECHNIQUES FOR SEPARATION OF ENANTIOMERS

When faced with a single-enantiomer target to synthesize, it is tempting to be drawn by the latest developments in asymmetric chemical methodology. However, it is often most important to be pragmatic and in this regard techniques based on resolution by crystallization are usually relatively simple to employ and can frequently be a first choice, especially where a racemate to be separated bears a salt-forming function (for example, amine or carboxylic acid) in the vicinity of a chiral centre, so that separation of enantiomers can be accomplished through formation of a diastereoisomeric salt with a resolving agent in the so-called classical resolution. Details of crystallization techniques are described in appropriate specialized texts (for example [11]). Unless optical purity in a synthesis can be guaranteed, then perhaps for any

synthesis of a single-enantiomer agent, it is of value to determine how crystals of the intermediate behave. A racemate might exist either in crystals containing equal amounts of both enantiomers (called a *racemic compound*) or as a mechanical mixture (*conglomerate*) of crystals of its separate enantiomers. In the latter case, recrystallization will give crystals of increased enantiomeric excess and it may be effective to resolve the racemate through selective entrainment of one enantiomer by seeding with that enantiomer. In the former case of a racemic compound, there will generally be a composition (enantiomeric excess) that is a eutectic of minimum melting point and highest solubility. Generally, material of higher enantiomeric excess than this can be recrystallized towards enantiomeric purity and that may be necessary after either an inefficient diastereoisomeric salt resolution or after an asymmetric synthesis of limited enantioselectivity.

STRATEGY IN SINGLE-ENANTIOMER SYNTHESIS

The strategy adopted in a single-enantiomer synthesis is to a large extent determined by whether the purpose is to produce a small amount of a material quickly for biological evaluation or whether it is for the production of kilograms or greater amounts of product. In the former case, it is often most convenient to modify a synthesis of a racemate through resolution of an appropriate intermediate or final product or through effecting diastereoselective control via attachment of a chiral auxiliary. In a medicinal chemistry programme based on developing an array of candidate entities around a core chiral framework (a chiral template approach), a route through a single generic chiral synthon is preferable to making each target as a racemate which is individually resolved. For a scaled-up process where economics becomes a consideration, a first question is whether the incorrect enantiomer can be recycled through racemization; if it can, then a late stage resolution is viable; otherwise resolution should be as early as possible or else an asymmetric synthesis should be developed. A particularly important case is when an entity contains more than one chiral centre that causes it to be formed as a mixture of diastereoisomers as well as enantiomers. In that event, early stage provision of single-enantiomer components which are then coupled becomes a better strategy than the use of racemic components which could lead to a complex mixture of isomers of the final product.

REQUIREMENTS OF CHIRAL METHODOLOGY FOR DRUG DISCOVERY OR FOR DRUG MANUFACTURE

The requirements of medicinal chemistry used for drug discovery and for

manufacture are quite distinct and are summarized as follows: a route used for the former will not generally be that preferred for manufacture. Indeed, the initial route is often not appropriate for provision of quantities of the order of a kilogram as needed for early clinical trials. It is important to consider this at an early stage of a drug's development as it is all too easy to push a medicinal chemistry-generated route too far so that either the route breaks down and causes delays to perhaps toxicology or a clinical trial, or that regulatory pressure to fix the route may cause a route to be adopted that is far from economical. However, what is common to both approaches is the requirement for a product of high purity.

Routes used in drug discovery should ideally be rapid, reliable, lead to unambiguous configuration for both isomers and be applicable to the synthesis of generic synthons. Manufacturing processes should ideally be safe, scaleable, give high yields at ambient conditions, use cheap materials and consist of a minimum number of steps from which the product may be easily isolated (preferably without using chromatography) with a minimum of effluent. It is appropriate to have the above issues in mind when considering the specific applications discussed in the following sections.

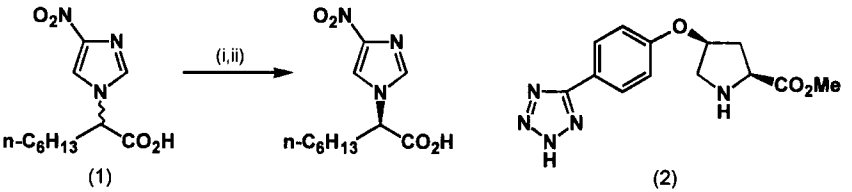
CHIRAL METHODOLOGIES USED IN MEDICINAL CHEMISTRY FOR DRUG DISCOVERY

CLASSICAL RESOLUTION

In cases where chiral synthons are not commercially available and have to be prepared by the medicinal chemist, classical resolution remains the most widely used of available methodologies. Despite its low technology image, in the research laboratory it is highly suited to the provision of multigramme quantities of single enantiomers from racemic compounds bearing a salt-forming residue proximal to an element of chirality. Advantages include the ready availability of cheap resolving agents, often in both enantiomeric forms, facilitating so-called mirror-image resolutions, as exemplified below. On the whole, synthetic resolving agents such as 1-phenylethylamine and the diaroyl tartaric acids have superseded many of the alkaloids, including toxic compounds such as brucine, which are prevalent in the early literature. In individual applications, preliminary screening is usually required in order to identify a combination of resolving agent and solvent which confer favourable crystallinity and solubility characteristics, and thereby maximize the separation of diastereomeric salts. Subsequently, preparative resolutions can be accomplished quickly, easily and at low cost.

The remainder of this section focuses on a variety of cases where classical resolution has been applied to good effect in drug discovery programmes (*Schemes 5.2–5.6*). For comprehensive information, including guidelines on screening for suitable resolving agent-solvent combinations, the reader is directed to the recent definitive monogragh by Eliel and Wilen [12] and an earlier review by Wilen *et al.* [13]. In addition, a compendium of resolution protocols from the primary literature is available [14].

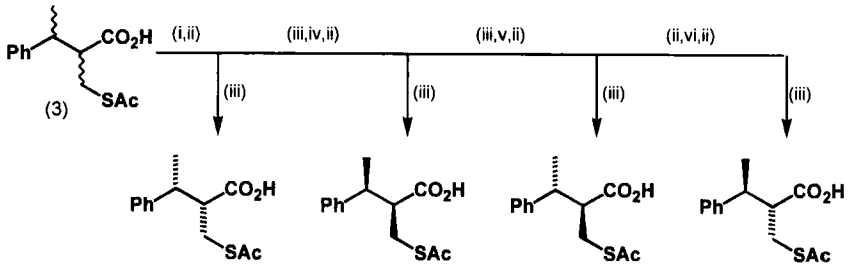
Palkowitz and coworkers [15] reported classical resolution of the imidazolyl carboxylic acid (1) with (-)-cinchonidine, as part of an integrated approach to triacid angiotensin II antagonists. The (*R*)-enantiomer of (1) thus obtained was then subjected to peptidic coupling with amino ester (2), prepared from *trans*-4-hydroxy-L-proline, a widely available chirality-pool material.



Reagents: (i) 0.5 mol. equivalent (-)-cinchonidine, crystallization; (ii) 1N HCl

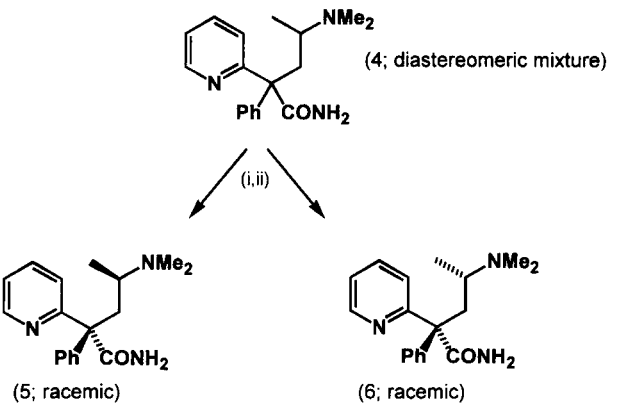
Scheme 5.2. Classical resolution of a synthon for angiotensin II antagonists.

Scheme 5.3 illustrates the principle of mirror-image resolution, as used by Roques and coworkers [16] in work directed at dual inhibitors of neural endopeptidase and ACE. 2-[Acetylthiomethyl]-3-phenylbutanoic acid (3) was



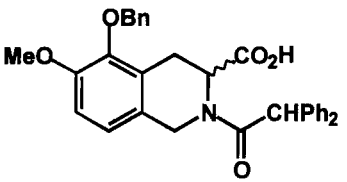
Reagents: (i) (*S*)-PhCHMeNH$_2$; (ii) crystallization; (iii) 1N HCl; (iv) (*R*)-PhCHMeNH$_2$; (v) (*S*)-(1-Naphthyl)CHMeNH$_2$; (vi) (*R*)-(1-Naphthyl)CHMeNH$_2$

Scheme 5.3. Mirror-image resolution of 2-acetylthiomethyl-3-phenylbutanoic acid.

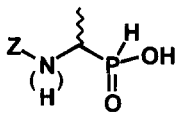


Scheme 5.4. Separation of diastereoisomers by crystallization of salt forms.

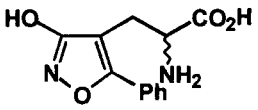
separated into its four constituent stereoisomers by iterative treatment with four different resolving agents, comprising antipodes of 1-phenylethylamine and 1-(1-naphthyl)ethylamine. Between crystallizations, the soluble fraction was washed with aqueous acid to recover the mixture of residual stereoisomers. Osayu *et al.* [17] faced the similar challenge of separating four diastereoisomers of the pyridyl pentanamide (4), an anticholinergic agent of po-



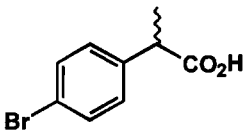
(*S*)-PhCHMeNH$_2$ gives (*R*)-enantiomer [21]



(*S*)-PhCHMeNH$_2$ gives (*R*)-enantiomer [22]

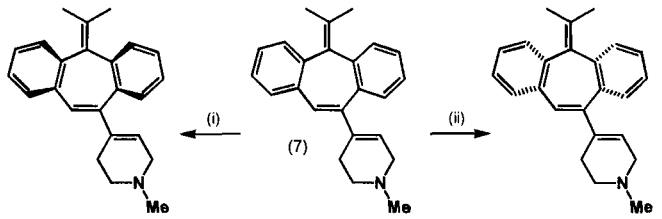


(*R*)-PhCHMeNH$_2$ gives (*S*)-enantiomer ;
(*S*)-PhCHMeNH$_2$ gives (*R*)-enantiomer [23]



(*R*)-PhCHMeNH$_2$ gives (-)-enantiomer;
(+)-enantiomer recovered from mother liquor [24]

Figure 5.3. Classical resolution of acids for use as pharmaceutical intermediates.



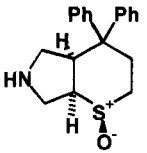
Reagents: (i) (2*S*,3*S*)-di-*p*-toluoyltartaric acid; (ii) (2*R*,3*R*)-di-*p*-toluoyltartaric acid

Scheme 5.5. Classical resolution of a racemate containing a chiral plane.

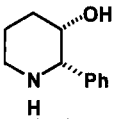
tential use in the treatment of overactive detrusor syndrome (*Scheme 5.4*). This was achieved by crystallization of the maleic acid salt to afford racemates (5) and (6), which were individually subjected to resolutions with (2*S*,3*S*)- and (2*R*,3*R*)-dibenzoyltartaric acids. Simpler cases of mirror image resolutions, as applied to racemic compounds with a single chiral centre, are included in *Figures 5.3 and 5.4*.

Classical resolutions are not restricted to racemic compounds containing tetrahedral chiral centres, a point exemplified by Phillips *et al.* [18] in work directed at clozapine analogues as potential neuroleptic agents. The 5*H*-dibenzo[*a*,*d*]cycloheptene (7), which exists as configurational enantiomers by virtue of planar chirality [19], was resolved using (2*S*,3*S*)- and (2*R*,3*R*)-di-*p*-toluoyltartaric acids (*Scheme 5.5*)

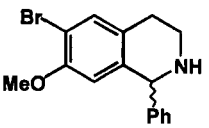
When a specific enantiomer is required, one way of improving atom utilization in classical resolution is by a process of asymmetric transformation, whereby crystallization of one diastereomeric salt is accompanied by contin-



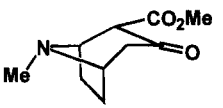
(*S*)-mandelic acid gives (-)-enantiomer [25]



(2*R*,3*R*)-dibenzoyltartaric acid gives (2*S*,3*S*)-enantiomer
(2*S*,3*S*)-dibenzoyltartaric acid gives (2*R*,3*R*)-enantiomer [26]

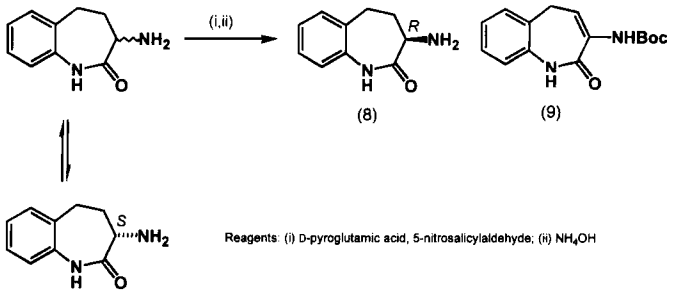


N-acetyl-D-leucine gives (+)-enantiomer
N-acetyl-L-leucine gives (-)-enantiomer [27]



(2*R*,3*R*)-tartaric acid gives (2*R*)-enantiomer
(2*S*,3*S*)-tartaric acid gives (2*S*)-enantiomer [28]

Figure 5.4. Classical resolution of amines for use as pharmaceutical intermediates.



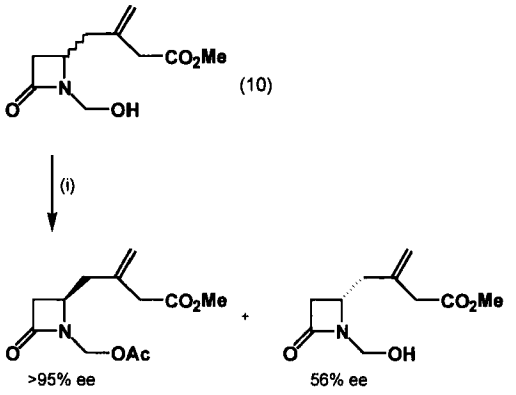
Scheme 5.6. Asymmetric transformation of a synthon for growth hormone secretatogues.

uous *in situ* racemization of the undesired enantiomer. In principle, it is thereby possible to convert a racemate to 100% of a single enantiomer. In practice, such a process is only feasible if the chiral centre is configurationally labile, or can be made more so by the presence of additives. This strategy was used by chemists at Merck who required the (*R*)-enantiomer of the bicyclic α-aminolactam (8) as a synthon for novel growth hormone secretagogues [20]. Optimum conditions for the desired racemization/resolution (*Scheme 5.6*) involved treatment of the racemate with D-pyroglutamic acid and 5-nitrosalicylaldehyde (3 mol %), the latter to assist racemization of the unwanted (*S*)-enantiomer via reversible formation of an imine intermediate. Compound (8) could also be prepared by asymmetric catalytic hydrogenation of the cyclic enamide (9), although this approach added several steps.

Further examples of the resolution of acids and amines are summarized in *Figures 5.3* and *5.4*

BIOCATALYTIC RESOLUTION

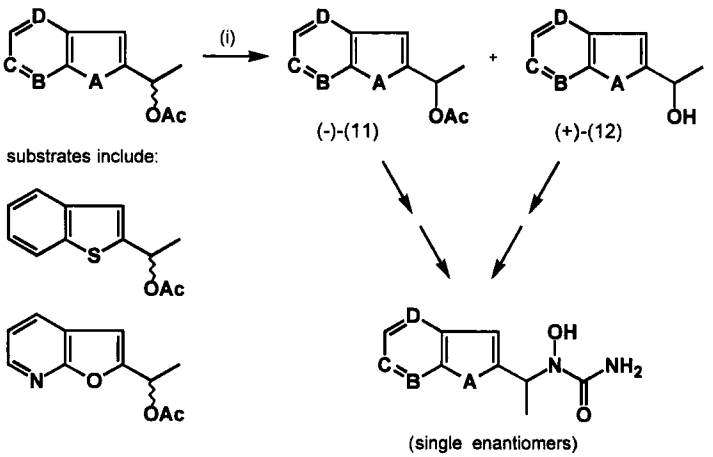
Biotransformations are now firmly established in the synthetic chemist's armoury, especially reactions employing inexpensive hydrolytic enzymes for the resolution of racemates and for the desymmetrization of prochiral substrates. From a practical viewpoint, biocatalytic resolution is arguably the simplest method available to obtain synthetically useful quantities of chiral synthons. As an illustration of this point, many racemic secondary alcohols ROH can be resolved without prior derivatization by combining with a lipase and a volatile acyl donor (usually vinyl acetate) in an organic solvent, to effect irreversible transesterification; once the desired degree of conversion has been reached, routine filtration to remove the enzyme and concentration of the filtrate affords the optically enriched products ROAcyl and ROH directly.



Reagents: (i) *Pseudomonas cepacia* lipase, vinyl acetate

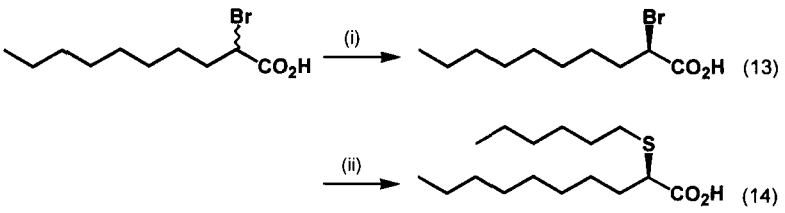
Scheme 5.7. Bioresolution of a 4-allylazetidinone.

The vast body of empirical information amassed, together with guidelines for predicting stereoselectivity based on active-site models, mean that biocatalytic resolution can be used with greater confidence and can be an important element during the planning of a synthesis. An exhaustive review by Santaniello *et al.* [29] and a recent monograph by Wong and Whitesides [30] attest to the broad applicability of biotransformations, and at the non-



Reagents: (i) *Candida cylindracea* lipase, aq. phosphate buffer, pH 6.9

Scheme 5.8. Bioresolution of zilueton congener precursors.



