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Mid-size Drugs Based on Peptides and Peptidomimetics

A New Drug Category

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ISSN 1864-8118 ISSN 1864-8126 (electronic)
SpringerBriefs in Pharmaceutical Science & Drug Development
ISBN 978-981-10-7690-9 ISBN 978-981-10-7691-6 (eBook)
https://doi.org/10.1007/978-981-10-7691-6

Library of Congress Control Number: 2017962023

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

This book describes studies conducted by the authors on mid-size drugs utilizing peptides and peptidomimetics, and on the development of anti-HIV agents. Peptides are important biological molecules having various physiological actions. Peptide-based drug discovery may help bring about the development of useful medicines that are highly safe and show potent pharmacological effects in small doses. Recently, it has been shown that there is an important space in the drugs market for drugs generated from middle sized molecular weight compounds, between low–molecular weight and high–molecular weight compounds. Thus, middle sized molecules, which are designated as mid-size drugs, including peptide compounds, which might have all the abovementioned advantages but fewer drawbacks associated with small and large drugs, are being focused on. To date, several peptidomimetics that mimic the primary, secondary, and tertiary structures of peptides have been developed to maintain and improve biological activities and the actions of peptides. In this book we consider the research on middle sized drugs in more detail, featuring peptide mimetics directed to high biological activity and structural stability, peptide derivatives with inhibitory activity, and middle sized molecules targeting protein–protein interactions. In addition, the merits of utilizing peptidomimetics in the development of mid-size drugs are referred to. Understanding such peptide-derived, mid-size drugs will lead to a comprehensive expansion of medicinal chemistry.

Tokyo, Japan — Hirokazu Tamamura

Acknowledgements

This work was supported in part by the "Research Program on HIV/AIDS: 17fk0410206h0302, 17fk0410102h0002, and 17fk0410204h0702" from the Japan Agency for Medical Research and Development (AMED); a "Grant-in-Aid for Scientific Research" from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; and a "Health and Labor Sciences Research Grant" from the Japanese Ministry of Health, Labor, and Welfare. It was also supported in part by the Japan Society for the Promotion of Science (JSPS) Core-to-Core Program, and the Platform Project for Supporting Drug Discovery and Life Science Research funded by AMED, Japan. T.K. was supported by JSPS Research Fellowships for Young Scientists (15J04754).

The authors wish to acknowledge their collaborators: Drs. Nobutaka Fujii (Kyoto University), Naoki Yamamoto (National University of Singapore), Tsutomu Murakami (National Institute of Infectious Diseases), Shuzo Matsushita (Kumamoto University), Kazuhisa Yoshimura (National Institute of Infectious Diseases), Shigeyoshi Harada (National Institute of Infectious Diseases), Hironori Sato (National Institute of Infectious Diseases), Hideki Nakashima (St. Marianna University), Michinori Waki (Kyushu University), Akira Otaka (The University of Tokushima), Hiroaki Mitsuya (Kumamoto University), Toshio Hattori (Tohoku University), John O. Trent (University of Louisville), Stephen C. Peiper (Thomas Jefferson University), Zixuan Wang (Thomas Jefferson University), Huangui Xiong (University of Nebraska Medical Center), Shuichi Kusano (St. Marianna University), Shigemi Terakubo (St. Marianna University), Shinya Oishi (Kyoto University), Kenichi Hiramatsu (Kyoto University), Satoshi Ueda (Kyoto University), Motoyoshi Nomizu (Tokyo University of Pharmacy and Life Sciences), Kentaro Hozumi (Tokyo University of Pharmacy and Life Sciences), Jun Komano (National Institute of Infectious Diseases), Kenji Ohba (National University of Singapore), Emiko Urano (National Institute of Infectious Diseases), Masayuki Fujino (National Institute of Infectious Diseases), Kasthuraiah Maddali (National Cancer Institute), Yves Pommier (National Cancer Institute), John A. Beutler (National Cancer Institute), Aikichi Iwamoto (The University of Tokyo),

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Dr. Tamamura won the Award for Distinguished Young Scientists from the Japanese Peptide Society in 1998 and the Award for Young Scientists from the Pharmaceutical Society of Japan in 2001.

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Abbreviations

aq.	Aqua
ADI	Alkene dipeptide isostere
Ac	Acetyl
Abu(2)	L-α-aminobutyric acid
Ab	Monoclonal antibody
AIDS	Acquired immune deficiency syndrome
AML	Acute myeloid leukemia
Bn	Benzyl
Boc	*Tert*-butoxycarbonyl
Bu	Butyl
Bus	*N-tert*-butylsulfonyl
*m*CPBA	3-chloroperbenzoic acid
CADI	Chloroalkene dipeptide isostere
CF_3-ADI	Trifluoromethyl alkene dipeptide isostere
CXCR4	CXC-chemokine receptor type 4
Clt	2-chlorotrityl
CHR	*C*-terminal heptad repeat
CC_{50}	50% cytotoxic concentration
CA	Capsid
dr	Diastereomeric ratio
DIBAL	Diisobutylaluminum hydride
DFT	Density functional theory
DIPCI	*N*,*N*'-diisopropylcarbodiimide
DMF	*N*,*N*-dimethylformamide
DIPEA	*N*,*N*-diisopropylethylamine
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
Dpa	Dipicolylamine
DKA	Diketoacid
Et	Ethyl
EADI	(*E*)-alkene dipeptide isostere

EDC	1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide
EC_{50}	50% effective concentration
Fmoc	9-fluorenylmethyloxycarbonyl
FADI	Fluoroalkene dipeptide isostere
FDA	The Food and Drug Administration
G-CSF	Granulocyte colony stimulating factor
GPCR	G protein-coupled receptor
h	Hour
HAART	Highly active anti-retroviral therapy
HAS	Human serum albumin
HATU	1-[bis(dimethylamino)methyliumyl]-1*H*-1,2,3-triazolo[4,5-b] pyridine-3-oxide hexafluorophosphate
HDF	Human dermal fibroblast
HIV-1	Human immunodeficiency virus-1
HMPA	Hexamethylphosphoramide
HOBt	1-hydroxy-1*H*-benzotriazole
HOAt	1-hydroxy-7-azabenzotriazole
HPLC	High-performance liquid chromatography
HR	Heptad repeat
^{i}Pr	Isopropyl
^{i}Bu	Isobutyl
IC_{50}	50% inhibitory concentration
IN	Integrase
LiHMDS	Lithium Bis(trimethylsilyl)amide
MeCN	Acetonitrile
Me	Methyl
min	Minute
Ms	Mesyl
M	$mol \cdot L^{-1}$
MDR	Multi-drug resistant
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MA	Matrix
NMR	Nuclear magnetic resonance
^{n}Bu	*Normal*-butyl
Nle	L-norleucine
Ns	2-nitrobenzenesulfonyl
Nva	5-hydroxy-L-norvaline
NCS	*N*-chlorosuccinimide
NHR	*N*-terminal heptad repeat
Nal	L-3-(2-naphthyl)alanine
NIR	Near infrared
Orn	L-ornithine
Ph	Phenyl
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PEM	Protein epitope mimetic