Reagents: (i) Lipase PS30, *n*-BuOH; (ii) hexanethiol, *t*-BuOK

Scheme 5.9. Bioresolution of (R)-2-bromodecanoic acid.

specialist level a laboratory manual by Roberts *et al.* [31] provides detailed experimental protocols.

In spite of these trends, cases in which novel biocatalytic resolutions (as opposed to utilization of established enzyme-substrate combinations) are reported as part of drug discovery programmes are relatively few compared with classical and other non-biological resolution techniques; *Schemes 5.7–5.11* show examples from the 1994 literature.

In research directed at chiral synthons for carbacepham antibiotics, Oumoch and Rousseau [32] demonstrated the non-destructive resolution of several 4-allylazetidinones, for example (10), by enantioselective acylation of the *N*-hydroxymethyl group (*Scheme 5.7*).

Preparation of optically enriched secondary alcohols by enzymatic hydrolysis of *O*-acyl derivatives was used by Galeazzi *et al.* [33] en route to several optically pure congeners of the racemic drug zilueton, an inhibitor of arachidonate 5-lipoxygenase. In their general approach (*Scheme 5.8*), running an initial biotransformation to at least 50% conversion provided enantiopure (-)-*O*-acetates (11; configuration unassigned), and further optical enrichment of the alcohol antipode (12) was achieved by reacylation and a second biotransformation under identical conditions.

McCarthy and coworkers [34] used two methods to prepare (*R*)-2-bromodecanoic acid (13), required as a synthon for inhibitors of acyl-coenzyme A:cholesterol acyl transferase (*Scheme 5.9*). As an alternative to diazotization of commercially obtained (*R*)-2-aminodecanoic acid, racemic bromo acid was esterified directly under lipase catalysis to provide unreacted (13) in 76% ee; the enantiomeric purity of the downstream thio acid (14) was improved by recrystallization of its dicyclohexylamine salt.

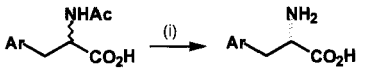
One of the most versatile classes of commercially available enzyme are the aminoacylases, which effect the cleavage of *N*-acetyl and *N*-haloacetyl derivatives of a large number of (*S*)-α-amino acids, thereby providing an efficient means of resolving the corresponding racemates [35]. Hagiwara *et al.* [36]

used biotransformations of this type to prepare novel (*S*)-arylalanine components of substance P antagonists (*Scheme 5.10*).

When planning a biotransformation, identification of a bifunctional prochiral substrate provides a clear benefit over conventional resolution since up to 100% of a desired enantiomer can be obtained by enzymatic desymmetrization. This feature is exemplified in the work of Achiwa and coworkers [37] in the area of 1,4-dihydropyridine calcium channel blockers (*Scheme 5.11*). Hydrolysis of the bis(pivaloyloxymethyl)ester (15) gave enantiopure monoacid (16) which was used to correlate the absolute configuration of the novel agent (17) with that of (*S*)-(-)-felodipine (18).

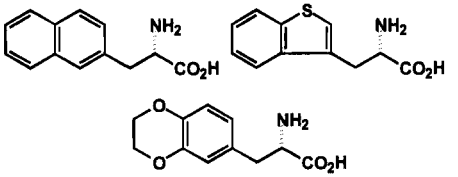
DIRECT SEPARATION OF ENANTIOMERS BY CHROMATOGRAPHY

One area of chirotechnology which is undergoing rapid development is chiral HPLC, whereby the use of chiral stationary phases (CSPs) permits the direct separation of racemic compounds into constituent enantiomers. Despite the capital outlay required, for example, for columns costing upwards of £ 3000, the use of preparative chiral HPLC in drug discovery has a number of benefits. After development of an appropriate method based on a previously defined analytical separation has been carried out, rapid and quantitative separation of racemates can be achieved, with evaporation of solvent from column fractions affording pure enantiomers directly. Although preparative chiral HPLC is less amenable to scale-up than other resolution techniques, it may be ideal for preliminary screening of both enantiomers in circumstances where manipulation of small quantities of material, for example, by crystallization, is impractical and prone to contamination problems.

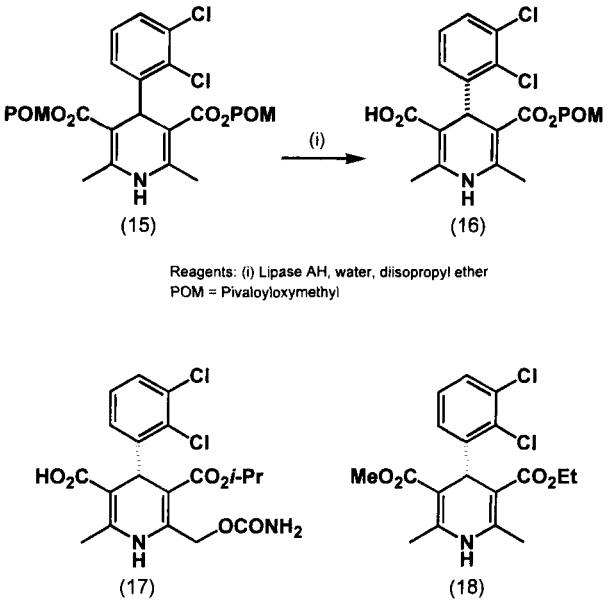


Reagents: (i) Amano acylase 15000, aq. phosphate buffer, pH 7.5

Products include:



Scheme 5.10. Synthesis of (S)-arylalanines by bioresolution.



Scheme 5.11. Enzymatic desymmetrization of 1,4-dihydropyridine-3,5-diesters.

Although a bewildering range of CSPs have been reported in the specialist literature since the mid 1960s [38], in practice only a relatively small number are utilized in commercially available preparative columns. The most widely used classes of CSP are those based on cellulose and cyclodextrin frameworks, with chemically modified hydroxyl substituents to allow operation in either normal phase or reverse phase mode, and so-called Pirkle CSPs, comprising a chiral recognition moiety linked via an achiral spacer to a silica carrier. The latter type encompass a range of structural subtypes, of which *N*-3,5-dinitrobenzoyl α-amino acids covalently bonded to 3-aminopropyl silanized silica are most frequently used in preparative applications. Since a detailed exposition of the area is beyond the scope of this article, the reader is directed to recent monographs [10, 39] and a review of the area [40].

Compared with other resolution techniques utilized in drug discovery, preparative chiral HPLC is normally applied to the separation of relatively complex materials such as polycyclic compounds containing two or more chiral centres. The method is frequently employed after preliminary biological screening of racemates and diastereomeric mixtures to identify lead compounds. After a second round of biological tests have identified a single en-

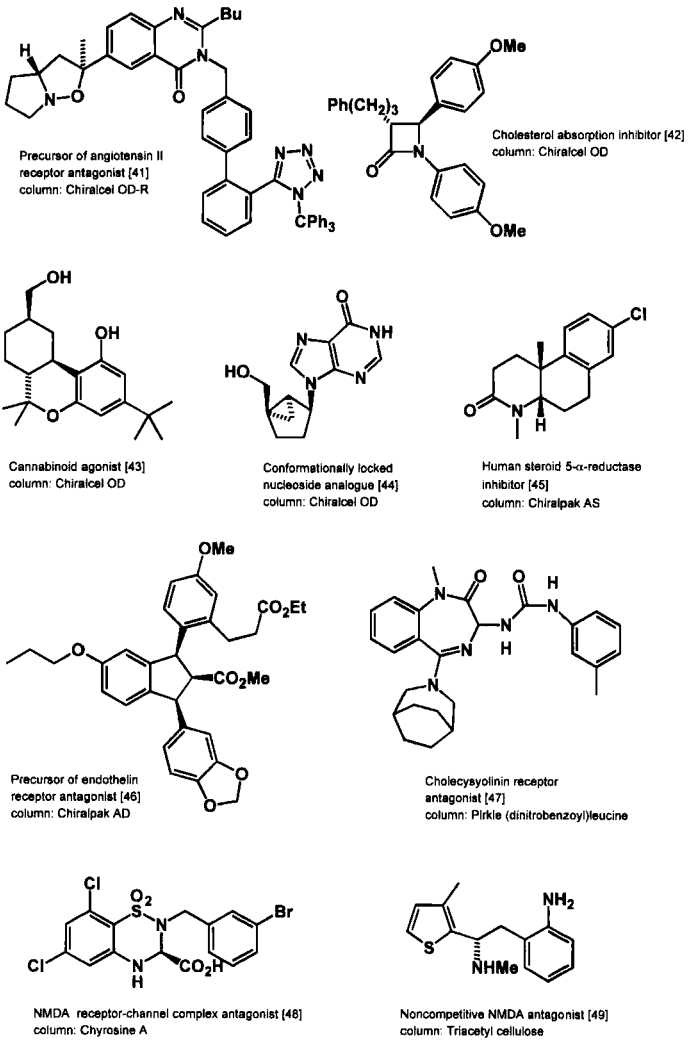
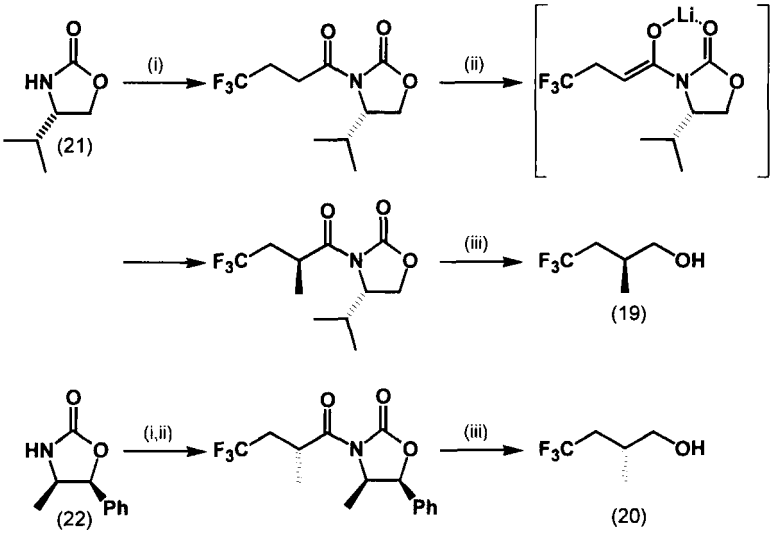


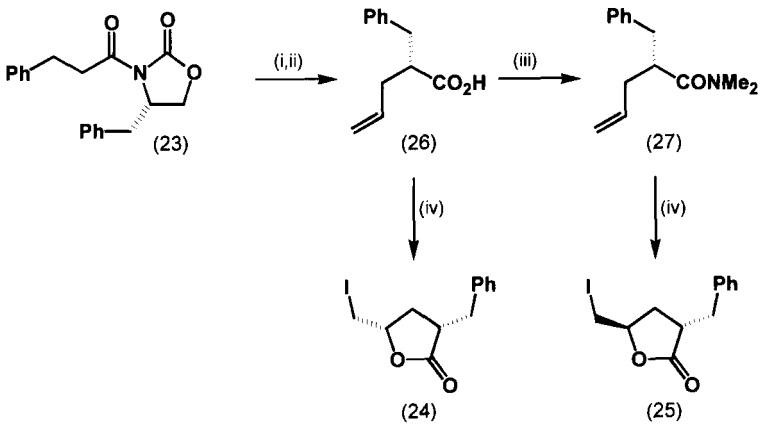
Figure 5.5. Examples of compounds separated by preparative chiral HPLC.

antiomer having favourable activities, only then is it necessary to devise new processes amenable to scale-up, where chirality is established at an earlier stage. *Figure 5.5* summarizes selective applications of preparative chiral HPLC from the 1994 medicinal chemistry literature for the provision of between 5 milligram and gram quantities of single enantiomers [41–49]; for

clarity, only one enantiomer is shown in each case. The recent review by Francotte [40] includes a more extensive listing.



Reagents: (i) *n*-BuLi, $CF_3(CH_2)_2COCl$; (ii) LDA, MeI; (iii) $LiAlH_4$



Reagents:
(i) allyl iodide, NaHMDS; (ii) aq. LiOH, H_2O_2; (iii) $Me_2NH.HCl$, BOP reagent; (iv) I_2, aq. $KHCO_3$; (v) I_2, H_2O

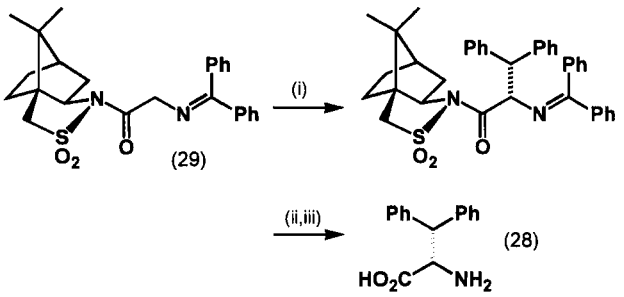
Scheme 5.12. Use of oxazolidinone-based chiral enolates to prepare pharmaceutical intermediates.

USE OF COVALENT CHIRAL AUXILIARIES

Stereoselective synthesis of individual enantiomers

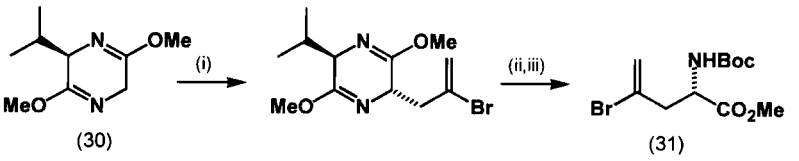
The strategy of using a covalently bound chiral auxiliary to influence the creation of further stereogenic centres can be a very effective one, allowing a chosen enantiomer of a target compound to be synthesized in a controlled and predictable fashion, without resort to laborious separation techniques. Arguably the most widely used class of auxiliary are those which allow the generation and face-selective reaction of geometrically defined chiral enolates, the best known examples being the prototype oxazolidinone auxiliaries of Evans, first reported in the early 1980's [50] and Oppolzer's camphorsulphonyl bornane-10,2-sultams [51]. Although the versatility of these chiral enolates has been demonstrated in diastereoselective aldol additions [52] and in reactions with heteroatom-based electrophiles [53], examples found in the medicinal chemisty literature have been largely concerned with simple *C*-alkylation reactions.

The synthesis of both (*R*)- and (*S*)-enantiomers of 4,4,4-trifluoro-3-methyl-1-butanol (19,20) by Jacobs *et al.* [54] as building blocks for leukotriene antagonists (*Scheme 5.12*), demonstrates how oxazolidinone auxiliaries (21) and (22), derived from L-valine and (1*S*,2*R*)-norephedrine, respectively, impart complementary selectivity in alkylation of chelated (*Z*)-enolates. Similarly, Trova *et al.* [55] have utilized the *N*-acyl oxazolidinone (23), from L-phenylalanine and 3-phenylpropanoyl chloride, for the construction of diastereomeric lactones (24) and (25) as synthons for HIV-1 protease inhibitors (*Scheme 5.12*). Following allylation and hydrolytic removal of the auxiliary, stereocomplementary iodolactonization reactions of



Reagents: (i) *n*-BuLi, Ph_2CHBr (>95% de); (ii) 0.5 N HCl; (iii) aq. LiOH

Scheme 5.13. Alkylation of a chiral glycinate equivalent derived from bornane-10,2-sultam.



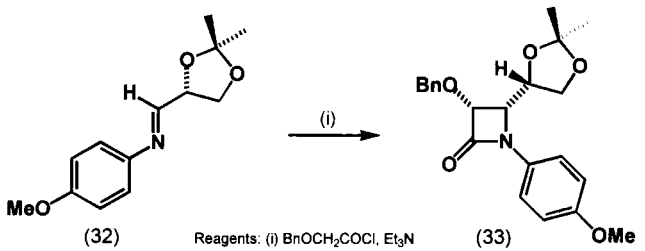
Reagents: (i) *n*-BuLi, bromoallyl bromide; (ii) 1N HCl, MeOH; (iii) Boc_2O

Scheme 5.14. Preparation of a functionalized (S)-α-amino ester using a bislactam ether glycinate equivalent.

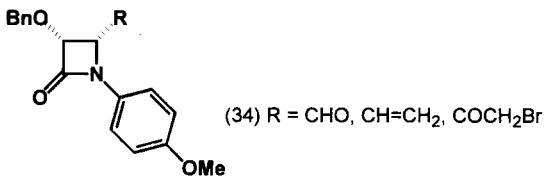
the acid (26) and its *N,N*-dimethyl carboxamide (27) gave synthons (24) and (25), respectively.

Oppolzer's bornane-10,2-sultams have been used by Josien *et al.* [56] in the synthesis of a series of conformationally restrained arylalanines [for example, (28), *Scheme 5.13*] as binding probes for the tachykinin NK-1 receptor. Highly diastereoselective alkylation of the chiral glycinate equivalent (29) was followed by sequential acid- and base-catalyzed hydrolysis reaction to yield the unprotected α-amino acid (28).

Schollkopf's bis-lactim ether (30) is one of the most versatile glycinate equivalents available, providing a ready-made template for the construction of unnatural (*S*)-α-amino acids [57]. For example, lithiation and alkylation of (30) followed by methanolysis and *N*-protection affords the vinyl bromide (31), which was used by Papageorgiou *et al.* [58] to construct a cyclosporin



Reagents: (i) $BnOCH_2COCl$, Et_3N

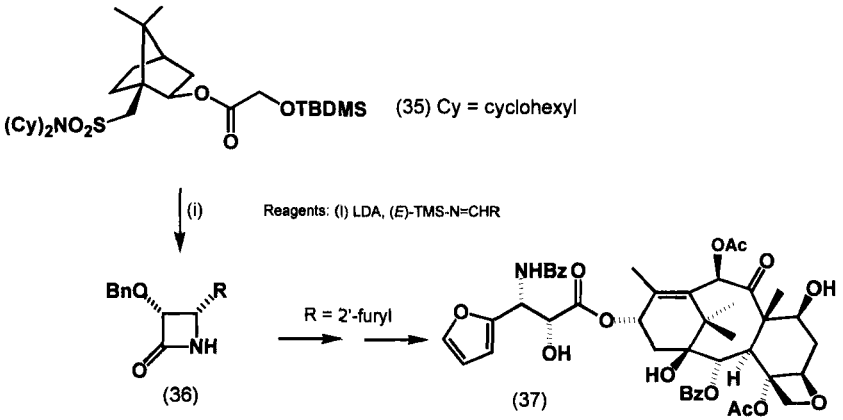


Scheme 5.15. Asymmetric ketene-imine cycloaddition used in the synthesis of C-3' taxol analogues.

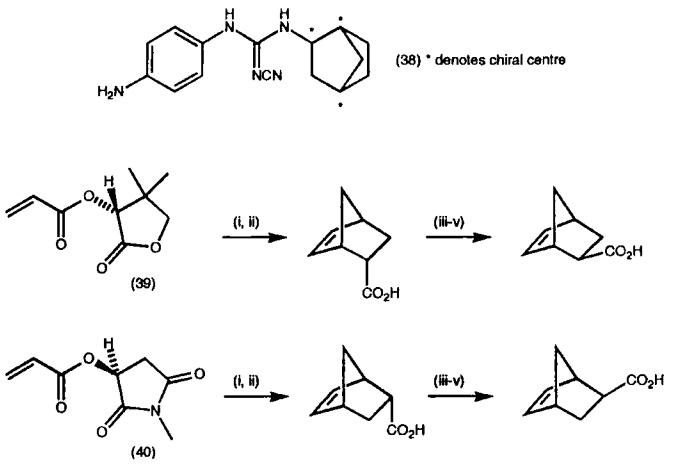
derivative with improved binding affinity for the receptor protein cyclophylin A (*Scheme 5.14*). Alternatively, the chloro-derivative of compound (30) can serve as a glycine cation equivalent for utilization in face-selective reactions with carbon nucleophiles [57].

Cycloadditions are another category of reactions for which covalently bonded chiral auxiliaries can confer impressive levels of asymmetric induction. For the preparation of a large series of taxol analogues in which the C-13 side-chain is modified at C-3′ whilst retaining a *syn*-α-hydroxy-β-aminoacyl structural motif (*Scheme 5.15*), Li *et al.* [59] used the thermal 2+2 cycloaddition of benzyloxyketene and the chiral imine (32), to afford the azetidinone (33) as a chiral template suitable for further elaboration *via* downstream intermediates (34).

Azetidinone precusors of taxol analogues have also been prepared by ester enolate-imine cyclocondensation, which can be regarded as non-concerted equivalent of the Staudinger cycloaddition in the case above [60]. An advantageous feature of these reactions is that the chiral auxilary initially borne on the ester component is displaced during ring formation and not in a separate operation. In an application reported by Georg *et al.* [61], directed at the synthesis of several C-3′-heteroaromatic taxol analogues (*Scheme 5.16*), the glycolate ester (35) of (-)-10-dicyclohexylsulphamoyl-D-isoborneol was condensed with *N*-TMS imines to afford azetidinones (36) as single diastereoisomers but with variable levels of enantioselectivity (31–95% ee). Of the analogues prepared by coupling *N*-benzoyl derivatives of (36) with the tetracyclic baccatin core, the 3′-(2-furyl) compound (37) exhibited higher *in vitro* activities than taxol itself.



Scheme 5.16. Asymmetric ester enolate-imine cyclocondensation used in the synthesis of C-3′ taxol analogues.



Scheme 5.17. Diels-Alder reactions of chiral acrylates en route to novel cyanoguanidines.

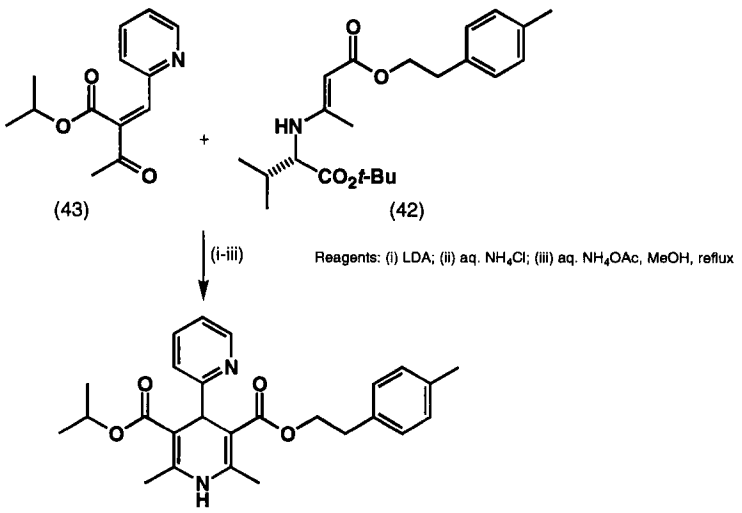
In the synthesis of a diastereomeric series of *N*-(6-amino-3-pyridyl)-*N'*-bicycloheptyl-*N''*-cyanoguanidine derivatives (38) as novel potassium-channel openers (*Scheme 5.17*), Eda *et al.* [62] utilized Diels-Alder reaction of butadiene with chiral acrylates (39) and (40) [63] to prepare individual antipodes of *endo*-bicyclo[2.2.1]hept-2-ene-5-carboxylic acid. The corresponding *exo*-isomers were obtained from the latter by base-catalyzed equilibration at C-5 and removal of residual *endo*-isomers by iodolactonization. Hoffman rearrangement of each acid, to afford the corresponding amine, was used *en route* to the target compounds (38). After biological screening, the (1*S*,2*R*,4*R*)-diastereoisomer of (38) was selected for development as an antihypertensive agent.

Asymmetric conjugate addition is a developing methodology [64], encompassing the use of chiral ligands with organocopper reagents, and more commonly, Michael acceptor-nucleophile combinations in which one component carries a chiral auxiliary. Iqbal *et al.* [65] have used the latter approach to prepare individual enantiomers of the novel 1,4-dihydropyridine calcium channel modulator (41) from (*S*)- and (*R*)-valine. In the sequence commencing from (*S*)-valine (*Scheme 5.18*), the aminocrotonate (42) is metallated and reacted with enone (43) to afford, after hydrolysis and cyclocondensation with ammonia, a single detectable enantiomer of (41). This degree of asymmetric induction is remarkable in view of the distance of five bonds between the chiral centres in the initially-formed Michael adduct.

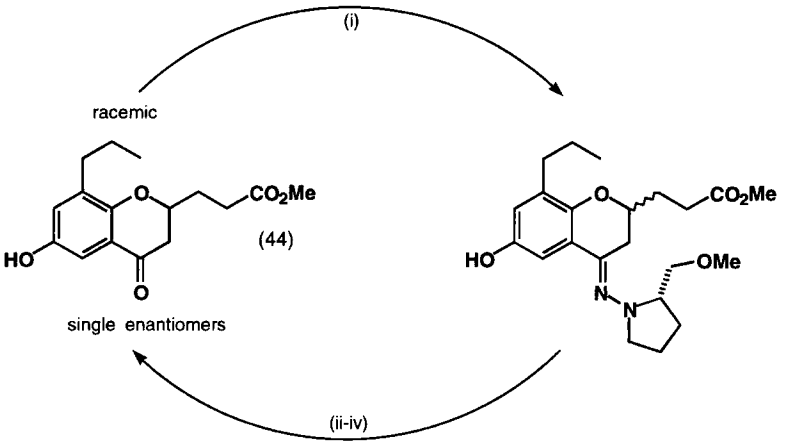
Separation of diastereoisomers by physical techniques

In contrast to the strategy described in the previous sub-section, in which specific enantiomers of target molecules are individually synthesized, chiral auxiliaries can also be used to resolve racemic mixtures by formation of a temporary covalent linkage and physical separation of the resultant pair of diastereoisomers, either by chromatography or less commonly by crystallization. This is especially valuable in cases where the absence of salt-forming ionisable moieties precludes separation of racemates by classical resolution, or where ionisable residues are present but are remote from asymmetric centres. In common with other resolution methods this strategy allows individual optical isomers to be accessed without having to devise elaborate synthetic routes which deviate from an established racemic synthesis. As the following cases demonstrate, it is imperative that the extra steps required to attach and subsequently remove the chiral auxiliary are high yielding and compatible with additional functionality present in the substance being resolved.

As part of studies directed at leukotriene B_4 receptor antagonists, Djuric *et al.* [66] resolved the chromanone (44) by derivatization of the ketone functionality with Enders' SAMP auxiliary [67] and separation of the resulting



Scheme 5.18. Synthesis of a 1,4-dihydropyridine calcium channel modulator via an asymmetric Michael reaction.

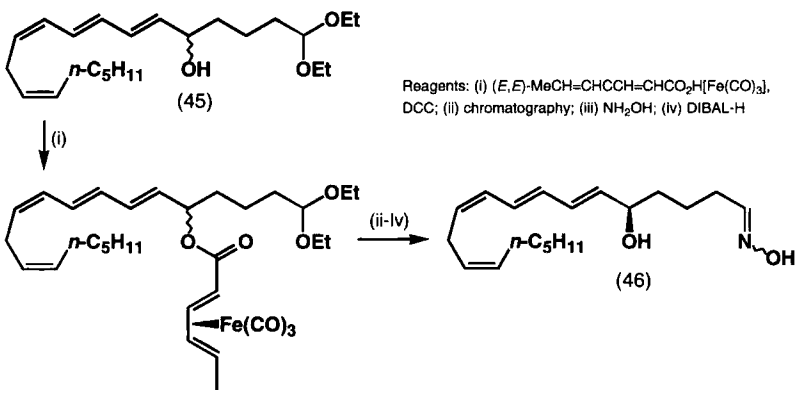


Reagents: (i) SAMP; (ii) chromatography; (iii) MeI; (iv) 2N HCl

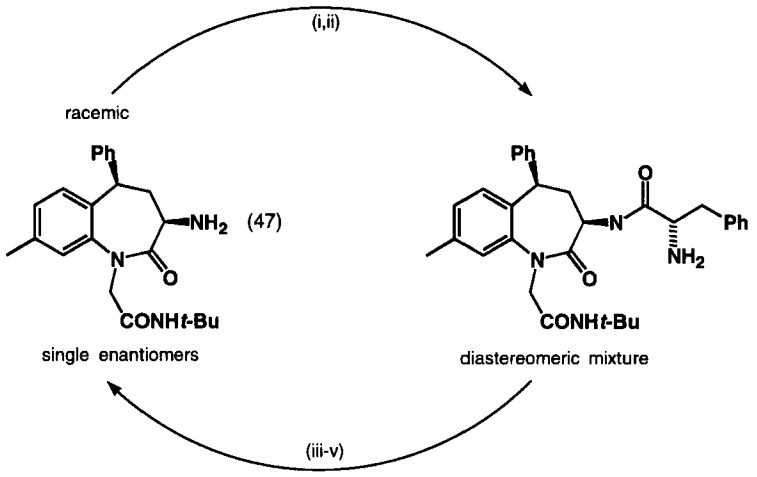
Scheme 5.19. Resolution of a chromanone via derivatisation with SAMP.

diastereomeric hydrazones by flash chromatography (*Scheme 5.19*). Regeneration of single enantiomers of (44) was effected by *N*-methylation and acid hydrolysis.

Resolution of a labile alcohol is exemplified in the synthesis of novel leukotriene analogues by Grée and coworkers [68] (*Scheme 5.20*). Acylation of compound (45) with the chiral tricarbonyliron complex of sorbic acid [69] gave a mixture of chromatographically separable diastereoisomers which



Scheme 5.20. Resolution of a racemic alcohol via acylation with chiral tricarbonyliron complex.



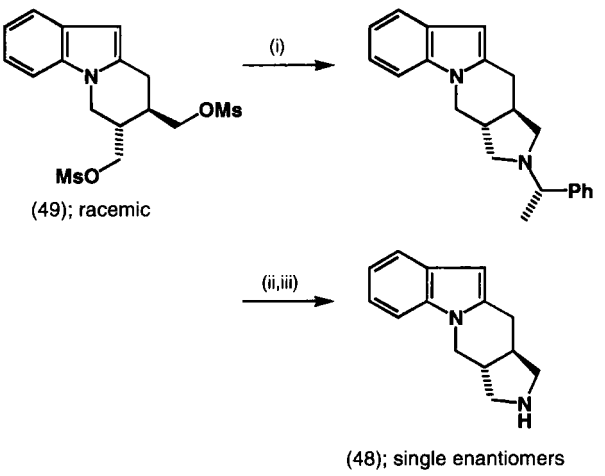
Reagents: (i) Boc-L-Phe, EDAC; (ii) HCl; (iii) chromatography; (iv) PhNCS; (v) TFA

Scheme 5.21. Resolution of an amino benzaepin-2-one via amidation with Boc-L-Phe.

were individually transformed to enantiopure oximes (46; only one enantiomer depicted).

For the synthesis of cholecystokinin-B receptor antagonists by Lowe III and coworkers [70], the amino benzazepin-2-one (47) was resolved into single stereoisomers by the protocol shown in *Scheme 5.21* (for clarity, only single stereoisomers depicted). The notable feature in this instance is the selective removal of the L-phenylalanine auxiliary by Edman degradation in the presence of additional amide linkages.

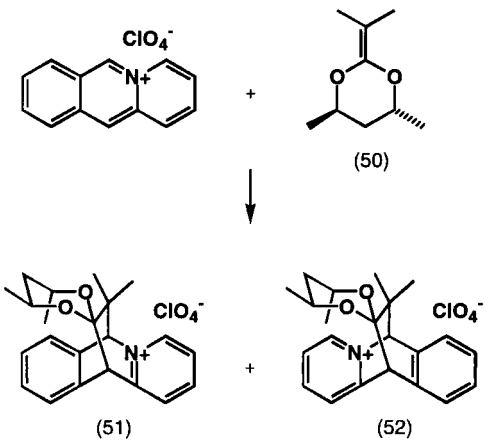
A tactical variant in the utilization of chiral auxiliaries is illustrated in cases where a portion of the auxiliary is used to form permanent skeletal bonds whilst the remainder provides a temporary chiral handle to facilitate separation of distereoisomers. Construction of the pyrrolidine ring in tetracycle (48) (*Scheme 5.22*; for clarity, only single stereoisomers depicted), required by Davis *et al.* [71] *en route* to bisindolylmaleimide inhibitors of protein kinase C, was accomplished by combining the racemic dimesylate (49) with (*S*)-1-phenylethylamine, prior to chromatographic separation of diastereoisomers and scission of the exocyclic N-C bond by transfer hydrogenation. A second example is found in the study of enantiopure 6,11-ethanobenzo[*b*]quinolizinium salts as *N*-methyl-D-aspartate antagonists, by Mallamo and coworkers [72]. As shown in *Scheme 5.23*, dienophile (50) incorporates a (2*R*,4*R*)-pentanediol residue to faciliate HPLC separation of diastero-



Reagents: (i) (*S*)-PhCHMeNH$_2$; (ii) chromatography; (iii) HCO_2NH_4, Pd-C

Scheme 5.22. Use of (S)-1-phenylethylamine to access single stereoisomers of a fused pyrrolidine.

meric cycloadducts (51) and (52), although to obtain multi-gramme quantities of the corresponding diethyl acetals this approach was superseded by classical resolution with antipodes of *O,O*-dibenzoyltartaric acid.



Scheme 5.23. Use of a chiral dienophile to access single stereoisomers of benzo[b]quinolizinium salts.

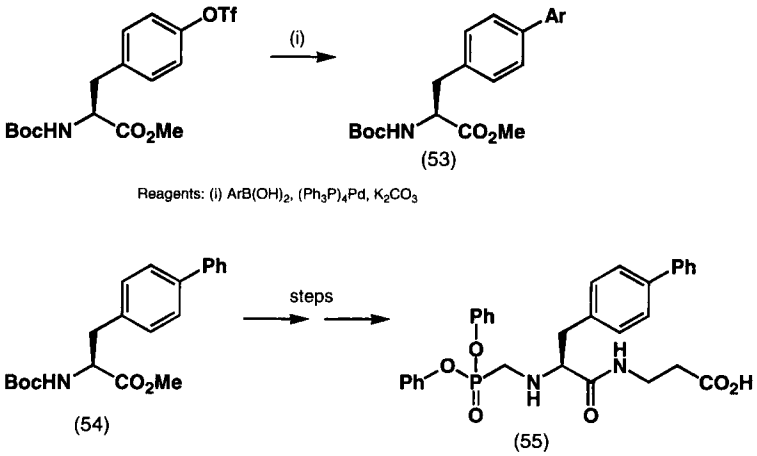
SYNTHESIS FROM CHIRALITY POOL MATERIALS

Of the three basic types of methodologies employed for the provision of chiral target molecules in optically enriched form, synthesis from chirality pool materials can often be the method of choice, providing that (i) suitable starting materials are readily available at reasonable cost, and (ii) it is not necessary to introduce extra steps which would otherwise be avoided, in, for example, the synthesis of an easily resolvable racemate. A distinct advantage of chirality pool synthesis over resolution techniques is the facility to correlate absolute configuration of the product with that of the starting material.

One strategy for the use of chirality pool material simply entails the maintenance of pre-existing chirality throughout a synthesis, with transformations of other regions of the molecule by either formation of new skeletal bonds or interconversion of functional groups. The obvious application of this approach, and one which will be excluded from the examples provided below, is the construction of synthetic peptides from protected derivatives of proteinogenic amino acids by standard coupling reactions [73]. Alternatively, pre-existing chirality can provide a control element for the creation of new stereogenic centres by so-called substrate-directable reactions [74]. Such reactions may either involve complete transfer of chirality from one centre to another, or non-sacrificial asymmetric induction to increase the number of chiral centres.