PM	Plasma membrane
rt	Room temperature
R5-HIV-1	Macrophage-tropic HIV-1
sat.	Saturated
SET	Single electron transfer
SPPS	Solid-phase peptide synthesis
SDF	Stromal cell-derived factor
SAR	Structure–activity relationship
THF	Tetrahydrofuran
TBS	*Tert*-butyldimethylsilyl
^{t}Bu	*Tert*-butyl
TMS	Trimethylsilyl
TFA	Trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TADI	(*E*)-tetrasubstituted alkene dipeptide isostere
Xaa	Amino acid
X4-HIV-1	T cell line-tropic HIV-1
Yaa	Amino acid

Chapter 1
Introduction to Mid-size Drugs and Peptidomimetics

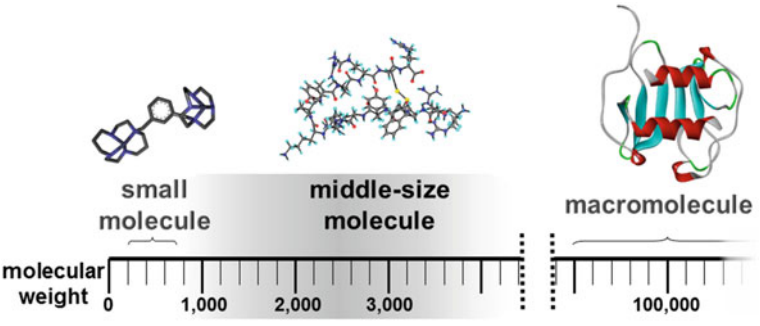
Abstract In the middle-size region, between small and macromolecules, there is an indispensable drug-like chemical space. These drugs with middle-size molecules are designated as mid-size drugs, which have the advantages that small and macromolecules possess and reduce the drawbacks. Peptide and peptide derivatives are mainly located in the above-mentioned middle-size region, and target not only the active centers of enzymes and pockets of receptors but also the protein–protein interactions because the mid-size drugs can cover target molecules broadly. Thus, the mid-size drugs might be expected as the next generation drugs. However, in therapeutical use of peptides, there is some limitation that involves several factors: low metabolic stability toward proteolysis, undesired activity resulting from interactions with several receptors, etc. Therefore, modifications of their structures for maintenance of biological activity have been considered and tried to develop peptidomimetics. Generally, peptidomimetics point peptide bond isosteres that mimic primary structures of peptides, such as transition-state mimics and ground-state mimics. As ground-state mimics, we have focused on the development of several alkene-type dipeptide isosteres (ADIs) such as chloroalkene dipeptide isosteres (CADIs). In the broad sense, peptidomimetics include mimetics of secondary and tertiary structures of peptides, which are useful for the development of inhibitors of protein–protein interactions.

Keywords Mid-size drug · Protein–protein interaction · Peptidomimetic
Ground-state mimic · Chloroalkene dipeptide isostere

1.1 Introduction to Mid-size Drugs

According to the progress of organic synthetic chemistry, low-molecular-weight drugs based on small organic compounds have been conventionally developed against several diseases. Small molecules have some advantages such as oral availability and low immunogenicity but some drawbacks such as low specificity and high possibility of side effects. Molecular target drugs, which target only

H. Tamamura et al., *Mid-size Drugs Based on Peptides and Peptidomimetics*,
SpringerBriefs in Pharmaceutical Science & Drug Development,
https://doi.org/10.1007/978-981-10-7691-6_1



	adaptability	cell permeability	antigenicity	specificity	side effect
small molecule	○	○	○ (low)	△	△
macro-molecule	×	×	× (high)	○	○ (a few)

Fig. 1.1 Advantages and drawbacks of small and macromolecules as drugs and location of middle-size molecules (mid-size drugs)

specific molecules relevant to corresponding diseases and inhibit their functions, have been in the spotlight, and high-molecular-weight biologics including human monoclonal antibodies have been developed in recent medicinal chemistry based on molecular targets. Macromolecules have some advantages such as high specificity and low possibility of side effects but some drawbacks such as low applicability and possible immunogenicity. Therefore, both low- and high-molecular-weight drugs have their advantages and drawbacks. Furthermore, there is an important drug-like chemical space in the mid-size region between low and high molecular weight (Fig. 1.1). Middle-size molecules, which are designated as mid-size drugs, might keep the above advantages and reduce the drawbacks involved by small and macromolecules. Peptide compounds, which are located in this middle-size region, are being focused on for the development of next generation drugs. Several peptidomimetics directed to high biological activity and structural stability, and peptide derivatives targeting protein–protein interactions are included in mid-size drugs.

1.2 Introduction to Peptidomimetics

During the last quarter-century, various biologically active peptides have been discovered and characterized. These bioactive peptides influence and control physiological functions through the interaction with their various receptors, and the number of natural and modified peptides that are used as therapeutics is still increasing. Many bioactive peptides have been developed and have led to the discovery of novel therapies.

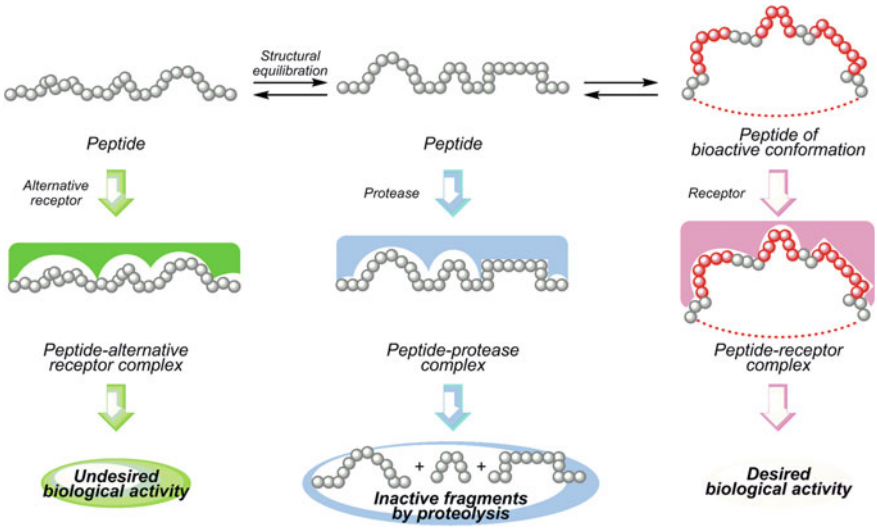


Fig. 1.2 The different conformations of peptides and their interactions

However, use of peptides as therapeutics is limited by several factors, which include low metabolic stability toward proteolysis and undesired activity resulting from interactions of flexible peptides with several receptors (Fig. 1.2) (Veber and Freidinger 1985; Giannis et al. 1993). In particular, flexibility is inherent in polypeptides and allows adoption of multiple conformations. The flexibility of each residue in a peptide results from two degrees of conformational freedom by N–C_α, C_α–CO, N–CO and C_α–R rotational bonds, which are described by φ, ψ, ω, and χ dihedral angles, respectively (Fig. 1.3). Despite these problems, medicinal research into the discovery of peptide-lead drugs has developed novel modifications of their structures for the maintenance of biological activity. The development of "*peptidomimetics*" (Gante 1994; Adessi and Soto 2002) matches these requirements. In this approach, peptides are considered as tools for the discovery of other classes of novel compounds. Peptidomimetics point peptide isosteres, which mimic primary structures of peptides, but in the broad sense, include mimetics that mimic secondary and tertiary structures of peptides, which are useful for the development of inhibitors of protein–protein interactions and described in Chaps. 4 and 5.