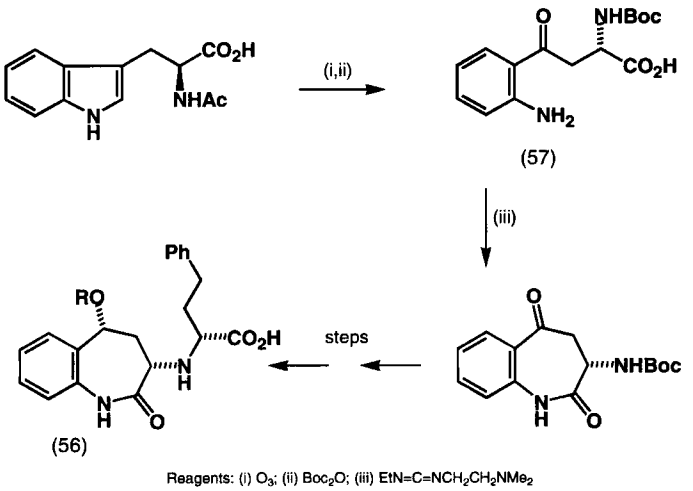
Chirality pool materials have been subdivided into five categories [75]: proteinogenic α-amino acids, hydroxy acids, carbohydrates, terpenes and alkaloids. The bulk of this section focuses on α-amino acids and carbohydrates, which have the broadest applicability as synthons for chiral NCEs. This list of naturally occurring substances can be augmented by considering the so-called new pool comprising man-made compounds which are produced in bulk as commodity chemicals, and also natural products for which industrial synthesis now constitutes the principal source. Examples of new pool materials employed recently during drug discovery are enantiopure C_3 and C_4 chiral synthons such as glycidyl tosylate [76] and derivatives of malic acid [77]. Since the demand for new medicines provides the impetus for development of manufacturing processes of such compounds, the range of new pool materials which will be available in the future is certain to increase.

α-Amino acids and their simple protected derivatives are extremely versatile synthons, especially when additional functionality is present in the sidechain. Synthetic transformations of individual α-amino acids have been reviewed in monographs by Coppola [78] and Jones [73]. Workers at Ciba-Geigy [79] have reported an asymmetric synthesis of (4-arylphenyl)alanines (53, Ar = arene, heteroaryl) by palladium(0) catalyzed cross-coupling of

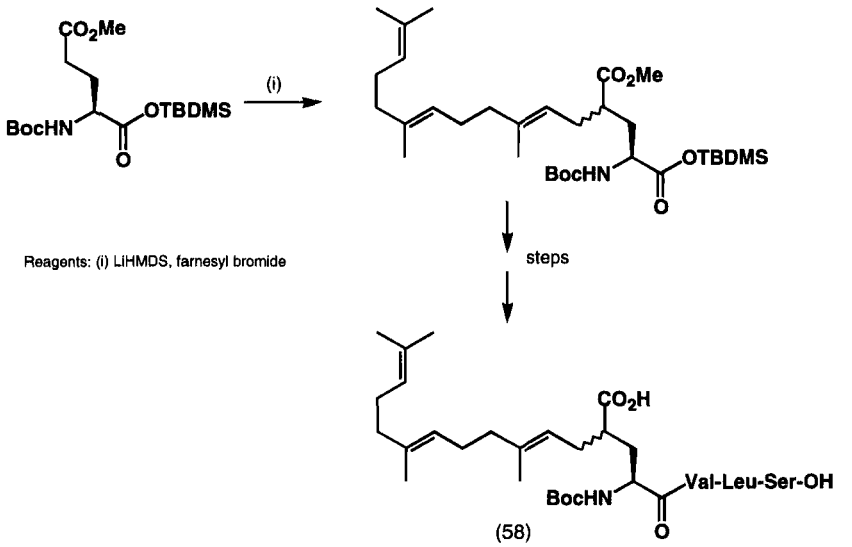


Scheme 5.24. Synthesis of (4-arylphenyl)alanines from L*-tyrosine by Pd(O)-catalyzed cross coupling.*

aryl boronic acids with diprotected L-tyrosine triflate (*Scheme 5.24*). (*S*)-(4-Phenylphenyl)alanine benzyl ester (54), obtained in this way, was used by De Lombaert *et al.* [80] as a chiral template for the synthesis of *N*-phosphonomethyl dipetides (for example, 55) as prodrugs for inhibition of neutral endopeptidase.



Scheme 5.25. Synthesis of benzazepines via ozonolysis of N-acetyl L*-tryptophan.*



Scheme 5.26. Homologation of protected L-*glutamic acid by alkylation at C-4.*

Another of the arylalanines, L-tryptophan, was used at the same laboratories [81] for the synthesis of novel benzazepines (56) as dual inhibitors of ACE and thromboxane synthase (*Scheme 5.25*). Ozonolysis of *N*-acetyl L-tryptophan to effect scission of the indole ring followed by *N*-protection gave the 4-keto acid (57) as a cyclization precursor. Downstream transformations included diastereoselective reduction of the ketone functionality and reductive alkylation of the amine substituent with ethyl 2-oxo-4-phenylbutanoate.

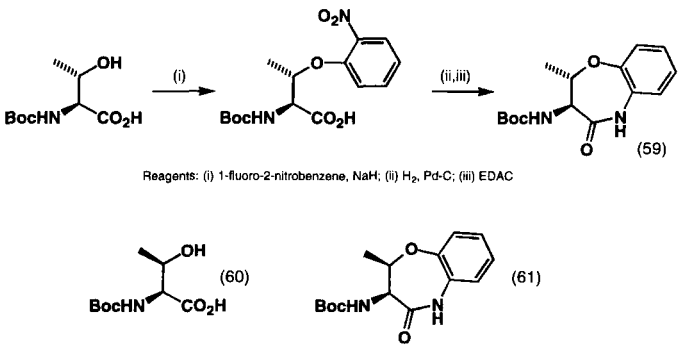
In suitably protected form, L-glutamic acid can be homologated by selective enolate generation and alkylation at C-4 [82]. This methodology has been used by Bhide and coworkers [83] to prepare the farnesyl derivative (58; 1:1 mixture of epimers) as an inhibitor of farnesyl protein transferase (*Scheme 5.26*). *In vitro* evaluation of compound (58) indicated only modest activity against this oncogenic protein, but provided the groundwork for the design of more potent analogues.

L-Threonine provides two chiral centres for synthetic manipulation, a feature demonstrated in work by Rohl and coworkers [84] directed at conformationally constrained inhibitors of ACE and neutral endopeptidase (*Scheme 5.27*). Nucleophilic substitution of 2-fluoro-1-nitrobenzene with the alkoxide generated from *N*-Boc-L-threonine, followed by hydrogenation and cyclocondensation gave the key benzoxazepine intermediate (59). An

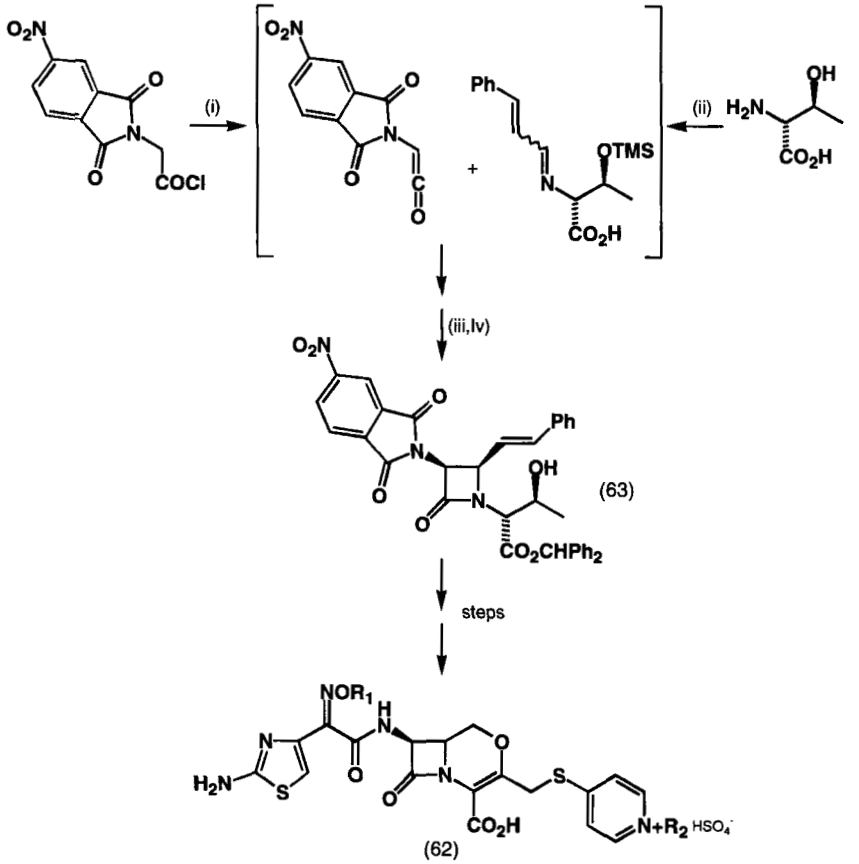
identical sequence commencing from *N*-Boc-*allo*-L-threonine (60) gave the epimer (61). D-Threonine has been used by Tsubouchi and coworkersl [85] to prepare novel 2-oxaisocephems (62) with improved activity against *Staphyococcus aureus* (*Scheme 5.28*). In a one-pot operation, the two components of ketene-imine cycloaddition were generated respectively from acyl chloride and amine precursors, and after desilylation and esterification the key β-lactam intermediate (63) was isolated as the major adduct (11:1 diastereomeric ratio).

trans-4-Hydroxy-L-proline (64), a commercially available product of collagen degradation, has found numerous applications in the synthesis of biologically active compounds and also as a source, via thermal decarboxylation [86], of widely used 3-substituted pyrrolidine synthons. Venkatraman *et al.* [87] have used compound (64) to prepare epimeric oxadiazolidines (65) and (66), as analogues of quisquailic acid, a potent neuroactive agent (*Scheme 5.29*). Conversion of the *N*-Boc methyl ester of compound (64) to the *cis*-4-hydroxy derivative (67) via sequential oxidation-reduction [88] was followed by a Mitsunobu reaction and further steps to produce *trans*-oxadiazolidine (65). In a separate sequence leading to *cis*-oxadiazolidine (66), diastereoselective reductive amination of the 4-keto ester (68) was the key step. The use of Mitsunobu reactions to access and derivatize individual optical isomers of *N*-Z-4-hydroxyproline was reported by Steinberg and coworkers [89] *en route* to the eight diastereoisomers of octanamide (70) for evaluation as angiotensin II receptor antagonists (*Scheme 5.30*; for clarity only single stereoisomers are depicted).

The carbohydrate pool provides a rich source of chiral templates for the construction of biologically active compounds. A large body of general synthetic methods in this area has been amassed in the literature [90,91], a useful starting point being Hanessian's influential monograph on The Chiron Ap-



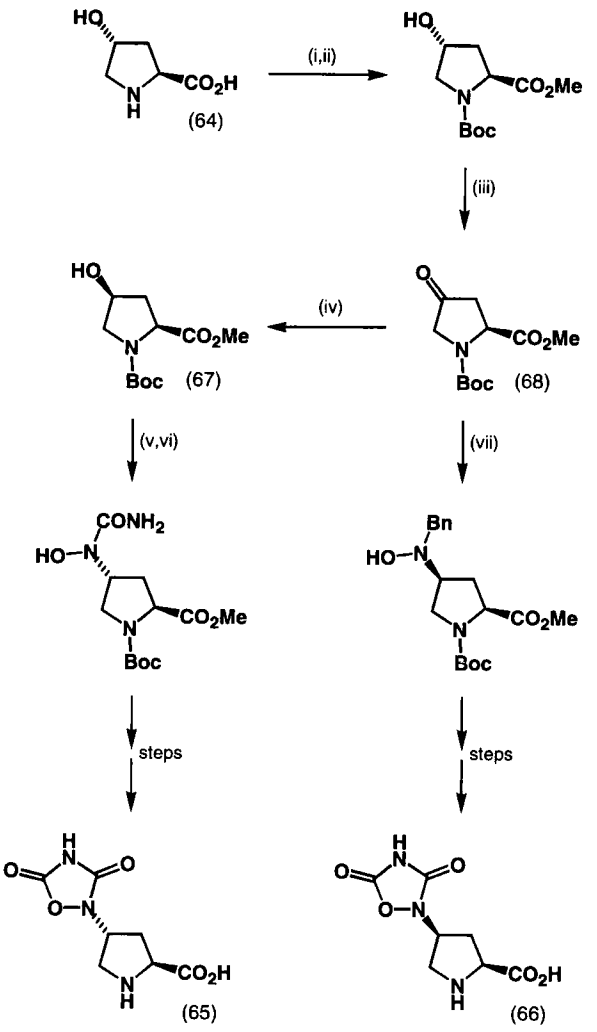
Scheme 5.27. Synthesis of benzoxazepines from L-*threonine and allo*-L-*threonine.*



Reagents: (i) Et_3N; (ii) PhCH=CHCHO, bis(trimethylsilyl)acetamide; (iii) H^+, MeOH; (iv) Ph_2CN_2

Scheme 5.28. Synthesis of 2-oxaisocephems via asymmetric ketene-imine cycloaddition.

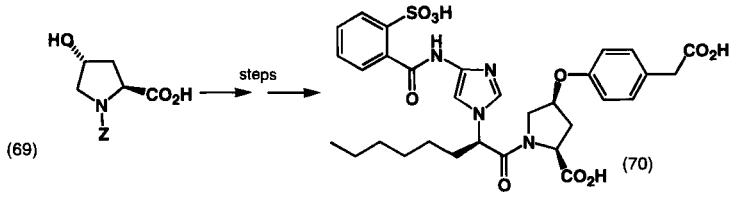
proach [91] published over ten years ago. Methodology concerned with the selective manipulation of polyol functionality, dependent on both conformational control factors and the differential reactivity of primary and secondary hydroxyl groups, is pivotal to the successful utilization of carbohydrate starting materials. Set against these benefits are the perceived over-reliance on protecting groups and the extra steps needed to remove chiral information, since in many applications the target molecule has fewer chiral centres than the starting material. Although such considerations may not augur well for the use of carbohydrate starting materials for manufacturing pur-



Reagents: (i) Boc_2O; (ii) MeOH, H^+; (iii) RuO_4, $NaIO_4$; (iv) $NaBH_4$;
(v) $PhCO_2NHOCO_2Ph$, DEAD, PPh_3; (vi) NH_4OH, MeOH; (vii) $BnNH_2$, $NaBH_3CN$

Scheme 5.29. Synthesis of epimeric oxadiazolidines from trans-4-hydroxy-L-proline.

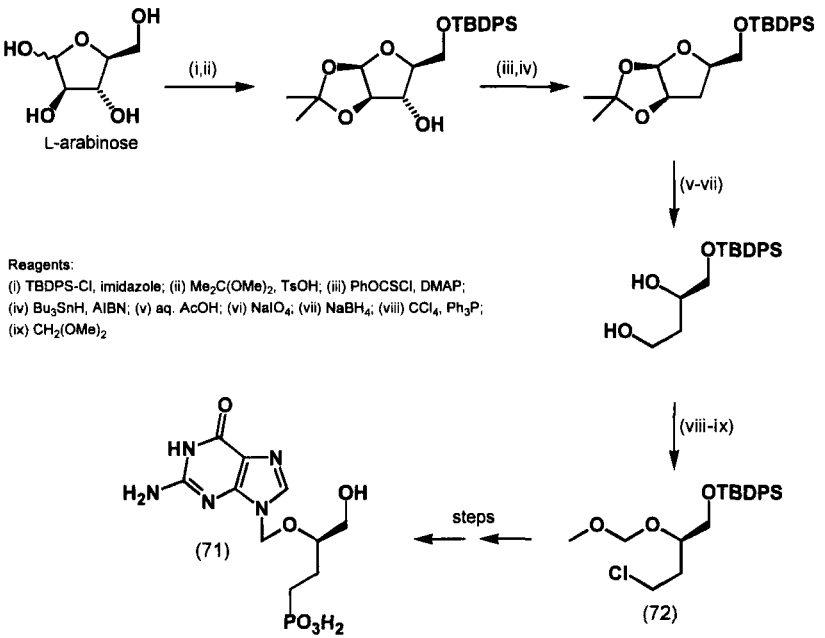
poses, they are less of a limitation during the NCE discovery phase, as exemplified in the following cases.



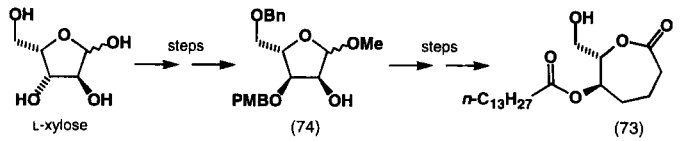
Scheme 5.30. Angiotensin II receptor antagonists from trans-4-hydroxy-L-proline.

Starting from L-arabinose, Chamberlain and coworkers [92] synthesized the (*R*)-enantiomer of ganciclovir monophosphate (71) (*Scheme 5.31*), the racemic form of which had been shown previously to be an effective inhibitor of human cytomegalovirus replication. Ten steps were required to convert L-arabinose to the key C_4 building block (72), prior to coupling with the diacetylguanine and additional steps.

Another of the pentoses, L-xylose, was used by Marquez and coworkers [93] in a seventeen-step synthesis of the heptanolide (73), for evaluation as a conformationally constrained diacylglycerol surrogate effecting activation of protein kinase C. Six steps were required to access the diprotected triol



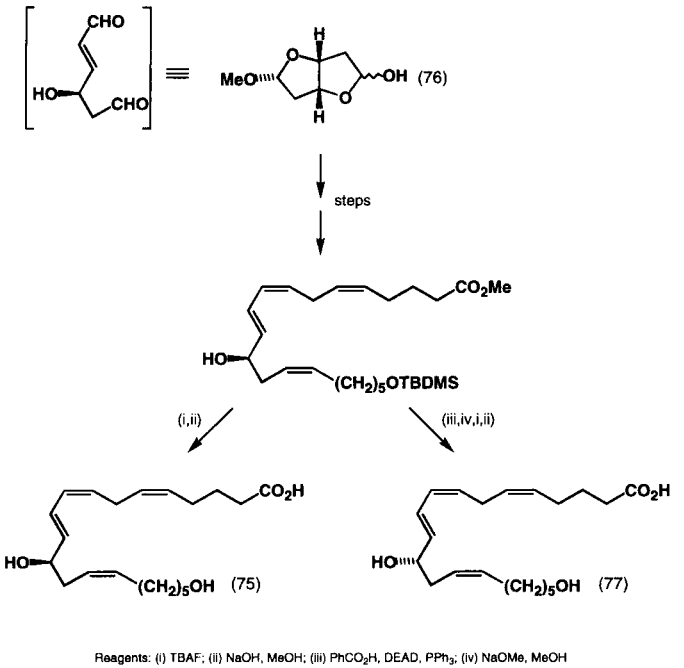
Scheme 5.31. Synthesis of (R)-ganciclovir monophosphate from L-arabinose.



Scheme 5.32. Synthesis of a constrained diacylglycerol analogue from L-*xylose.*

(74) (*Scheme 5.32*), suitable for transformation of each of the functional groups in a controlled sequence, including radical deoxygenation at C-2 and Wittig homologation at C-1.

Falck's synthesis of the (*R*)-enantiomer (75) of the eicosanoid 12,20-di-HETE [94], for evaluation of its vasodilatory properties, required a masked synthetic equivalent of (2*E*,4*R*)-4-hydroxy-2-hexenedial (*Scheme 5.33*). For this purpose the masked bislactol (76), derived from D-glucuronic acid [95], was used. 12(*S*)-20-DiHETE (77), which proved to be the source of all vasodilatory activity of the racemate, was secured by Mitsunobu inversion at the penultimate stage of the synthesis.



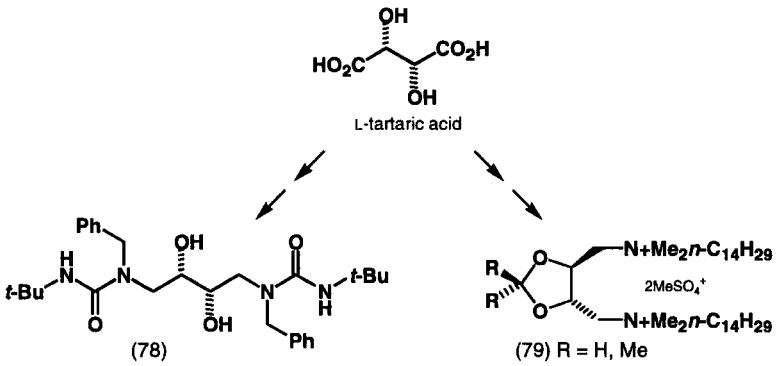
Scheme 5.33. Use a masked bislactol derived from D-*glucuronic acid for the synthesis of (R)- and (S)-12,20-diHETE.*

Outside the amino acid and carbohydrate pools, derivatives of natural and unnatural tartaric acid (L- and D- respectively) merit attention as chiral synthons, in addition to their more familiar applications in classical resolution and asymmetric synthesis. Biologically active compounds prepared from tartrates (*Scheme 5.34*) include the symmetry-based HIV protease inhibitor (78) [96] and the endotoxin inhibitors (79) [97].

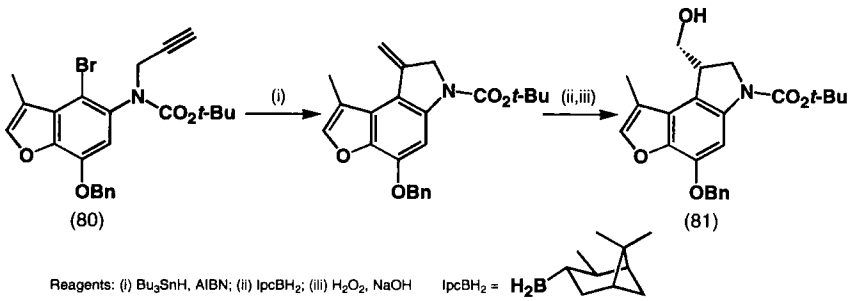
ASYMMETRIC SYNTHESIS WITH CHIRAL REAGENTS AND CHIRAL CATALYSTS

Preparation of enantiopure chiral molecules by transformation of prochiral substrates can offer the most elegant of available approaches, especially when the source of chirality is a man-made chemical catalyst rather than a reagent used in stoichiometric quantitites. Tremendous effort has been devoted to the development of asymmetric synthesis methodology, with notable success in the fields of asymmetric hydrogenation [98], hydride reduction of ketones [99], epoxidation [100] and dihydroxylation [101] of alkenes. In constrast to the enzymes which are used in organic synthesis, man-made chiral catalysts [102] are much simpler molecular entities and are routinely available in both enantiomeric forms. Since reactions employing such catalysts usually follow a predictable course, the correct form can be chosen for the desired product configuration.

Development of an individual methodology needs to reach a certain efficiency before application to new substrates will reliably result in high enantiomeric purities. For applications in drug discovery, this criterion is paramount, since any shortfall in enantiomeric purity will often be carried through to the product, unless additional manipulation is carried out to boost ee levels. If in such a case the product is a solid and the ratio of enan-



Scheme 5.34. Biologically active compounds prepared from tartrates.

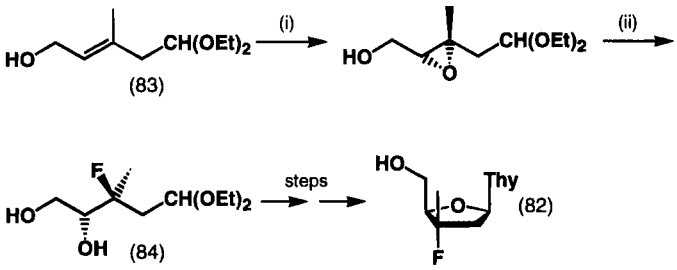


Scheme 5.35. Synthesis of a furanoindoline via asymmetric hydroboration.

tiomers is higher than that defined by its eutectic composition [103], recrystallization provides the simplest expedient. Otherwise, less convenient measures may be needed, which will further reduce the efficiency of the process. For example, in work by Mohamadi and coworkers [104] directed at novel antineoplastic agents, radical-mediated cyclization of the alkynyl benzofuran (80) gave a 3-methylene indoline, which without purification was subjected to asymmetric hydroboration using a stoichiometric quantity of monoisopinocamphenylborane (*Scheme 5.35*). This process was only moderately enantioselective, giving a 3:1 mixture (50% ee) of (*R*)- and (*S*)-enantiomers, and crystallization of the (*R*)-*O*-acetylmandelate derivative was required to obtain an enantiopure sample of (*R*)-(81).

Because the priority during the first phase of drug discovery is rapid access to target molecules, the use of chiral reagents and chiral catalysts is fairly uncommon, with successful applications confined to a small number of well developed methodologies. Pre-eminent amongst these is the Sharpless epoxidation of allylic alcohols, which was first reported as a stoichiometric method in 1980 [105] and later adapted into a practical catalytic variant [106].

In a collaborative programme directed at novel deoxynucleosides as inhibitors of HIV reverse transcriptase, Shutalev *et al.*[107] employed an asymmetric approach to 3′-fluoro-3′-methyl-2′,3′-dideoxythimidine (82) (*Scheme 5.36*). Key steps in the construction of the fluoropentose moiety were Sharpless epoxidation of the (*E*)-hydroxy acetal (83) under catalytic conditions [108] followed by ring opening of the resultant epoxide (95% ee) with fluoride, although the latter step gave only 38% of the desired regioisomer (84). Since epoxidation of (*E*)-alkenes is known to proceed at a faster rate than the corresponding (*Z*)-isomers, the presence of residual (*Z*)-hydoxy acetal (ca. 2%) in the starting material (83) was not detrimental to the efficiency of the process.

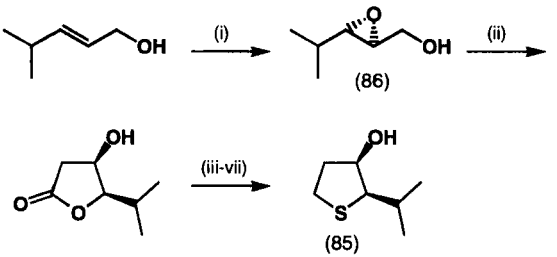


Reagents: (i) *t*-BuOOH, 15 mol% di-isopropyl D-tartrate, $Ti(O\textit{i}\text{-}Pr)_4$, molecular sieves; (ii) $Ti(O\textit{i}\text{-}Pr)_2F_2$

Scheme 5.36. Synthesis of 3'-fluoro-3'-methyl-2',3'-dideoxythimidine via Sharpless epoxidation.

Ghosh and coworkers [109] have reported the development of a novel class of HIV-1 protease inhibitors which incorporate a cyclic sulphone moiety for binding to the P_2 subsite. For the provision of *cis*-2-alkyl-3-hydroxytetrahydrothiophene building blocks (for example, 85), Sharpless epoxidations of (*E*)-4-methyl-2-penten-1-ol under catalytic conditions with (+)-diethyl L-tartrate and gave epoxy alcohol (86) of 90% ee (*Scheme 5.37*). Several steps were then required to access compound (85), including cyanide-induced Payne rearrangement and introdution of sulphur by a double nucleophilic substitution. The (*S,S*)-antipode of compound (85) was accessed via Sharpless epoxidation using (-)-diethyl D-tartrate.

As a final example, enantiocomplementary Sharpless epoxidations of the allyl alcohol (87) were used by Saksena *et al.* [110] to prepare epoxyalcohols (88) and (89) of around 90% ee (*Scheme 5.38*). Elaboration of these synthons to furnish the individual stereoisomers of broad-spectrum azole antifungals

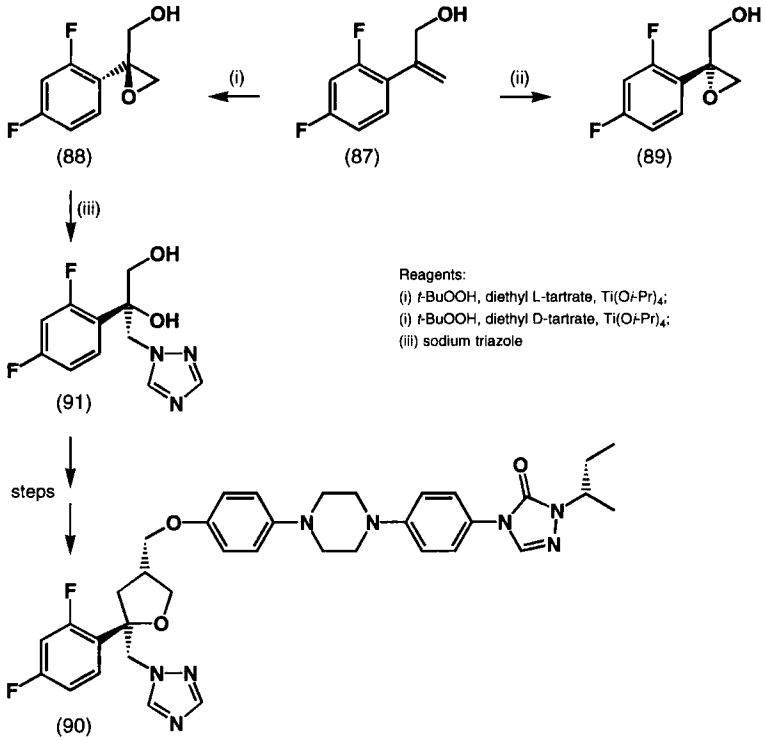


Reagents:

(i) *t*-BuOOH, 6 mol% diethyl L-tartrate, $Ti(O\textit{i}\text{-}Pr)_4$, molecular sieves;

(ii) NaCN, H_2O, then H^+; (iii) MOM-Cl; (iv) LAH; (v) MsCl; (vi) Na_2S; (vii) PhSH, $BF_3.Et_2O$

Scheme 5.37. Use of the Sharpless epoxidation to prepare tetrahydrothiophene synthons for HIV-1 protease inhibitors.



Scheme 5.38. Use of the Sharpless epoxidation to prepare azole antifungal agents.

(for example, 90) included epoxide ring opening with sodium triazole and recrystallization of the resultant diols (for example, 91) to boost enantiomeric purity to >98% ee. Development of processes based on enzymatic transesterification to target compound (90), the most efficacious stereoisomer, have subsequently been reported by workers at the same laboratories [111].

CHIRAL METHODOLOGIES USED FOR MANUFACTURE

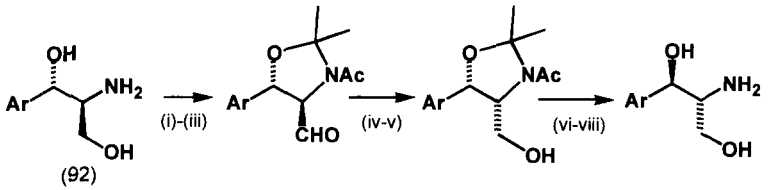
ISSUES IN MANUFACTURE

As introduced above in the first section, the requirements for a manufacturing process to a single-enantiomer pharmaceutical agent can differ widely from those of initial lead generation in medicinal chemistry. Thus the primary consideration should be the scaleability and economy of a route which

can depend on maximizing yields and minimizing waste streams. Often a choice of synthesis is made on the basis of availability of raw materials specific to the required target such that two structurally closely related pharmaceutical agents might best be made by entirely different routes. The principles applied to the industrial synthesis of single enantiomers have been presented in detail in recent texts [112,113] and the purpose of this review in this regard is to summarize the key issues with regard to a range of important examples.

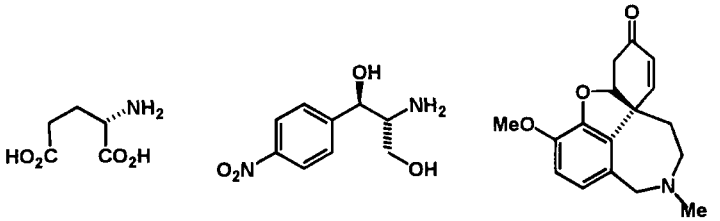
Generally for maximizing economy and minimizing waste streams, catalytic processes are preferred over ones that use stoichiometric reagents or auxiliaries. However, such processes may take substantial resource to develop and this has to be weighed against the need to have a process fixed before a drug can be marketed. Even for a given route it has to be borne in mind that the process development involved will generally far outweigh the laboratory work that led to the design of that process. Often it is worthwhile to commence process research for route selection well before it is certain that the pharmaceutical agent is going to progress to a clinical trial. Consider for instance the use of biocatalysis. Often, readily obtainable enzymes are expensive and might not give the enantiocontrol or be sufficiently active to give a viable process. However, with screening of microbial strains, a better activity might be identified, and the enzyme recovery and use, which could include immobilization, optimized to give a highly economic bulk process. Similar considerations can apply to the development of a chemical catalyst for asymmetric synthesis.

For large scale processes the emphasis is on simplicity and a result is that excellent processes might use techniques developed over a century ago; thus classical resolution with diastereoisomeric salts plays a large part in manufacturing processes. However, an important issue in all resolution-based processes when used in manufacure is the utilization of the incorrect enantiomer. If possible, it should be racemized and recycled and this is straightforward for instance for esters of 2-arylpropionic acid (anti-inflammatory agents) that are racemizable with base. Where this is not possible, a resolution should be effected as early as possible in a synthesis as it is uneconomic to carry material through process steps where half is eventually discarded. An interesting case of the drive to utilize the incorrect enantiomer is with the anti-infective thiamphenicol where a route has been developed to invert both of the chiral centres in an intermediate [114] (*Scheme 5.39*), though the economics of such a sophisticated procedure are questionable. The issue of wrong-enantiomer utilization is a recurring issue in the manufacture of single-enantiomer pharmaceuticals and is considered in many of the examples to follow.



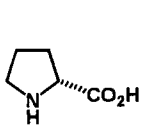
Ar = MeS-C_6H_4-; Reagents: (i) Me_2CO, (ii) MeCOCl, (iii) $(COCl)_2$, Me_2SO, (iv) DABCO, (v) $NaBH_4$, (vi) CH_3COCl, NEt_3, (vii) $(CH_3CO)_2O$, $MeSO_3H$, (viii) NaOH (Aq)

Scheme 5.39. Inversion of a thiamphenicol precursor.



TYPES OF CHIRAL PROCESSES AMENABLE TO SCALE-UP

The types of chiral processes that have been most often used in manufacture are (i) fermentation, (ii) biotransformation by enzymes, (iii) crystallization-based methods and recently (iv) asymmetric chemical catalytic processes. As indicated above, a preferred method for enantiomer manufacture may take a substantial resource to develop. Natural amino acids are now mostly made by fermentation methods [115]. Biocatalytic methods using isolated enzymes or microbial strains are also often appropriate to manufacture since the methods are catalytic and readily scaled up if the biocatalyst is derived from a microbial strain that can be grown up to whatever biomass is necessary [116]. Crystallization methods continue to be developed, for example, where a racemate exists as a conglomerate of its enantiomers. While only a fraction of a single isomer may be recovered in a cycle by entrainment with seeds of a single isomer, repetition of cycles can be automated; such a process has the benefit of its simplicity as there is no resolving agent to recycle; recycling can be costly in terms of vessel utilization on a plant. Examples of compounds that exist as conglomerates separable by entrainment are: glutamic acid (93) [117], the chloramphenicol intermediate (94) [118,119] and the alkaloid narwedine (95) [120]. In the latter case, the compound undergoes spontaneous racemization in the presence of an amine base, so that upon entrainment the *in situ* racemization allows all the materi-



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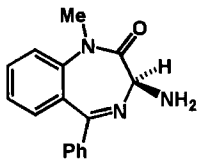


(97)

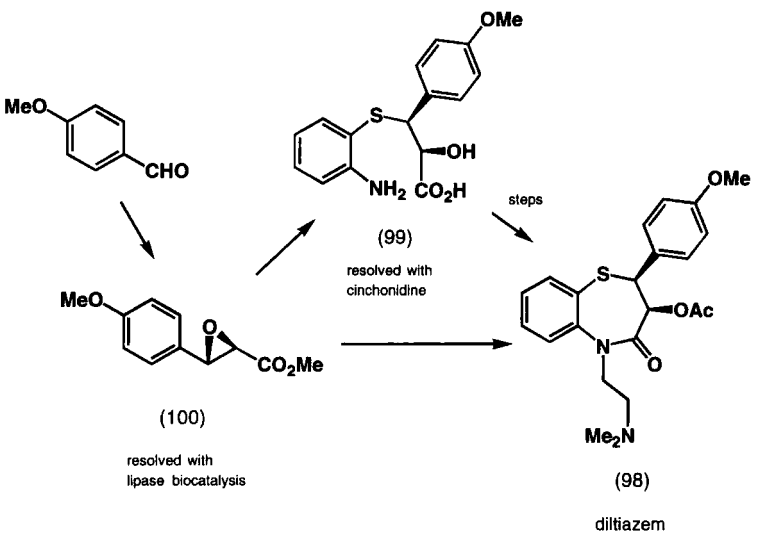
al to be converted into a single enantiomer in a single cycle. Such a process is known as a crystallization-induced asymmetric transformation and has been used mostly with classical diastereoisomer resolution. Examples of this are in production of D-proline (96) from L-proline [121] with tartaric acid and for a benzodiazepinone (97) [122] with camphorsulphonic acid, in each case in the presence of an aldehyde to effect racemization through imine formation with the amine function.