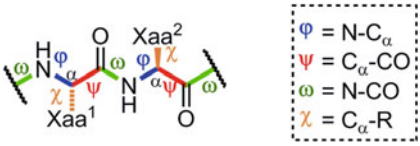


Fig. 1.3 Dihedral angles that are correlated to conformational flexibility in peptides

1.3 Peptide Isosteres in Medicinal Chemistry

1.3.1 History of Isosteres in Peptidomimetics

The term of "isosterism" for compounds was designated in 1919 by Langmuir, who pioneered studies on isosteres by investigating the similarities of various physical properties of atoms in molecules (Langmuir 1919). Subsequently, widespread application of isosteres for biological activity led to the term "bioisosterism" which was proposed by Friedman (1951) and by Thornber (1979) and diversified by Burger as: Bioisosteres are compounds or groups that possess very similar molecular shapes and volumes, and approximately the same distribution of electrons. Bioisosteres exhibit similar physical properties (Burger 1991). Practically, the concept of isosterism has been applied to the development of peptidomimetics in medicinal chemistry (Gante 1994; Oliyai 1996; Patani and Lavoie 1996; Vagner et al. 2008; Grauer and König 2009; Avan et al. 2014).

1.3.2 Modifications of Peptides

Peptidomimetics are modifications of the parent peptide sequences and accordingly, the following classification based on the chemical modifications has been proposed (Adessi and Soto 2002).

(i) *Modified peptides*: These compounds contain small modifications, particularly to amino acid side chains, which do not modify the peptide bond itself, thus they still possess the general structures of parent peptides.
(ii) *Pseudopeptides*: These compounds contain partial modifications of either peptide bonds or side chains, which lead to the derivation of molecules possessing chemical structures of partial parent peptides.
(iii) *Peptidomimetics*: These compounds do not possess any amide bonds of the corresponding parent peptides, and the structural similarity leads to dependence on the pharmacophore and on the pharmacological activity of the bioactive peptide conformation. As described in the previous section, in the broad sense, peptidomimetics include mimetics that mimic secondary and tertiary structures of peptides.

1.3.3 Local Modification in Peptidomimetics

A general approach to modifications of peptides involves the exchange of local structures. Local modification by the introduction of conformational restrictions,

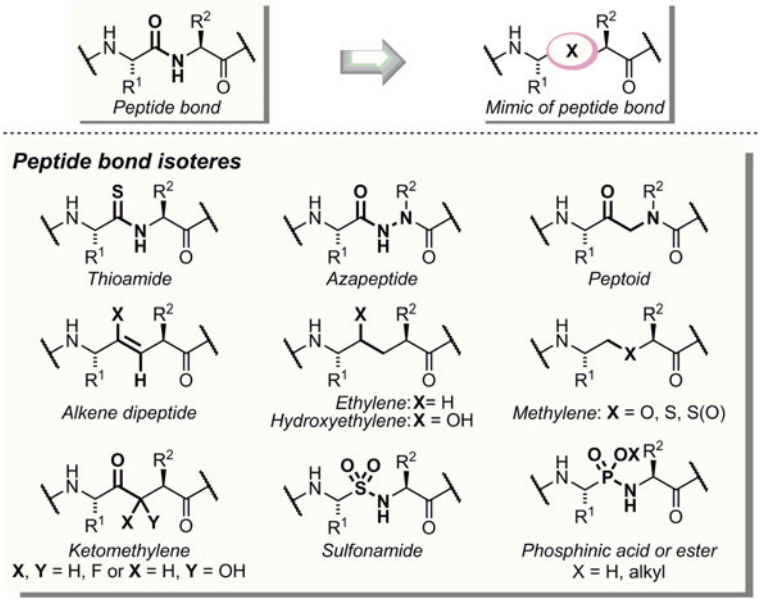


Fig. 1.4 Examples of peptide bond isosteres

such as the exchange of the backbone in side chain structures with the corresponding isosteric fragments, can have a significant effect on an increase of affinity for target receptors or stabilization of the molecules against degradation by proteases. This approach was conceived to constrain the rotational freedom of the backbone.

To date, several peptide bond isosteres have been proposed based on the conformational profiles of peptides alone or involving interactions with other molecules (Fig. 1.4) (Choudhary and Raines 2011). For example, the introduction of aliphatic moieties increases a peptide's conformational flexibility and on the other hand, the introduction of alkene-type isosteres does not significantly alter the topology, and augments conformational restrictions in possible intramolecular structures.

1.4 Peptide Bond Isosteres: Transition-State Mimics

In the special case of protease inhibitors, peptide bond isosteres have been designed as compounds with stable and non-hydrolyzable functional groups, which mimic the tetrahedral structures of transition state of hydrolysis of peptide bonds by proteases. Incorporation of a transition-state mimic into a peptide has led to the

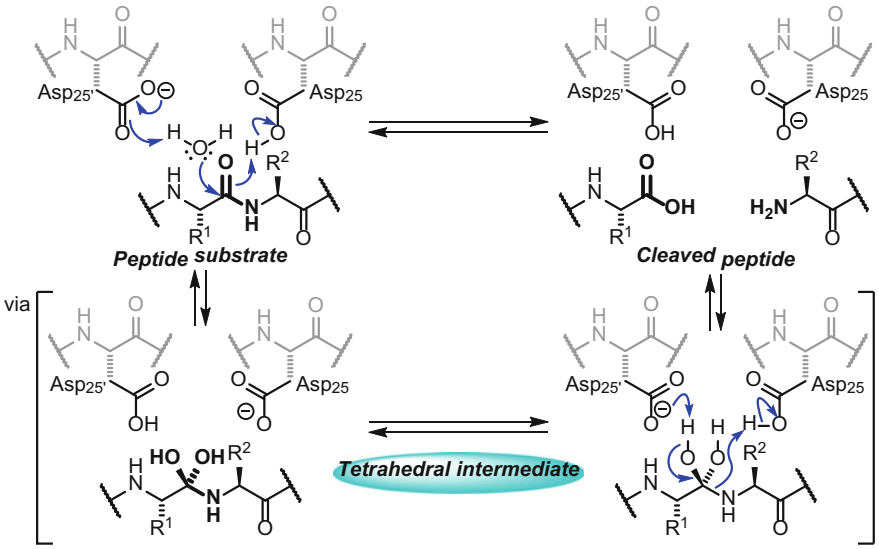


Fig. 1.5 Mechanism of a peptide bond cleavage by an aspartic protease, HIV-1 protease

development of aspartic protease inhibitors based on reactions mediated by proteases, which proceed via a tetrahedral transition state resulting from the nucleophilic attack by a water molecule at the scissile carbonyl group in the peptide bond (Fig. 1.5) (Bursavich and Rich 2002; Brik and Wong 2003). This strategy has led to the development of several HIV protease inhibitors based on replacement of the hydrolyzable peptide bond by non-hydrolyzable transition-state mimics (De Clercq 1995). Most reported inhibitors are characterized by the introduction of non-hydrolyzable transition-state mimics at positions R^1 or R^2 in place of the peptide bond. Statine, a representative transition-state mimic, is a non-proteinogenic β-hydroxy-γ-amino acid, which is found in natural protease inhibitors. This and various other transition-state analogs have been developed and explored as protease inhibitors (Fig. 1.6) (Müller 2005; Gupta et al. 2010). Other types of transition-state mimics, including hydroxyethylamine isosteres (Tamamura et al. 2002a, 2003a, b; Akaji et al. 2003; Gautier et al. 2006; Sova et al. 2009; Marcin et al. 2011; Kale et al. 2011), phosphinates (Drąg et al. 2007; Yamagishi et al. 2008; Grzywa and Oleksyszyn 2008), hydroxyl-methylcarbonyls (Stockel-Maschek et al. 2005; Weik et al. 2006; Nakatani et al. 2008), and hydroxyethylene isosteres (Hanessian et al. 2006; Yamaguchi et al. 2009; Björklund et al. 2010) have been reported (Fig. 1.6).

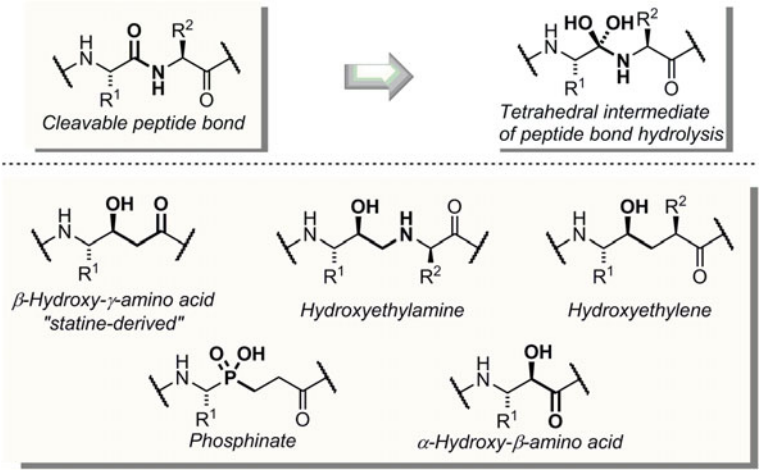


Fig. 1.6 Transition-state mimics of hydrolysis of amide bonds by proteases

1.5 Peptide Bond Isosteres: Ground-State Mimics

1.5.1 Overview of Alkene-Type Dipeptide Isosteres

Alkene-type dipeptide isosteres (ADIs) are considered as ideal amide bond mimetics due to a design based on the partial double-bond character of natural peptide bonds in the ground-state conformation (Fig. 1.7). Practically, several groups have tried to displace amide bonds in peptides with normal (*E*)-ADIs (EADIs) (Tamamura et al. 1997, 2002b, 2005; Oishi et al. 2002; Fu et al. 2005; Sasaki et al. 2006; Brouwer et al. 2008; Proteau-Gagne et al. 2011; Misu et al. 2014), and have developed fluoroalkene dipeptide isosteres (FADIs) (Narumi et al. 2008, 2010; Oishi et al. 2009; McKinney and Urban 2010; Watanabe et al. 2011;

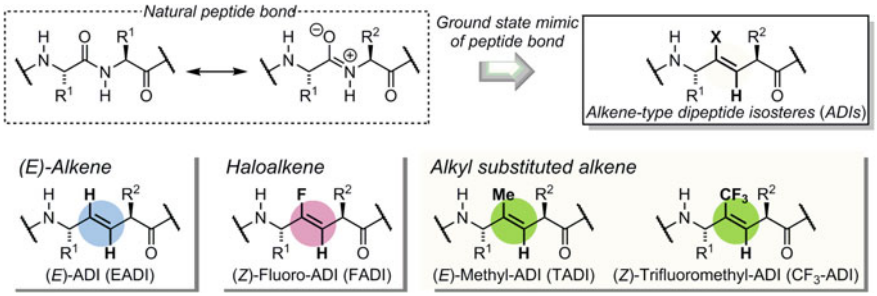


Fig. 1.7 Natural peptide bonds and alkene-type dipeptide isosteres

Villiers et al. 2015) or other alkene-type dipeptide isosteres (Reddy et al. 2007; Inokuchi et al. 2011; Kobayashi et al. 2012; Lenci et al. 2015).

1.5.2 *Representative Synthetic Methods and Bioactive Evaluation of Alkene and Fluoroalkene Dipeptide Isosteres*

Fujii and coworkers have reported the synthesis of (*E*)-alkene- and (*Z*)-fluoroalkene dipeptide isosteres, and an application for introduction to FC131 **1.1** possessing CXCR4 antagonistic activity (Fig. 1.8) (Tamamura et al. 2005; Narumi et al. 2008, 2010). The Fujii group has synthesized Fmoc–Orn(Cbz)–ψ[(*E*)–CH=CH]–Orn (Cbz)–OH (**1.6**) as an (*E*)-alkene dipeptide isostere (EADI), which is contained in an FC131 analog (**1.2**) (Scheme 1.1). Diastereoselective and (*Z*)-selective synthesis of Ns–Orn(Cbz)–ψ[(*E*)–CH=CH]–L–Nva(5–OTBS)–O^{t}Bu (**1.5**) was achieved from β-aziridinyl-α,β-enoate (**1.4**), which was synthesized from Boc–Orn(Cbz)–OMe (**1.3**) in eight steps, by an organocopper-mediated *anti*-S_N2' reaction followed by derivation to Fmoc–Orn(Cbz)–ψ[(*E*)–CH=CH]–Orn(Cbz)–OH (**1.6**) for Fmoc-based solid-phase peptide (SPPS) synthesis in five steps (Oishi et al. 2002, 2009; Narumi et al. 2010). An EADI-containing FC131 (**1.2**) was produced from the resulting **1.6** utilizing SPPS. A synthetic method for the (*Z*)-fluoroalkene dipeptide isosteres (FADIs) was also reported by the same group. In case of synthesis of FADIs, treatment of γ,γ-difluoro-α,β-enoyl sultam (**1.8**) with the Gilman reagent ($Me_2CuLi{\cdot}LiI{\cdot}2LiBr$), which was supplied from the starting material (**1.7**) in six steps, obtained a dienolate intermediate (**1.9**) by carbon-fluorine cleavage via a single electron transfer (SET) mechanism followed by asymmetric alkylation to yield **1.10**. Compound **1.10** was converted to Fmoc–Orn(Cbz)–ψ[(*E*)–CH=CH]–Orn(Cbz)–OH (**1.11**) and this was followed by SPPS to obtain the desired FADI-containing FC131 (**1.3**) (Scheme 1.2).

The biological activity of EADI- or FADI-containing FC131 was evaluated by its inhibition of [^{125}I]-SDF-1-binding to CXCR4. Although the potency was