SPECIFIC EXAMPLES OF THE MANUFACTURE OF CHIRAL DRUGS

A selection of syntheses used to produce chiral drugs and their intermediates is discussed in this section. These syntheses serve to illustrate the points discussed in previous sections but are not intended to be comprehensive.

Diltiazem

Diltiazem (98) is an antihypertensive agent marketed as the single enantiomer. The earliest route by Tanabe chemists [123] uses a relatively late stage resolution of the carboxylic acid intermediate (99) with cinchonidine (*Scheme 5.40*). This means that a substantial amount of material is lost as the incorrect enantiomer which cannot be cyclized. It is more economical to resolve earlier in the synthesis and this has been done at the glycidate ester intermediate (100) by means of a lipase-mediated kinetic resolution, whereby the unwanted isomer forms the carboxylic acid [124]. A complication with this procedure is that the carboxylic acid rearranges with decarboxylation to give an aldehyde product that inhibits the enzyme. However, process development was able to solve this problem through the inclusion of sulphite in the biotransformation medium which converts the byproduct to a non-inhibitory bisulphite complex. Even then, half of the material from the resolution is wasted. The racemic glycidate ester is produced cheaply by Darzens condensation between methyl chloroacetate and *p*-anisaldehyde; it would therefore be attractive to have an asymmetric Darzens condensation. How-



Scheme 5.40. Synthesis of diltiazem.

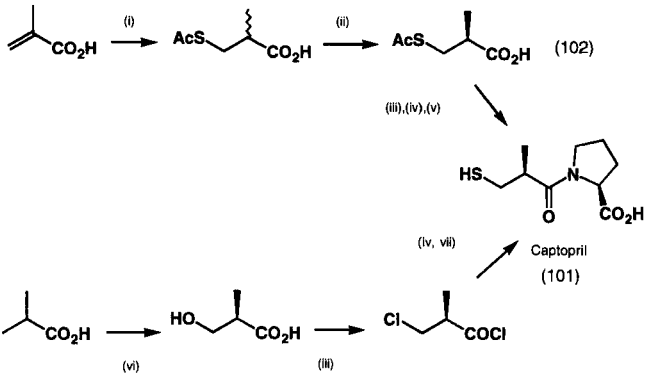
ever, application of this method using the enantiomeric 2-phenylcyclohexyl chloroacetate [125] gave a moderate diastereoisomeric excess and the auxiliary serves as much to allow the required isomer to be crystallized to purity. It is important to note that the costs of using and recycling the auxiliary are likely to make the process more expensive than the enzymic resolution method. The ideal synthesis of diltiazem could be envisaged to start with a catalytic asymmetric Darzens condensation but this has not yet been achieved.

Captopril

Captopril (101) is an antihypertensive ACE inhibitor. Syntheses of note involve classical resolution of the acetyl β-mercaptoisobutyric acid to provide the (*R*)-enantiomer (102) [126], and secondly, generation of the isobutyrate component following the asymmetric hydroxylation of isobutyric acid by a microbial strain [127] (*Scheme 5.41*). The important point is to have the isobutyrate component resolved prior to the coupling with proline, so that the proline is fully utilized.

Enalapril

Enalapril (103) is another ACE inhibitor though its L-homophenylalaninyl component is common to many other ACE inhibitors such as benzapril



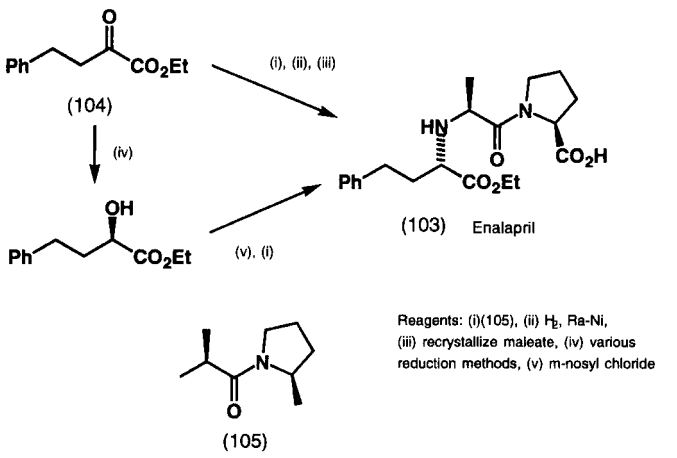
Reagents: (i) AcSH, (ii) resolution, (iii) $SOCl_2$, (iv) L-proline
(v) deacylate, (vi) C. rugosa cells, (vii) NaSH

Scheme 5.41. Syntheses of captopril.

and lisinopril. The original Merck route [128, 129] involves condensation of the ketoester (104) with the alanine-proline dipeptide (105) and then cyanoborohydride or Raney nickel-catalyzed hydrogenation of the resulting imine (*Scheme 5.42*). Usefully, this gives a reasonable excess (70%de) of the required diastereoisomer which can be recystallized as its maleate salt to give enalapril. Of many other known routes, an alternative is to couple the alanine alone and attach the proline unit last [130]. Another interesting approach is addition of the alanine unit onto $PhCOCH{=}CHCO_2Et$ which also gives the required diastereoisomer [131]. For a fuller range of ACE inhibitors of this type, a generic synthon for the phenylbutyrate component has been sought. A mass of work by Ciba Geigy directed towards benzapril on asymmetric reduction of the ketoester [132] is noteworthy, either through microbial reduction, heterogeneous asymmetric hydrogenation over platinum-alumina-cinchonidine or homogeneous asymmetric hydrogenation with rhodium-phosphines. Replacement of the hydroxyl function by the ala-pro component is carried out effectively via the *m*-nitrobenzenesulphonate [132]. A complementary approach starts with L-homophenylalanine [133] but then the issue is to make this amino acid in single-enantiomer form.

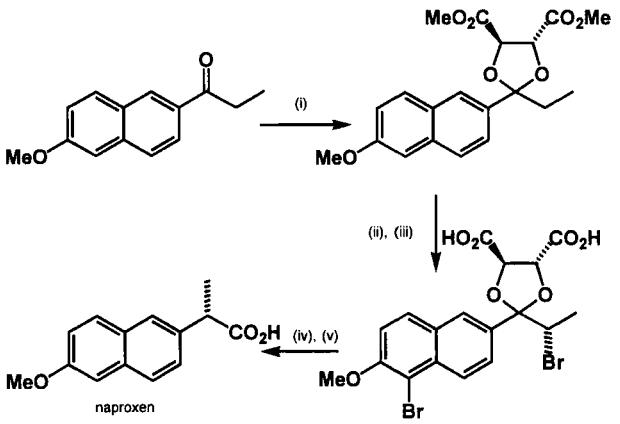
Naproxen

Naproxen (106) is one of a family of arylpropionic acid anti-inflammatory agents including also ibuprofen and ketoprofen, though naproxen is the only one of these that has always been marketed as the single (*S*)-enantio-



Scheme 5.42. Syntheses of enalapril.

mer. It has been accessed by quite a variety of resolution and asymmetric synthesis methods. Late-stage resolution in this case is a viable economic proposition since the undesired enantiomer is readily racemized, for instance, as an ester with base. Also the racemate is readily produced, for example, following reaction of the Grignard reagent from 2-bromo-6-methoxynaphthalene with the magnesium salt of 2-bromopropionate [134]. Thus, a resolution of naproxen with alkylglucamines has been reported by the original manufacturers Syntex [135]. Enzymic resolution of the ester has been shown with *Candida cylindracea* lipase [136] which provides the required (*S*)-naproxen directly, leaving the (*R*)-ester that is readily recycled. Esterases with the opposite enantiospecificity are easily found but yield a less efficient process. An alternative enzymic approach uses a nitrile hydrolysing microbial strain though this yields the (*R*)-amide and (*S*)-acid rather than leaving (*R*)-nitrile that would be preferred for recycling [137]. Amongst asymmetric approaches, there is catalytic asymmetric hydrogenation of the 2-arylacrylate [138] and asymmetric hydroformylation of the arylethene [139]. In such cases, the substrate for the asymmetric methodology tends to be less easily accessed by synthesis than the racemic substrate for resolution. Ideally, the asymmetric step should replace a step in a synthesis rather than add to it. In this connection, an interesting and elegant process has been developed by Zambon, as depicted in *Scheme 5.43*, whereby a tartrate acetal of the arylpropiophenone is asymmetrically brominated and rearranged to the arylpropionate (106) [140].



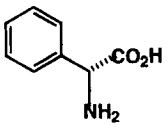
(106)

Reagents: (i) dimethyl tartrate, (ii) Br_2, (iii) hydrolysis, (iv) H^+, H_2O, (v) H_2, Pd

Scheme 5.43. The Zambon asymmetric synthesis of naproxen.

D-*Phenylglycine and* D-*4-hydroxyphenylglycine*

The unnatural amino acids D-phenylglycine (107) and D-4-hydroxyphenylglycine (108) are used in large quantities attached to penicillanic acid to provide the semisynthetic antibiotics ampicillin and amoxicillin, respectively. Resolution can be by salt crystallizaton methods [141,142]. However, excellent routes to both of these proceed through hydantoin intermediates prepared from benzaldehyde, cyanide and urea for the phenylglycine hydantoin, and from phenol, glyoxylic acid and urea for the 4-hydroxyphenylhydantoin. The enantiospecific hydrolysis of the hydantoins can be effected with D-hydantoinases which are abundant in micro-organisms where naturally they cleave the pyrimidine nucleoside bases. A useful feature of the hydantoins is that once one of its enantiomers is hydrolyzed, that remaining undergoes facile racemization via an achiral (aromatized) tautomer. The racemization is catalyzed either by base or by racemase enzymes; either way, the result is that all the substrate is converted into D-isomer product in a dynamic resolution (a second order asymmetric transformation). As indicated in the earlier sections, a dynamic resolution is as good as an asymmetric synthesis. The product from hydantoinase action is the carbamoylamino acid which may be cleaved either chemically by nitrous acid diazotization [143] or microbiologically with a strain containing a carbamoylase. For this latter case, a microbial system has been identified by Recordati chemists that effects both



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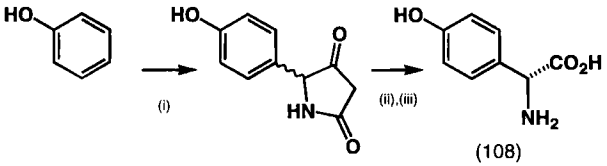


(108)

Reagents: (i) H_2NCONH_2, CN^-, (ii) D-hydantoinase, (iii) carbamoylase

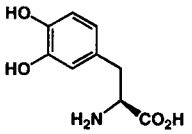
Scheme 5.44. A synthesis of D-*(4-hydroxyphenyl)glycine.*

the hydantoinase and carbamoylase steps [144] and thus a complete synthesis of D-hydroxyphenylglycine is achieved as represented in *Scheme 5.44*.

Levodopa and other arylalanines

Levodopa (109) is used in the treatment of Parkinson's disease and its industrial synthesis demonstrates the emergence of asymmetric chemical hydrogenation catalysis in manufacturing chemistry. A process was developed in 1975 by Monsanto [145] of hydrogenating the enamide (110) (*Scheme 5.45*) by the rhodium complexes of the bisphosphine ligand DiPAMP (111), which gives much improved specificities over the early catalytic asymmetric hydrogenation work with the ligand DIOP [146]. The ligand DiPAMP is difficult to prepare and in this regard it is noteworthy that other ligands have emerged in recent years that are much easier to obtain such as a bisphosphinite derived from glucose [147].

There are of course other ways of obtaining single-enantiomer unnatural



levodopa

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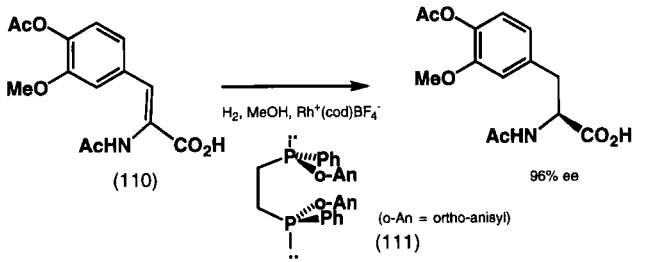
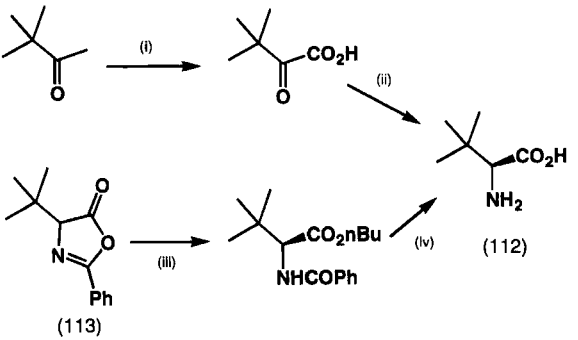


Scheme 5.45. Monsanto's asymmetric hydrogenation approach to levodopa.

amino acids like levodopa. Enzymes that modify amino acids and peptides are abundant and an effective means to resolve amino acids is through acylase treatment of the racemic acetyl amino acid that gives the L-amino acid generally in high enantiomeric excess [148,149]. Alternatively, an amino acid ester can be cleaved with a protease.

tert-Leucine

tert-Leucine (L-tert-butylglycine) (112) is an unnatural hydrophobic and hindered amino acid that has applications in oligopeptide chemistry [150]. An effective route to this compound, as with other amino acids, is dependent on the availability of raw materials. A route developed by Degussa starts with the readily available pinacolone which is oxidized to the ketoacid and this converted to the L-tert-leucine through a dehydrogenase action on the imine in a coupled enzyme reaction to recycle the enzyme's cofactor (*Scheme 5.46*) [151]. An alternative route is a lipase biotransformation on the racemic



Reagents: (i) $KMnO_4$, (ii) NH_3, dehydrogenase, (iii) lipase, n-BuOH, (iv) KOH

Scheme 5.46. Routes to L-*tert-leucine.*

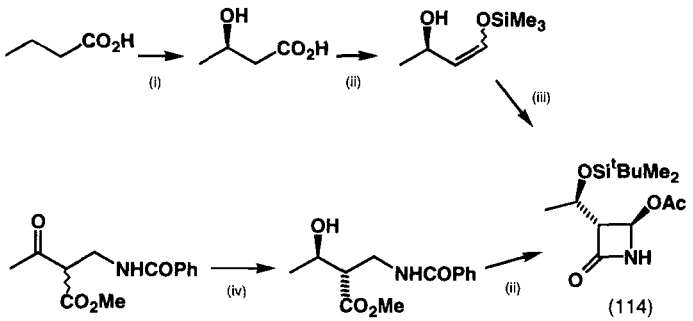
oxazolinone (azlactone) (113) with n-butanol where the L-enantiomer is transformed and the D-isomer undergoes *in situ* racemization to provide a dynamic resolution comparable to that mentioned above with hydantoins [152].

Carbapenem intermediate

The intermediate (114), for which there are two competitive syntheses (*Scheme 5.47*), is the core building block for carbapenem antibiotics such as thienamycin. The Kanegafuchi method (cf. captopril above)[127,153] begins with a microbial hydroxylation of n-butyric acid to the (*R*)-3-hydroxybutyrate which is modified in preparation for the key diastereoselective [2+2] cycloaddition with chlorosulphonyl isocyanate. Takasago's route is based on the asymmetric hydrogenation methodology of Noyori using catalysis by a BINAP-ruthenium complex. The key reaction is notably not only an asymmetric ketone reduction but is a dynamic resolution in converting only one enantiomer of the ketoester precursor of the target [154].

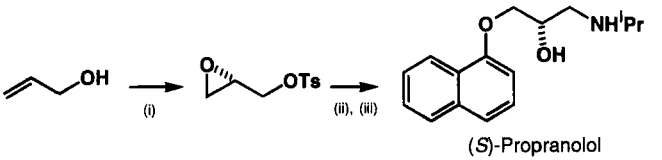
β-Adrenergic antagonists

Most β-adrenergic antagonists such as propranolol are presently used as racemates but technology is available for their manufacture in single-enantiomer form. The key building block is a glycidol arylsulphonate, where the sulphonate and epoxide functions can be respectively regioselectively displaced by a phenolate and then the amine component [155]. A biocatalytic route to the glycidol by a lipase resolution of the butyrate [156] has been developed industrially, as also has the Sharpless epoxidation of allyl alcohol.



Reagents: (i) microbial strain, (ii) various steps, (iii) $ClSO_2NCO$, (iv) Ru-BINAP, H_2

Scheme 5.47. Routes to a carbapenem synthon.



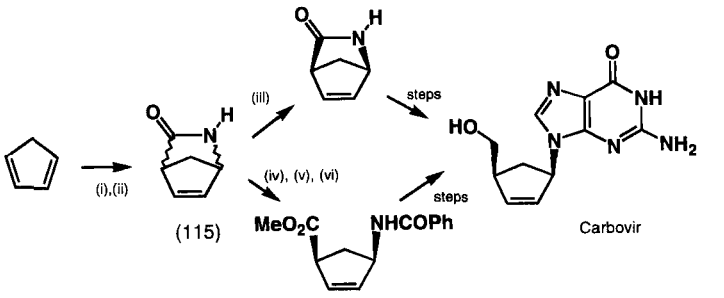
Reagents: (i) t-BuOOH, $Ti(OiPr)_4$, di-isopropyltartrate, (ii) 1-naphtholate, (iii) iPr_2NH

Scheme 5.48. A route to (S)-propranolol.