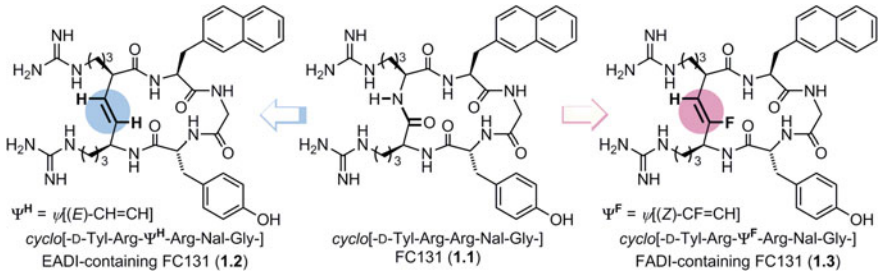
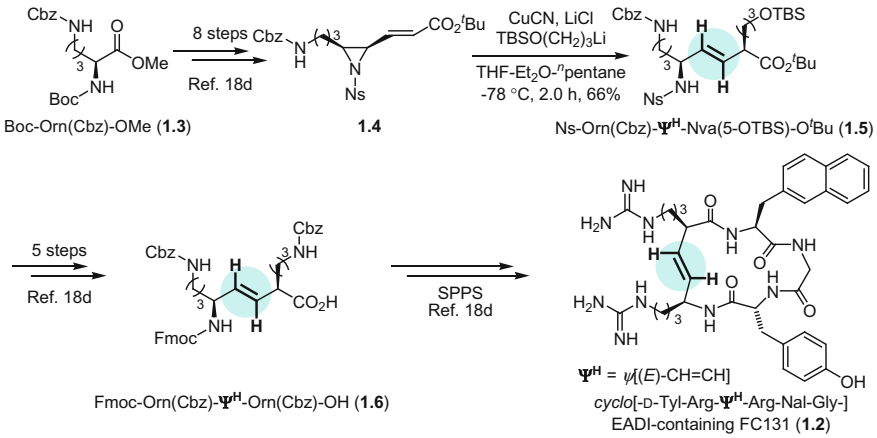
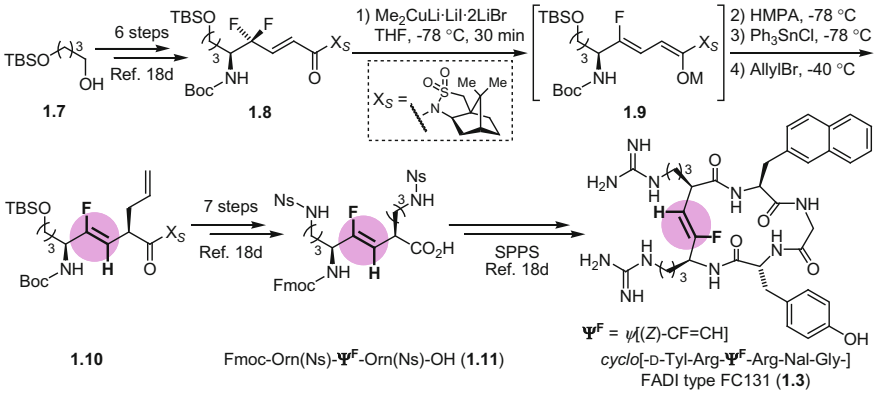


Fig. 1.8 Structural modifications of FC131 by peptide isosteres



Scheme 1.1 Synthesis of EADI-containing FC131



Scheme 1.2 Synthesis of FADI-containing FC131

approximately 20-fold lower than that of the parent peptide FC131 (**1.1**), both EADI- and FADI-containing FC131s (**1.2** and **1.3**) significantly inhibited the SDF-1 binding to CXCR4 with IC_{50} values of 1.46 μM (for **1.2**) and 1.78 μM (for **1.3**) as shown in Fig. 1.9 (Narumi et al. 2010).

1.5.3 Design and Concept of Chloroalkene Dipeptide Isosteres

Several alkene-type dipeptide isosteres (ADIs), which are associated with some issues such as multistep synthesis and low total yields, have been developed and

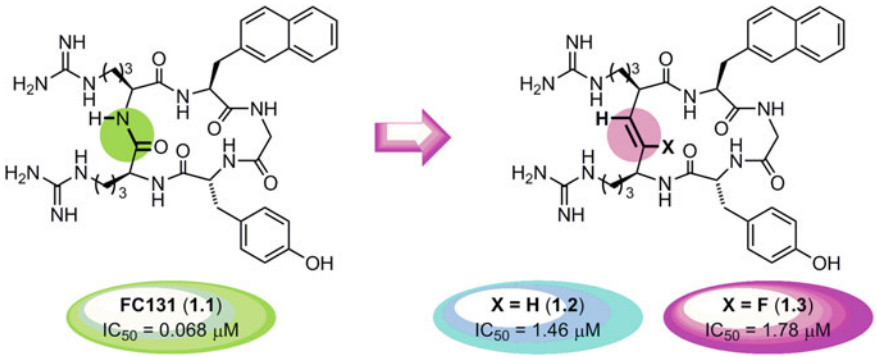
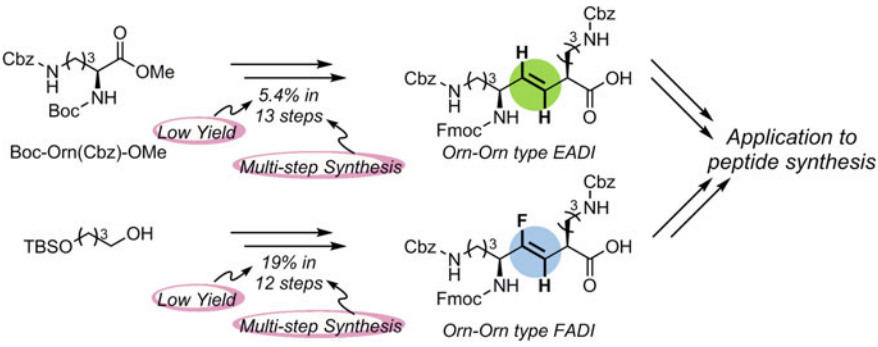


Fig. 1.9 Biological evaluation of EADI- and FADI-containing FC131s

applied to bioactive peptides (Scheme 1.3). ADIs are difficult to prepare using conventional methods of peptide synthesis. Furthermore, ADI-containing peptides might not always be as bioactive as peptidomimetics because traditional ADIs might possess a smaller dipole moment resulting from electronegativity. In addition, these ADIs lack the steric restriction between the carbonyl oxygen atom and the side chain on the β-carbon in natural peptides due to smaller van der Waals radius (VDR) (Bondi 1964; Batsanov 2001) compared to that of a natural amide bond.

Our research group has focused on the chloroalkene structures in chloroalkene dipeptide isosteres (CADIs) which replace an amide bond in peptides as shown in Fig. 1.10. CADIs are considered to be superior peptide bond mimetics because the VDR and the electronegativity value (Pauling 1932; Mulliken 1934; Allred and Rochow 1958; Sanderson 1983; Allen 1989) of the chlorine atom are similar to those of the oxygen atom which it replaces (Table 1.1). Replacement of the peptide bond by the chloroolefin conformation can be also conceived as mimicking steric restriction by the pseudo-1,3-allylic strain due to a chlorine atom that is larger than the carbonyl oxygen (Fig. 1.11).



Scheme 1.3 Issues of ADIs or FADIs for application to peptide synthesis

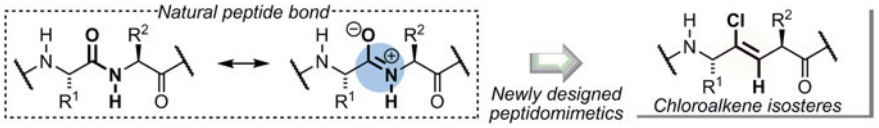


Fig. 1.10 Newly designed chloroalkene dipeptide isosteres as peptidomimetics

Table 1.1 Van der Waals radius (VDR) and the electronegativity

X	VDR (pm)	Electronegativity
O	152	3.4[a] (3.5)[b]
H	120	2.2[a] (2.3)[b]
F	147	4.0[a] (4.1)[b]
Cl	175	3.2[a] (2.8)[b]

[a]Pauling value
[b]Allred-Rochow value

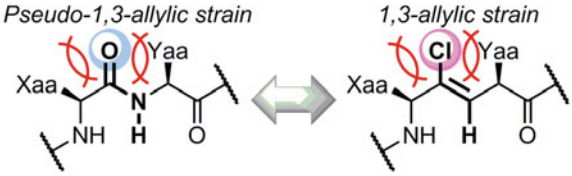


Fig. 1.11 Mimetic of pseudo-1,3-allylic strain

In addition, the direction of the vector of the dipole moment in the chloroolefin is similar with that of an amide, but in fluoroolefins, the vector of the dipole moment is significantly different (Fig. 1.12). The optimized geometries calculated by PM3 Density Functional with the Spartan'14 (version 1.1.9: Wavefunction Inc., Irvine, California) were used as starting geometries for DFT calculations. DFT calculations

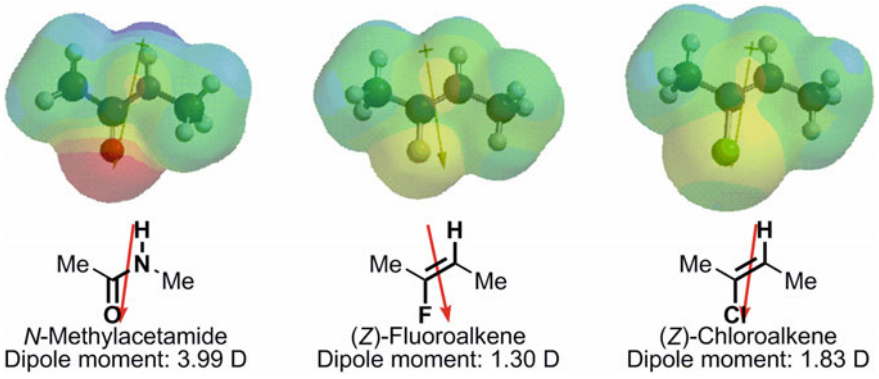


Fig. 1.12 Direction of the vector of the dipole moment of amide and haloolefin

were carried out with the same program. The geometries were fully optimized in vacuo by using the B3LYP/6-31G* level of theory. We expect in this way to compensate for the drawbacks of ADIs. Few reports, however, are available on application of chloroalkene structures as peptidomimetics (Waelchli et al. 1996). This possibly is due to a lack of efficient methods of synthesis.

Consequently, the development of efficient synthetic methods for the chloroalkene structure, which are useful for available versatile synthetic intermediates (Song et al. 2005; Tan and Negishi 2006; Matveenko et al. 2008; Geary and Hultin 2010; Sawant et al. 2015) and marine natural products (Wu et al. 2007; White et al. 2008; Ueda et al. 2009; Guinchard and Roulland 2011; Martín et al. 2013; Brockway et al. 2015; Tanaka-Yanuma et al. 2015) and stereoselective synthetic methods for various Xaa-Yaa type CADIs, is to be undertaken for the discovery of new peptidomimetics.

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Chapter 2
Chloroalkene Dipeptide Isosteres as Peptidomimetics

Abstract In drug discovery, the development of "isosteres", in which a part of molecules is changed to their mimetic, is required to enhance the activity of seed or lead compounds. Alkene-type dipeptide isosteres (ADIs), which have been designed based on the ground-state mimics of amide bonds, are expected as useful "peptide bond isosteres" because of their high structural homology with natural dipeptides. Recently, our group has focused on the chloroalkene structure as a chemical equivalent of amide bond, and developed chloroalkene dipeptide isosteres (CADIs). Treatment of a γ,γ-dichloro-α,β-unsaturated ester, which was synthesized from a chiral sulfonamide and an aldehyde corresponding to the alkyl side chain of an amino acid, with higher order organocuprates has led to L-Xaa-Gly type isosteres. Treatment of allylic *gem*-dichlorides with lower order organocuprates provided the allylic alkylated compound in high yield and excellent diastereoselectivity. In addition, we have succeeded in the synthesis of several (L,D)- and (L,L)-type CADIs by switching olefin geometry of the substrate. Furthermore, we have succeeded in the application of a CADI. Utilizing this methodology, a CADI was incorporated into a cyclic RGD peptide. In addition, this cyclic RGD peptide mimic has shown higher activity than the parent cyclic peptide.

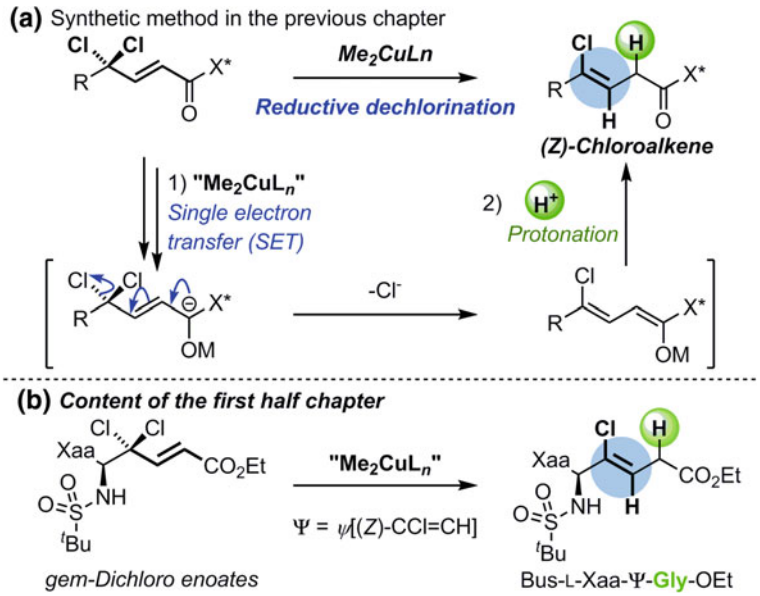
Keywords Chloroalkene dipeptide isostere · Higher order organocuprate Lower order organocuprate · Cyclic RGD peptide

2.1 Stereoselective Synthesis of (*Z*)-Chloroalkene Dipeptide Isosteres

2.1.1 Concept of Xaa-Gly Type (Z)-Chloroalkene Dipeptide Isosteres

In the previous chapters, the reaction with a Gilman cuprate was shown to lead to reduced compounds by a carbon–chlorine bond cleavage via a single electron transfer (SET) mechanism (Scheme 2.1a) (Narumi et al. 2008; Otaka et al. 2000).

H. Tamamura et al., *Mid-size Drugs Based on Peptides and Peptidomimetics*, SpringerBriefs in Pharmaceutical Science & Drug Development, https://doi.org/10.1007/978-981-10-7691-6_2



Scheme 2.1 Synthesis of Xaa-Gly type chloroalkene dipeptide isosteres utilizing the organocuprate-mediated reduction