It is noteworthy that while the Sharpless method gives only a little over 90% ee glycidol, the tosylate of the product can be recrystallized to optical purity [157] (*Scheme 5.48*).

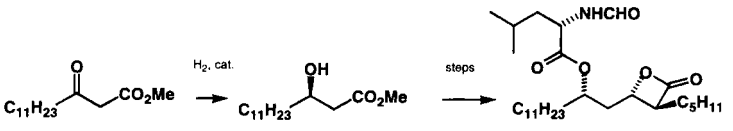
Carbocyclic nucleosides

Carbocyclic nucleosides where the ribose oxygen has been replaced by a methylene group are increasing in importance as therapeutic antivirals and as cardiac vasodilators. They have the advantage of greater metabolic stability than the ribose-based nucleosides but as total synthesis is generally necessary, the synthesis must accommodate the need for a single enantiomer. This has been done through biocatalytic resolution [158] of the key bicyclic lactam (115) starting material (*Scheme 5.49*). It is important to note that as in this example, where effective recycling of the undesired enantiomer is not available, it is best to perform the resolution as early in the synthesis as possible. Resolution has also been moved slightly further down the pathway by enzyme resolution on the methyl ester benzoylamide (116)[159]. A third approach uses a lipase resolution of a bicyclic hydroxylactone where palla-



Reagents: (i) Ts-CN, (ii) AcOH, (iii) microbial lactamase, (iv) MeOH, H^+, (v) (vi) lipase

Scheme 5.49. Routes to the antiviral agent (-)-carbovir.



(117)

Scheme 5.50. Synthesis of tetrahydrolipstatin

dium-catalyzed allylic substitution methodology can access the required substitution pattern [160].

Tetrahydrolipstatin

Tetrahydrolipstatin (117) is a pancreatic lipase inhibitor for the treatment of obesity and is being developed by Hoffman La Roche. A variety of approaches to this target have been published [161] but of particular utility and interest is the introduction of the chirality is through asymmetric reduction of 3-ketomyristate [162] (*Scheme 5.50*). For catalytic homogeneous asymmetric hydrogenation, Roche developed their own ligand as an alternative to BINAP; in either case the ruthenium complex gives a high enantiomeric excess of the alcohol. However, the preferred approach [162] was to use Raney-Nickel modified by tartaric acid [163]. Although this gives a lower ee (86%), the product can be recrystallized to optical purity and the process overall is simpler to operate.

CONCLUDING REMARKS

Synthetic methods for enantiomers in a drug discovery programme differ appreciably from those used in their manufacture but the same basic principles are useful in both, for example, resolution by physical, chemical, or biocatalytic means, asymmetric synthesis, or use of the chirality pool. Generally, the method of choice ultimately used in manufacture of a pharmaceutical will be quite different from that first used to obtain the single-enantiomer material in a drug-discovery programme.

From our survey of the recent literature, classical resolution is probably the most widely used method in drug discovery by virtue of its simplicity. Perhaps for this reason it has attracted a low-technology image, but it can be utilized in sophisticated ways, for instance to separate complex mixtures of diastereoisomers as well as enantiomers, and its usefulness should never be underestimated when compared with the lure of the latest exotic chemis-

try. Biocatalysis is a powerful method for separation which is broadly applicable and often a method of choice for separating chiral secondary alcohols, but it is probably underused in drug discovery, perhaps because the synthetic training of medicinal chemists tends to underestimate this technique. Direct separation by chromatography is another technique which is developing rapidly with the increasing availability of effective chiral stationary phases based on cellulose or cyclodextrins. Although presently beset by high equipment and stationary phase costs, this technique is bound to increase in importance as could other physical methods of separation such as those based on membrane systems. Covalently bound chiral auxiliaries are valuable either for giving derivatives of enantiomers which are separable diastereoisomers, or by mediating asymmetric chemistry. The auxiliaries of Evans and Oppolzer are the most widely used in the latter context. However, the use of auxiliaries adds complexity to any synthesis and the increasing effectiveness of more direct enantiomer separation methods may reduce the popularity of auxiliary-based methods in the future. The chirality pool, on the other hand, stands to become more important as a source of single enantiomers simply because this pool is in effect growing with the increasing commercial availabilty of single-enantiomer materials from synthetic sources. Lastly, we see the emergence methods of catalytic chemical asymmetric synthesis where those that have proven reliability such as the Sharpless oxidation methods are attracting increasing use by medicinal chemists.

For scale-up and manufacture, quite a wide variety of synthetic methodologies are amenable. Resolution methods involving crystallization will continue in importance with perhaps more emphasis on methods of *in situ* recycling of undesired enantiomer, in which case the approach becomes as effective as an asymmetric synthesis. Catalytic methods have obvious economic advantage over the use of stoichiometric reagents or auxiliaries, and in this respect, both biocatalytic and asymmetric chemical methods are effective. The use of asymmetric catalytic hydrogenation (Noyori) and asymmetric epoxidation (Sharpless) in scaled-up processes is significant although there is need for additional scaleable asymmetric methods and it is hoped that these will emerge. Also, the availability of increasingly reliable methods should overcome the long process development time associated with chemical asymmetric catalysis, a problem that may compromise a pharmaceutical development programme. There is a desire to be able to directly extend the methods used in medicinal chemistry to scale-up and this will further propel the growth of physical methods of enantiomer separation including chromatography on chiral stationary phases (for example, [164]), use of membrane systems, or separation using chiral inclusion complex formation [165]. Perhaps completely new methodologies for enantiomers of pharmaceuticals

will be discovered and put to practical use in the coming years, but already we are at the position where it can be concluded that in almost all cases, synthetic inaccessiblity is a poor excuse for developing a racemate.

REFERENCES

1 Ariens, E.J. (1984) Eur. J. Clin. Pharmacol. 26, 663–668.
2 DeCamp, W.H. (1989) Chirality 1, 2–6.
3 Rauws, A.B and Groen, K. (1994) Chirality 6, 72–75.
4 Millership, J.S. and Fitzpatrick, A. (1993) Chirality 5, 573–576.
5 Pfeiffer, C.C. (1956) Science (Washington, D.C.) 124, 29–30.
6 Bain, J.D., Wacker, D.A., Kuo, E.E. and Chamberlin, R. (1991) Tetrahedron 47, 2389–2400.
7 Selke, R., Facklam, C., Foken, H. and Heller, D. (1993) Tetrahedron: Asymmetry 4, 369–382.
8 Kagan, H.B. and Fiaud, J.C. (1988) in Topics in Stereochemistry (Eliel, E.L. and Wilen, S., eds.) Vol 18, pp. 249–330.
9 Chen, C.-S., Fujimoto, Y., Girdaukas, G. and Sih, C.J. (1982) J. Am. Chem. Soc. 104, 7294–7299.
10 Allenmark, S. (1991) Chromatographic Enantioseparation: Methods and Applications, 2nd edn (Ellis Horwood, New York).
11 Jacques, J., Collet, A. and Wilen, S.H. (1981) Enantiomers, Racemates and Resolutions (Wiley-Interscience, New York)
12 Eliel, E.L. and Wilen, S.H. (1994) Stereochemistry of Organic Compounds, Wiley, New York.
13 Wilen, S.H., Collet, A. and Jacques, J. (1977) Tetrahedron 33, 2725–2736.
14 Newman, P. (1981) Optical Resolution Procedures for Chemical Compounds (Volume 1, Amines; Volume 2, Acids), Manhattan College Publications, New York.
15 Palkowitz, A.D., Steinberg, M.I., Thrasher, K.J., Reel, J.K., Hauser, K.L., Zimmerman, K.M., Wiest, S.A., Whitesitt, C.A., Simon, R.L., Pfeifer, W., Lifer, S.L., Boyd, D.B., Barnett, C.J., Wilson, T.M., Deeter, J.B., Takeuchi, K., Riley, R.E., Miller, W.D. and Marshall, W.S. (1994) J. Med. Chem. 37, 4508–4521.
16 Fournié-Zalkuski, M.-C., Coric, P., Turcaud, S., Rousselet, N., Gonzalez, W., Barbe, B., Pham, I., Jullian, N., Michel, J.-B., and Roques, B.P. (1994) J. Med. Chem. 37, 1070–1083.
17 Osayu, H., Nagano, M., Akahane, A., Tomoi, M., Tada, T. and Matsuo, M. (1994) J. Med. Chem. 37, 1378–1381.
18 Phillips, S.T., de Paulis, T., Baron, B.M., Siegel, B.W., Seeman, P., Van Tol, H.H.M. and Guan, H.-C. (1994) J. Med. Chem. 37, 2686–2696.
19 Ref. 12, chapter 14.
20 Scheoen, W.R., Ok, D., DeVita, R.J., Pisano, J.M., Hodges, P., Cheng, K., Chan, W.W.-S., Butler, B.S., Smith, R.G., Wyvratt, M.J. and Fisher, M.H. (1994) Bioorg. Med. Chem. Lett. 4, 1117–1122; Armstrong III, J.D., Eng, K.K., Keller, J.K., Purick, R.M., Hartner Jr., W.F., Choi, W.-B., Askin, D. and Volante, R.P. (1994) Tetrahedron Lett. 35, 3239–3242.
21 Klutcho, S., Hamby, J. M. and Hodges, J. C. (1994) Bioorg. Med. Chem. Lett. 4, 57–62.
22 Goulet, J. L., Kinneary, J. F., Durette, P. L., Stein, R. L., Harrison, R. K., Izquierdo-Martin, M., Kuo, D. W., Lin, T.-Y. and Hagmann, W. K. (1994) Bioorg. Med. Chem. Lett.

4, 1221–1224; Baylis, E. K., Campbell, C. D. and Dingwall, J. G. (1994) J. Chem. Soc., Perkin Trans. 1, 2845–2853.
23 Ebert, B., Lenz, S., Brehm, L., Bregnedal, P., Hansen, J. J., Frederiksen, K., Bøgesø, K. P. and Krogsgaard-Larsen, P. (1994) J. Med. Chem. 37, 878–884.
24 Gualtieri, F., Conti, G., Dei, S., Giovannoni, M. P., Nannucci, F., Romanelli, M. N., Scapecchi, S., Teodori, E., Fanfasi, L., Ghelardini, C., Giotti, A. and Bartolini, A. (1994) J. Med. Chem. 37, 1704–1711.
25 Achard, D., Truchon, A. and Peyronel, J. F. (1994) Bioorg. Med. Chem. Lett. 4, 669–672.
26 Harrison, T., Williams, B. J., Swain, C. J. and Ball, R. G. (1994) Bioorg. Med. Chem. Lett. 4, 2545–2550.
27 Minor, D. L., Wyrick, S. D., Charifson, P. S., Watts, V. J., Nichols, D. E. and Mailman, R. B. (1994) J. Med. Chem. 37, 4317–4328.
28 Meltzer, P. C., Liang, A. Y. and Madras, B. K. (1994) J. Med. Chem. 37, 2001–2010.
29 Santaniello, E., Ferraboschi, P., Grisenti, P. and Manzocchi, A. (1992) Chem. Rev. 92, 1071–1140.
30 Wong, C.-H. and Whitesides, G.M. (1994) Enzymes in Synthetic Organic Chemistry, Elsevier, Oxford.
31 Roberts, S.M., Wiggins, K., Casy, G. and Phythian, S. (1992 and supplements, 1993–1995) Preparative Biotransformations, John Wiley, Chichester.
32 Oumoch, S. and Rousseau, G. (1994) Bioorg. Med. Chem. Lett. 4, 2841–2844.
33 Galeazzi, E., Guzman, A., Kluge, A., Morgans Jr., D., Muchowski, J., Jamil-Panah, S., Saavedra, P., Salazar, M.A., Talamas, F.X., Teitelbaum, P., Young, J. and Yamamoto, D. (1994) Bioorg. Med. Chem. Lett. 4, 1437–1442.
34 McCarthy, P.A., Hamanaka, E.S., Marzetta, C.A., Bamberger, M.J., Gaynor, B.J., Chang, G., Kelly, S.E., Inskeep, P.B., Mayne, J.T., Beyer, T.A., Walker, F.J., Goldberg, D.I., Savoy, Y.E., Davis, K.M., Diaz, C.L., Freeman, A.M., Johnson, D.A., LaCour, T.G., Long, C.A., Maloney, M.E., Martingano, R.J., Pettini, J.L., Sand, T.M. and Wint, L.T. (1994) J. Med. Chem. 37, 1252–1255.
35 Ref. 30, pp. 42–43.
36 Hagiwara, D., Miyake, H., Igari, N., Karino, M., Maeda, Y., Fujii, T. and Matsua, M. (1994) J. Med. Chem. 37, 2090–2099.
37 Yamazaki, Y., Ebike, H. and Achiwa, K. (1994) Chem. Pharm. Bull. 42, 1968–1970
38 Ref. 12, pp. 249–264.
39 Subramanian, G., ed. (1994) A Practical Approach to Chiral Separations by Liquid Chromatography, VCH, Weinheim.
40 Francotte, E. (1994) J. Chromatogr. A 666, 565–601.
41 Levin, J.I., Chan, P.S., Coupet, J., Thibault, L., Venkatesan, A.M., Bailey, T.K., Vice, G., Cobuzzi, A., Lai, F. and Mellish, N. (1994) Bioorg. Med. Chem. Lett. 4, 1709–1714.
42 Burnett, D. A., Caplen, M. A., Davis Jr., H.R., Burrier, R.E. and Clader, J.W. (1994) J. Med. Chem. 37, 1733–1742.
43 Yan, G., Yin, D., Khanolkar, A.D., Compton, D.R., Martin, B.R. and Makriyannis, A. (1994) J. Med. Chem. 37, 2619–2622.
44 Rodriguez, J.B., Marquez, V.E., Nicklaus, M.C., Mitsuya, H. and Barchi Jr., J.J. (1994) J. Med. Chem. 37, 3389–3399.
45 Erhard, K.F., Yen, H.-K., Yamashita, D.S., Brandt, M., Mohammed, H., Levy, M.A. and Holt, D.A. (1994) Bioorg. Med. Chem. Lett. 4, 1365–1368.
46 Elliot, J.D., Lago, M.A., Cousins, R.D., Gao, A., Leber, J.D., Erhard, K.F., Nambi, P., Elshourbagy, N.A., Kumar, C., Lee, J.A., Bean, J.W., DeBrosse, C.W., Eggleston, D.S., Brooks, D.P., Feuerstein, G., Ruffulo Jr., R.R., Weinstock, J., Gleason, J.G., Peishoff, C.E. and Ohlstein, E.H. (1994) J. Med. Chem. 37, 1553–1557.

47 Showell, G.A., Bourrain, S., Neduvelil, J.G., Fletcher, S.R., Baker, R., Watt, A.P., Fletcher, A.E., Freedman, S.B., Kemp, J.A., Marshall, G.R., Patel, S., Smith, A.J. and Matassa, V.G. (1994) J. Med. Chem. 37, 719–721.
48 Jimonet, P., Audiau, F., Aloup, J.-C., Barreau, M., Blanchard, J.-C., Bohme, A., Boireau, A., Cheve, M., Damour, D., Doble, A., Lavayre, J., Dutruc-Rosset, G., Randle, J.C.R., Rataud, J., Stutzmann, J.-M. and Mignani, S. (1994) Bioorg. Med. Chem. Lett. 4, 2735–2740.
49 Martin, L.L., Lee, G.E., Lee, T.B.K., Rush, D.K., Wilmot, C.A., Paulus, E., Elben, U., Grome, J.J., Porsche-Wiebking, E., Rudolph, K.A. and Stoll, P. (1994) J. Med. Chem. 37, 3008–3015.
50 Evans, D.A. (1982) Aldrichim. Acta 15, 23–32 and references cited therein.
51 Oppolzer, W. (1990) in Chirality in Drug Design and Synthesis (ed. Brown, C.), chapter 11, Academic Press, London and references cited therein.
52 Evans, D.A., Bartroli, J. and Shih, T. (1981) J. Am. Chem. Soc. 103, 2127–2129; Oppolzer, W. (1988) Pure. Appl. Chem. 60, 39–48.
53 Evans, D.A., Morrissey, M.M. and Dorow, R.L. (1985) J. Am. Chem. Soc. 107, 4346–4348; Oppolzer, W. and Tamura, O. (1990) Tetrahedron Lett. 31, 991–994.
54 Jacobs, R.T., Bernstein, P.R., Cronk, L.A., Vacek, E.P., Newcomb., L.F., Aharony, D., Buckner, C.K. and Kusner, E.J. (1994) J. Med. Chem. 37, 1282–1297.
55 Trova, M.P., Wissner, A., Casscles Jr., W.T. and Hsu, G.C. (1994) Bioorg. Med. Chem. Lett. 4, 903–906.
56 Josien, H., Lavielle, S., Brunissen, A., Saffroy, M., Torrens, Y., Beaujouan, J.-C., Glowinski, J. and Chassaing, G. (1994) J. Med. Chem. 37, 1586–1601.
57 Williams, R.M. (1989) Synthesis of Optically Active α-Amino Acids, chapter 1, Pergamon, Oxford and references cited therein.
58 Papageorgiou, C., Florineth, A. and Mikol V. (1994) J. Med. Chem. 37, 3674–3676.
59 Li, L., Thomas, S.A., Klein, L.L., Yeung, C.M., Maring, C.J., Grampovnik, D.J., Lartey, P.A. and Plattner, J.J. (1994) J. Med. Chem. 37, 2655–2663.
60 Ojima, I. (1993) in The Organic Chemistry of β-Lactams (Georg., G.I., ed.), pp. 231–242, VCH, New York.
61 Georg, G.I., Harriman, G.C.B., Hepperle, M. and Himes, R.H. (1994) Bioorg. Med. Chem. Lett. 4, 1381–1384.
62 Eda, M., Takemoto, T., Ono, S., Okada, T., Kosaka, K., Gohda, M., Matzno, S., Nakamura, N. and Fukaya, C. (1994) J. Med. Chem. 37, 1983–1990.
63 Poll, T., Abdel Hardy, A.F., Karge. R., Linz, G., Weetman, J. and Helmchen, G. (1989) Tetrahedron Lett. 30, 5595–5598.
64 Leonard, J. (1994) Contemp. Org. Synth. 1, 387–415.
65 Iqbal, N., Vo, D., McEwen, C.-A., Wolowyk, M.W. and Knaus, E.E. (1994) Chirality 6, 5151–520.
66 Djuric, S.W., Docter, S.H., Yu, S.S., Spangler, D., Tsai, B.-S., Anglin, C.P., Gaginella, T.S., Kachur, J.F., Keith, R.H., Maziasz, T.J., Villani-Price, D., Rao, T.S., Walsh, R.E., Widomski, D.L. and Fretland, D.J. (1994) Bioorg. Med. Chem. Lett. 4, 811–816.
67 Enders, D., Kipphardt, H. and Fey., P. (1987) Org. Synth. 65, 192–202.
68 De Montarby, L., Mosset, P., Grée, R., Millet, J., Sepulchre, C. and Bellamy, F. (1994) Bioorg. Med. Chem. Lett. 4, 261–266.
69 De Montarby, L., Tourbah, H. and Grée, R. (1989) Bull. Soc. Chim. Fr. 419–432.
70 Lowe III, J., Hageman, D.L., Drozda, S.E., McLean, S., Bryce, D.K., Crawford, R.T., Zorn, S., Morrone, J. and Bordner, J. (1994) J. Med. Chem. 37, 3789–3811.
71 Davis, P.D., Hallam., T.J., Harris, W., Hill, C.H., Lawton, G., Nixon, J.S., Smith, J.L., Vesey, D.R. and Wilkinson, S. (1994) Bioorg. Med. Chem. Lett. 4, 1303–1308.