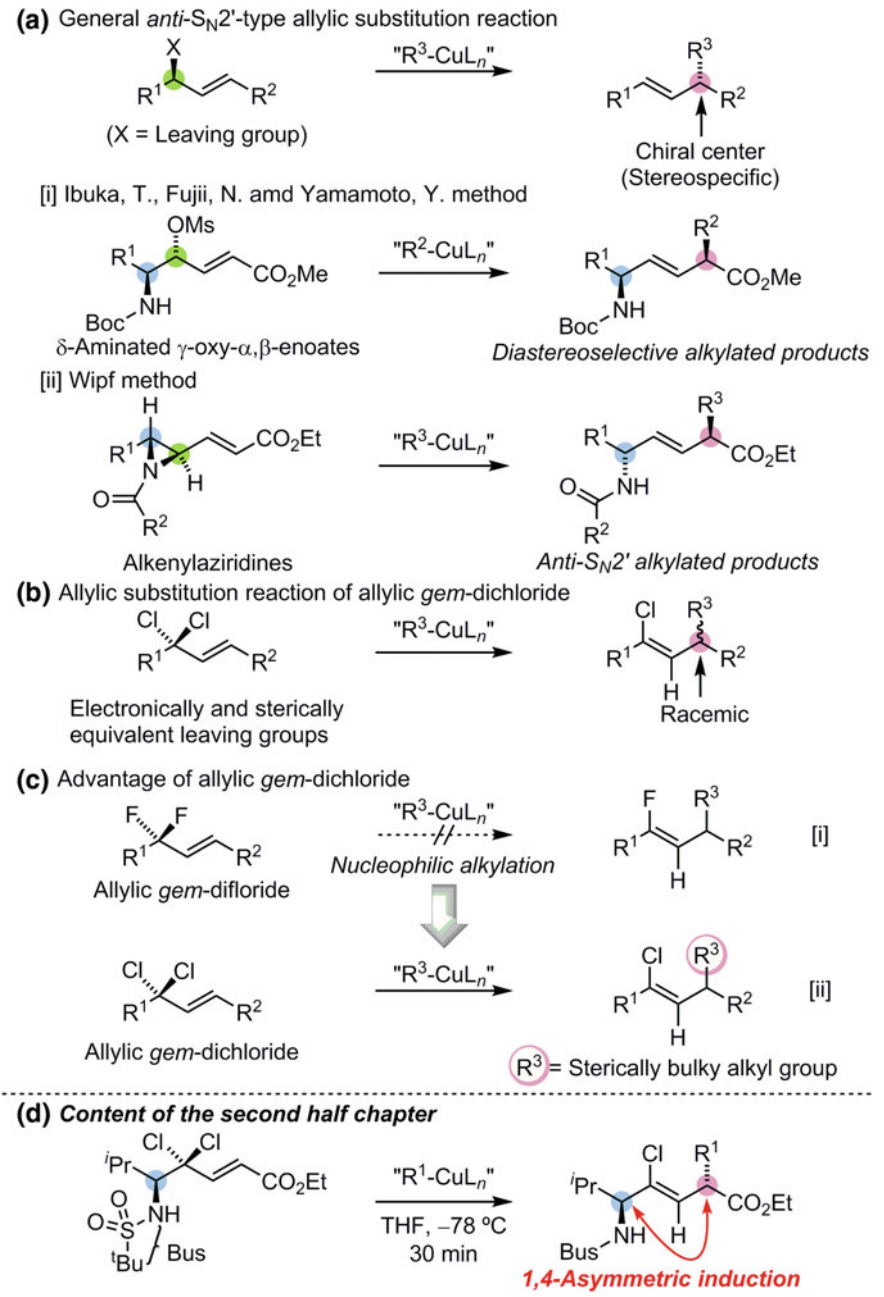
This SET reaction produces only the reduced dechlorinated compounds, which correspond to Xaa-Gly type CADIs. In this chapter, as the extension of the scope of application, a useful method for the stereoselective synthesis of Bus–L–Xaa–ψ[(*Z*)–CCl=CH]–Gly–OEt utilizing organocuprate-mediated reduction is described (Scheme 2.1b).

2.1.2 *Concept of Xaa-Yaa Type (Z)-Chloroalkene Dipeptide Isosteres*

The discovery of novel synthetic methods for the stereoselective formation of structurally complex organic molecules with high levels of efficiency and selectivity remains a challenge in organic synthesis. Asymmetric induction by preexisting stereogenic centers provides reliable methods for the facile construction of synthetically valuable building blocks bearing multiple chiral centers (O'Brien et al. 2011; Geary 2009; Mengel et al. 1999) and remote asymmetric inductions such as 1,4- (Acocella et al. 2013; Moustafa et al. 2012; Hayashi et al. 2010a, b; Pellicena et al. 2008; Huang et al. 2007; Sugimoto et al. 2006; Pedrosa et al. 2006), 1,5- (Yamaoka and Yamamoto 2010; Paton and Goodman 2008; García Ruano et al. 2005; Denmark et al. 2005; Dias and Aguilar 2008; Yeung and Paterson 2005), and 1,6-stereorelationships (Takahashi et al. 2014; Hayashi et al. 2012; Mukaeda et al.

2012; Hayashi et al. 2010a, b) are of particular interest because of the synthetic utility that can provide potentially intriguing synthetic strategies for the synthesis of complex molecules (Hayashi et al. 2010a, b; Dias and Aguilar 2008; Yeung et al. 2005; Takahashi et al. 2014; Hayashi 2012).

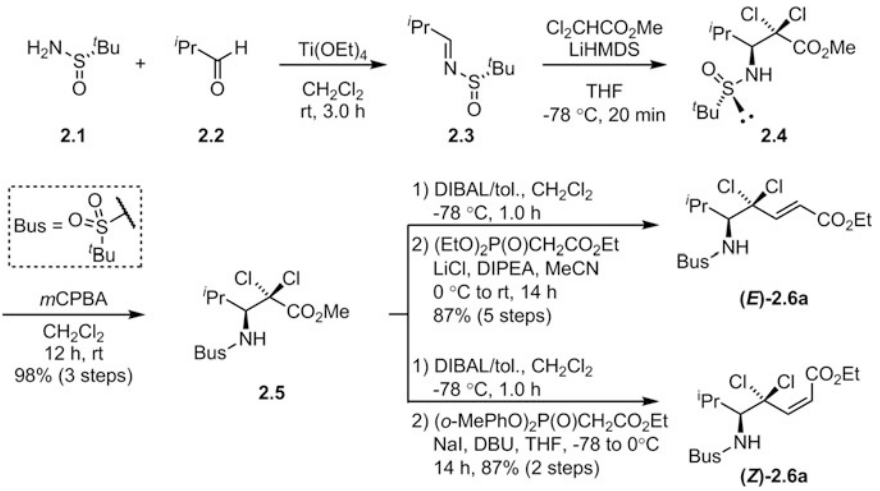
The significance of chloroalkenes and the synthetic and application studies of chloroalkenes (Geary and Hultin 2010; Bell et al. 2007; Jones et al. 2000; Alami et al. 1995; Guinchard et al. 2011; Ando et al. 2010) were discussed in the previous chapters. The allylic substitution reaction with an organocopper reagent takes place stereospecifically on the face *anti* to the leaving group of allylic electrophiles (*anti*-S_N2' manner, Scheme 2.2a) (Goering and Kantner 1984; Corey and Boaz 1984; Yoshikai and Nakamura 2012). As a representative *anti*-S_N2' reaction utilizing organocopper reagents, Ibuka, Fujii, Yamamoto, and coworkers reported that the treatment of the substrates of δ-aminated γ-oxy-α,β-enoates with suitably prepared copper reagents led to a diastereoselective *anti*-S_N2' reaction (Scheme 2.2a-[i]) (Ibuka et al. 1991). Wipf and coworkers also showed that the treatment of alkenylaziridines with organocopper reagents afforded *anti*-S_N2' alkylated products (Scheme 2.2a-[ii]) (Wipf and Fritch 1994). However, the stereoselective reaction of allylic *gem*-dichlorides is particularly challenging because allylic *gem*-dichlorides have two electronically and sterically equivalent leaving groups that impose severe limitations on the *anti*-S_N2' strategy and depend on the stereochemistry of the leaving group (Scheme 2.2b). In this situation, the observed moderate diastereoselectivity in the allylic alkylation of allylic *gem*-dichlorides described in the previous chapters has been achieved by means of induction exerted by the chiral center at C5 bearing noncoordinating phenyl and siloxy groups. These results suggest the possibility that the introduction of a coordinating group such as a sulphonamide (Furuya et al. 2010; Musio et al. 2009; Guo et al. 2009; Hui et al. 2006; Duncan et al. 2004; Garcia et al. 2002) at C5 could control the facial attack of allylic electrophiles and provide the trisubstituted (*Z*)-chloroalkene flanking two stereogenic centers bearing the amino functionality. These motifs are highly attractive for peptidomimetics with a functionalized alkene as a peptide bond surrogate (alkene-type dipeptide isosteres: ADIs), which are thought of as one of the most ideal dipeptide mimetics in medicinal chemistry and chemical biology (Namanja et al. 2010; Tamamura et al. 2005; Narumi et al. 2010). In addition, the development of a synthetic method for nucleophilic alkylation, using a substrate of allylic *gem*-difluorides, has not been reported because a fluorine atom is not a better leaving group (Scheme 2.2c-[i]) (Watanabe et al. 2011; Fujita et al. 2016). It is also considered that alkylation with sterically bulky groups might be performed nucleophilically in an allylic *gem*-dichloride substrate (Scheme 2.2c-[ii]). This chapter describes diastereoselective 1,4-asymmetric induction in the allylic alkylation of allylic *gem*-dichlorides utilizing organocopper reagents to obtain (*Z*)-chloroalkene dipeptide isosteres (CADI) (Scheme 2.2c). This synthetic method provides efficient access to either L,L-type or L,D-type (*Z*)-CADIs by simply switching the olefin geometry of the allylic *gem*-dichlorides.