72 Mallamo, J.P., Earley, W.G., Kumar, V., Subramanyam, Dority Jr., J.A., Miller, M.S., DeHaven-Hudkins, D.L., Ault, B., Herrmann Jr., J.L., Dung J.-S., McMullen, L.A., Jaeger, E., Kullnig, R. and Magee, L. J. (1994) J. Med. Chem. 37, 4438–4448.
73 Jones, J.H. (1991) The Chemical Synthesis of Peptides, Oxford University Press, Oxford.
74 Hoveyda, A.H., Evans, D.A. and Fu, G.C. (1993) Chem. Rev. 93. 1307–1370.
75 Crosby, J. (1991) Tetrahedron 47, 4789–4846.
76 Bittman, R., Byun H.-S., Mercier, B. and Salari, H. (1994) J. Med. Chem. 37, 425–430; Morie, T., Kato, S., Harada, H., Yoshida, N. and Matsumoto, J. (1994) Chem. Pharm. Bull. 42, 877–882.
77 Naylor, A., Judd, D.B., Scopes, D.I.C., Hayes, A.G. and Birch, P.J. (1994) J. Med. Chem. 37, 2138–2144; Ghosh, A.K., Thompson, W.J., Fitzgerald, P.M.D., Culberson, J.C., Axel, M.G., McKee, S.P., Huff, J.R. and Anderson, P.S. (1994) J. Med. Chem. 37, 2506–2508.
78 Coppola, G.M. and Schuster, H.F. (1987) Asymmetric Synthesis: Construction of Chiral Molecules using Amino Acids, John Wiley, New York.
79 Sheih, W.-C. and Carlson, J.A. (1992) J. Org. Chem. 57, 379–381.
80 De Lombaert, S., Erion, M.D., Tan, J., Blanchard, L., El-Chehabi, L., Ghai, R.D., Sakane, C.B. and Trapani, A.J. (1994) J. Med. Chem. 37, 498–511.
81 Ksander, G.M., Erion, M., Yuan, A.M., Diefenbacher, C.G., El-Chehabi, L., Cote, D. and Levens, N. (1994) J. Med. Chem. 37, 1823–1832.
82 Baldwin, J.E., North, M., Flinn, A. and Moloney, M.G. (1988) J. Chem. Soc., Chem. Commun. 828–829.
83 Bhide, R.S., Patel, D.V., Patel, M.M., Robinson, S.P., Huniham, L.W. and Gordon, E.M. (1994) Bioorg. Med. Chem. Lett. 4, 2107–2112.
84 Rohl, J.A., Simpkins, L.M., Stevenson, J., Sun, C.-Q., Murugesan, N., Barrish, J.C., Asaad, M.M., Bird, J.E., Schaeffer, T.R., Trippodo, N.C., Petrillo, N.C. and Karanewsky, D.S. (1994) Bioorg. Med. Chem. Lett. 4, 1789–1794.
85 Ishikawa, H., Tsubouchi, H. and Yasumura, K. (1994) Bioorg. Med. Chem. Lett. 4, 1147–1152; Tsubouchi, H., Tsuji, K., Yasumura, K., Tada, N., Nishitani, S., Minamikawa, J. and Ishikawa, H. (1994) Tetrahedron: Asymmetry 5, 441–452.
86 Wallbaum, S., Mehler, T. and Martens, J. (1994) Synth. Commun. 24, 1381–1387.
87 Venkatraman, S., Roon, R.J., Schulte, M.K., Koerner, J.F. and Johnson, R.L. (1994) J. Med. Chem. 37, 3939–3946.
88 Dormoy, J.-R. and Castro B. (1986) Synthesis 81–82.
89 Steinberg, M.I., Palkowitz, A.D., Thrasher, K.J., Reel, J.K., Zimmerman, K.M., Whitesitt, C.A., Simon, R.L., Hauser, K.L., Lifer, S.L., Pfeifer, W., Takeuchi, K., Wiest, S.A., Vasudevan, V., Bemis, K.G., Deeter, J.B., Barnett, C.J., Wilson, T.M., Marshall, W.S. and Boyd, D.B. (1994) Bioorg. Med. Chem. Lett. 4, 51–56.
90 For a review with experimental procedures, see Lichtenthaler, F.W. (1992) in Modern Synthetic Methods 1992 (Scheffold, R., ed.) pp. 273–376, VCH, Weinheim. For recent progress in the field, see (1994) Tetrahedron: Asymmetry 5, issues 11 and 12.
91 Hanessian, S. (1983) Total Synthesis of Natural Products: The Chiron Approach, Pergamon, Oxford.
92 Chamberlain, S.D., Biron, K.K., Dornsife, R.E., Averett, D.R., Beauchamp, L. and Koszalka, G.W. (1994) J. Med. Chem. 37, 1371–1377.
93 Lee, J., Marquez, V.E., Lewin, N.E. and Blumberg, P.M. (1994) Bioorg. Med. Chem. Lett. 4, 543–548.
94 Falck, J.R., Chauhan, K., Rosolowsky, M. and Campbell, W.B. (1994) Bioorg. Med. Chem. Lett. 4, 2113–2116.
95 Dax, K. and Weidmann, H. (1976) Adv. Carbohydr. Chem. Biochem. 33, 189–234.

96 Randad, R.S., Naik, R.H., Gulnik, S.V., Yu, B. and Erickson, J.W. (1994) Bioorg. Med. Chem. Lett. 4, 2343–2346.
97 Kim, K.-I., Lill-Elghanian, D.A. and Hollingsworth, R.I. (1994) Bioorg. Med. Chem. Lett. 4, 1691–1696.
98 Noyori, R. (1994) Asymmetric Catalysis in Organic Synthesis, chapter 2, Wiley, New York.
99 Singh, V.K. (1992) Synthesis, 605–617; Wallbaum, S. and Martens, J. (1992) Tetrahedron: Asymmetry 3, 1475–1504.
100 Johnson, R.A. and Sharpless, K.B., Jacobson, E.N. (1993) in Catalytic Asymmetric Synthesis (Ojima, I., ed.), chapters 4.1 and 4.2, VCH, New York.
101 Kolb, H.C., VanNieuwenhze, M.S. and Sharpless, K.B. (1994) Chem. Rev. 94, 2483–2547.
102 Brunner, H. and Zettlmeier, W. (1993), Handbook of Enantioselective Catalysis, Volumes 1 and 2, VCH, Weinheim.
103 Sheldon, R.A. (1993) Chirotechnology: Industrial Synthesis of Optically Active Compounds, pp. 182–183, Marcel Dekker, New York.
104 Mohamadi, F., Spees, M.M., Staten, G.S., Marder, P., Kipka, J.K., Jonhson, D.A., Boger, D.L. and Zarrinmayeh, H. (1994) J. Med. Chem. 37, 232–239.
105 Katsuki, T. and Sharpless, K.B. (1980) J. Am. Chem. Soc. 102, 5974–5976.
106 Gao, Y., Hanson, R.M., Klunder, J.M., Ko, S.Y., Masamune, H. and Sharpless, K.B. (1987) J. Am. Chem. Soc. 109, 5765–5780.
107 Shutalev, A.D., Mikerin, I.E., Arshava, B.M., Nikitenko, A.A., Raifield, Y.E., Vid, G.Y., Lee, V.J., Gurskaya, G.V., Viktorova, L.S., Jas'ko, M.V., Semizarov, D.G. and Zavodnik, V.E. (1994) Bioorg. Med. Chem. Lett. 4, 761–768.
108 Raifeld, Y.E., Vid, G.Y., Mikerin, I.E., Arshava, B.M. and Nikitenko, A.A. (1992) Carbohydr. Res. 224, 103–109.
109 Ghosh, A.K., Lee, H.Y., Thompson, W.J., Culberson, C., Holloway, M.K., McKee, S.P., Munson, P.M., Duong, T.T., Smith, A.M., Darke, P.L., Zugay, J.A., Emini, E.A., Schleif, W.A., Huff, J.R. and Anderson, P.S. (1994) J. Med. Chem. 37, 1177–1188.
110 Saksena, A.K., Girijavallabhan, V.M., Lovey, R.G., Pike, R.E., Desai, J.A., Ganguly, A.K., Hare, R.S., Loebenberg, D., Cacciapuoti, A. and Parmegiani, R.M. (1994) Bioorg. Med. Chem. Lett. 4, 2023–2028.
111 Lovey, R.G., Saksena, A.K. and Girijavallabhan, V.M. (1994) Tetrahedron Lett. 35, 6047–6050.
112 Sheldon, R.A. (1993) Chirotechnology: Industrial Synthesis of Optically Active Compounds, Marcel Dekker, New York.
113 Collins, A.N., Sheldrake, G.N. and Crosby, J. (eds.) (1992) Chirality in Industry: The Commercial Manufacture and Applications of Optically Active Compounds, John Wiley, Chichester.
114 Giordano, C., Cavicchioli, S., Levi, S. and Villa, M. (1991), J. Org. Chem. 56, 6114–6118.
115 Kamphuis, J., Boesten, W.H.J., Kaptein, B., Hermes, H.F.M., Sonke, T., Broxterman, Q.B., van den Tweel, W.J.J. and Schoemaker, H.E. Ref. 113, chapter 8.
116 Furuhashi, K. Ref. 113, chapter 7.
117 Wakamatsu, H. (1968) Food Eng. 92–95; (1969) Chem. Abstr. 70, 88226.
118 Amiard, G. (1959) Experientia 15, 1–7.
119 Nedbal, J., Hert, C.J. and Spacek, M. (1982) Czech Patent CS208915; (1983) Chem. Abstr. 98, 197759.
120 Barton, D.H.R. and Kirby, G.W. (1962) J. Chem. Soc. 806–817; Shieh, W.-C. and Carlson, J.A. (1994) J. Org. Chem. 59, 5463–5465.
121 Shiraiwa, T., Shinjo, K. and Kurokawa, H. (1989) Chem. Lett. 1313–4.

122 Reider P.J., Davis, P., Hughes, L.D. and Grabowski, E.J.S. (1987) J. Org. Chem. 52, 955–957
123 Inoue, H., Takeo, S., Kawazu, M. and Kugita, H. (1973) Yakugaku Zasshi 93, 729–732.
124 Dodds, D.R., Lopez, J.L., Zepp, C.M., Brandt, S. and Rossi, R.F. (1990) PCT Int. Pat 9004643; (1990) Chem Abstr, 113, 76620.
125 Schwartz, A., Madan, P.B., Mohacsi, E., O'Brien, J.P., Todaro, L.J. and Coffen, D.L. (1992) J. Org. Chem. 57, 851–856.
126 Sawayama, T., Tsukamoto, M., Sasagawa, T., Naruto, S., Matsumoto, J. and Uno, H. (1989) Chem. Pharm. Bull. 37, 1382.
127 Ohashi, T and Hasegawa, J. (1992) Ref. 113, pp. 249–267 and 269–278.
128 Wyvratt, M.J., Tristram, E.W., Ikeler, T.J., Lohr, N.S., Joshua, H., Springer, J.P., Arison, B.H. and Patchett, A.A. (1984) J. Org. Chem. 49, 2818–2819.
129 Blacklock, T.J., Snuman, R.F., Butcher, J.W., Budavari, J. and Grenda, V.J. (1988) J. Org. Chem. 53, 836–844,
130 Takahashi, S., Inoue, K., Yanagida, Y., Ohashi, T. and Watanabe, K. (1987) Eur. Patent 215335; (1988) Chem. Abstr. 108, 22288.
131 Uriach, J. and y Cia, S.A. (1989) Spanish Patent 2007679; (1991) Chem. Abstr. 114, 207834.
132 Blaser, H.U. (1991) Tetrahedron: Asymmetry 2, 843–866.
133 Castellet, I.L. and Mir, M.O. (1986) Spanish Pat. 538784; (1987) Chem. Abstr. 106, 50652.
134 Arnold, R.A. and Matthews, G.J. (1978) German Patent 2805488; (1979) Chem. Abstr. 90, 6106.
135 Felder, E., Pitre, D. and Zutter, H. (1980) U.K. Patent 2025968; (1980) Chem. Abstr. 93, 132285.
136 Gu, Q.M., Chen, C.S. and Sih, C.J. (1986) Tetrahedron Lett. 27, 1763.
137 Layh, N., Stolz, A., Bohme, J., Effenberger, F. and Knackmuss, H.J. (1993) J. Biotechnol. 33, 175–182.
138 Ohta, T., Takaya, H., Kitamura, M., Nagai, K. and Noyori, R. (1987) J. Org. Chem. 52, 3176–3178.
139 Alper, H. and Hamel, N. (1990) J. Am. Chem. Soc. 112, 2803–2804.
140 Giordano, C., Villa, M. and Panossian, S. (1992) Ref. 113, pp. 303–312.
141 Sheldon, R.A. (1990) Chem. Ind. 212–219.
142 Chibata, I., Yamada, S., Hongo, C. and Yoshioka, R (1982) Eur. Patent 70114; (1983) Chem. Abstr. 99, 6039.
143 Takahashi, S. (1986) Prog. Ind. Microbiol. 24, 269–279.
144 Olivieri, R., Fascetti, E., Angelini, L. and Degen, L. (1985) Biotechnol. Bioeng. 23, 2173.
145 Knowles, W.S., Sabacky, M.J., Vineyard, B.D. and Winekauf, D.J. (1975) J. Am. Chem. Soc. 97, 2567.
146 Kagan, H.B. and Dang, T.P. (1968) J. Chem. Soc., Chem. Commun. 1445.
147 Selke, R. and Pracejus, H. (1986) J. Mol. Catal. 37, 213–225.
148 Chibata, I., Tosa, T., Sato. T. and Mori, T. (1976) Methods Enzymol. 44, 756–759.
149 Chenault, H.K., Dahmer, J. and Whitesides, G.M. (1989) J. Am. Chem. Soc. 111, 6354–6364.
150 Fauchere, J.-L. and Petermann, C. (1980) Helv. Chim. Acta 63, 824–831.
151 Drauz, K., Bommarius, A., Knaup, G., Kottenhahn, M. and Schwarm, M. (1994) Presented at Chiral-Europe 94 Symposium (Spring Innovations Ltd) Nice, France.
152 Turner, N.J., Winterman, J.R., McCague, R., Parratt, J.S. and Taylor, S.J.C. (1995) Tetrahedron Lett. 36, 1113–1116.
153 Ohashi, T. and Hasegawa, J. (1987) J. Synth. Org. Chem. Jpn. 45, 331–345.

154 Kitamura, M., Ohkuma, T., Tokunaga, M. and Noyori, R. (1990) Tetrahedron Asymmetry 1, 1–4; Akutagawa, S. (1992) Ref. 113, pp. 325–339.
155 Hanson, R.M. (1991) Chem. Rev. 91, 437–475.
156 Ladner, W.E. and Whitesides, G.M. (1984), J. Am. Chem. Soc. 106, 7250–7251.
157 Klunder, J.M., Onami, T. and Sharpless, K.B. (1989) J. Org. Chem. 54, 1295–1304.
158 Ikbal, M., Cerceau, C., Le Goffic, F. and Sicsic, S. (1989) Eur. J. Med. Chem. 24, 415.
159 Taylor, S.J.C., McCague, R., Wisdom, R., Lee, C., Dickson, K., Ruecroft, G.R., O'Brien, F., Littlechild, J., Bevan, J., Roberts S.M. and Evans, C.T. (1993) Tetrahedron Asymmetry 4, 1117–1128.
160 MacKeith, R.A., McCague, R., Olivo, H.F., Roberts, S.M., Taylor, S.J.C. and Xiong, H. (1994) Bioorg. Med. Chem. 2, 387–394.
161 Prous, J., Mealy, N. and Castaner, J. (1994) Drugs Future 19, 1003–1010.
162 Schmid, R. and Broger, E.A. (1994) Presented at Chiral-Europe 94 Symposium (Spring Innovations Ltd) Nice, France.
163 Izumi, Y (1983) Adv. Catal. 32, 215.
164 Gattuso, M., McCulloch, B. and Priegnitz, J.W. (1994) Presented at Chiral-Europe 94 Symposium (Spring Innovations Ltd) Nice, France.
165 Toda, F. (1987) Top. Curr. Chem. 140, 43.

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