Scheme 2.2 Allylic substitution reaction with organocopper reagents

2.1.3 *Preparation of Allylic* Gem-*Dichlorides as Precursors of Chloroalkene Dipeptide Isosteres*

In seeking to develop an effective approach to the preparation of a key intermediate, the (2*E*)-γ,γ-dichloro-α,β-enoate [**(*E*)-2.6**] with the chiral center at C5 bearing a sulfonamide group, an *N*-*tert*-butylsulfonyl (Bus) group (Sun and Weinreb 1997) was employed for the protection of amine functionality in a manner that would be tolerated in the multistep synthesis. The Bus group can be easily prepared by oxidation of an *N*-sulfinyl group (Sun and Weinreb 1997) and removed by treatment with $AlCl_3$ (Enders et al. 2010; Mita et al. 2013). As shown in Scheme 2.3, nucleophilic addition of the lithium enolate of methyl dichloroacetate to the chiral *N*-sulfinyl aldimine (**2.3**), which was prepared from (*S*)-*tert*-butylsulfinamide (**2.1**) (Robak et al. 2010) and isobutyl aldehyde (**2.2**) corresponding to the side chain of an amino acid, provided the corresponding ester (**2.4**) (Davis and Deng 2007) with high diastereoselectivity (>20:1) through a six-membered chair-like transition-state model (Tang and Ellman 1999, 2002). Oxidation of the *N*-sulfinyl group with *m*-CPBA provided the *N*-Bus-protected-α,α-dichloro-β-amino ester (**2.5**) as a single enantiomer. Reduction of **2.5** with DIBAL-H followed by the Horner–Wadsworth–Emmons reaction afforded the desired (*E*)- or (*Z*)-enoate [**(*E*)-2.6a** or **(*Z*)-2.6a**]. Because of the need for fewer purifications by flash chromatography, the desired products were easily produced in five steps from readily available starting materials. Other precursors of CADIs are possible to be supplied by exchanging the aldehyde of the starting material and using a similar synthetic method.



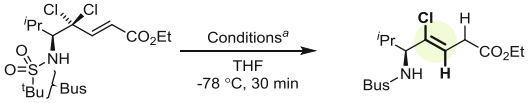
Scheme 2.3 Preparation of γ,γ-dichloro-α,β-enoate **(*E*)**- and **(*Z*)-2.6a**

2.1.4 Synthesis of Xaa-Gly Type (Z)-Chloroalkene Dipeptide Isosteres by Organocuprate-Mediated Reduction

In the synthesis of Bus–L–Val–ψ[(*Z*)–CCl=CH]–Gly–OEt (**2.7a**), with a suitable substrate in hand, the reactivity of ester (***E*)-2.6a** with a Gilman cuprate was examined (Table 2.1). All of the reactions produced (*Z*)-chloroalkene products with undetectable levels of the (*E*)-chloroalkene isomers (*Z*/*E* = >20:1). Subsequent reaction with a Gilman cuprate afforded the reductive dechlorinated compound (**2.7a**) in excellent yield (entry 1). The reactions with a higher order cuprate (Me_3CuLi_2·LiI·3LiBr) and with the cyano Gilman reagent (Me_2CuLi_2·LiCN·2LiBr) produced **2.7a** in slightly lower but still high yields (entries 2, 3). Changing the methyl group of alkyl ligands to an *n*-butyl group resulted in a decreased yield (entry 4). On the other hand, Gilman cuprates with additives such as TMSCl, BF_3·OEt_2, and HMPA did not lead to significant differences in chemical yields, but small amounts of α-methylated compounds were observed (entries 5–7). Based on these results, the Gilman reagent (entry 1) was adopted as most suitable.

The optimal reduction condition is identified in Table 2.1 (entry 1), and next, the substrate scope of this reductive dechlorination was explored (Table 2.2). Treatment of various (*E*)-enoates [**(*E*)-2.6a–e**] as Xaa-Gly precursors with the optimized Gilman cuprates in a similar manner to that in Table 2.1 (entry 1) led to

Table 2.1 Reactivity of a γ,γ-dichloro-α,β-enoate with organocopper reagents



(*E*)-2.6a Bus-Val-ψ[(*Z*)-CCl=CH]-Gly-OEt (**2.7a**)

Entry	Reagents	Additives[b]	**2.7a**, *Z*/*E*[c]	**2.7a**, yield (%)[d]
1	Me_2CuLi·LiI·2LiBr	–	>20:1	99
2	Me_3CuLi_2·LiI·3LiBr	–	>20:1	78
3	$Me_2Cu(CN)$·2LiBr	–	>20:1	80
4	^nBuCuLi·LiI	–	>20:1	41
5	Me_2CuLi·LiI·2LiBr	TMSCl	>20:1	94
6	Me_2CuLi·LiI·2LiBr	BF_3·OEt_2	>20:1	88
7	Me_2CuLi·LiI·2LiBr	HMPA	>20:1	94

[a]All reactions were carried out at −78 °C for 30 min on a 0.1 mmol scale with 4 equiv of organocuprates in the presence of Li salts
[b]4 equiv
[c]Determined by ^1H NMR with the unpurified reaction mixture
[d]Yields were determined by ^1H NMR of the unpurified reaction mixture using 1,4-dinitrobenzene as an internal standard

Table 2.2 Scope of functional group tolerance

(*E*)-**2.6a**: R = ^{i}Pr, (*E*)-**2.6b**: R = ^{i}Bu
(*E*)-**2.6c**: R = ^{n}Bu, (*E*)-**2.6d**: R = Bn
(*E*)-**2.6e**: R = TBSO(CH$_2$)—≀

2.7a: R = ^{i}Pr, **2.7b**: R = ^{i}Bu
2.7c: R = ^{n}Bu, **2.7d**: R = Bn
2.7e: R = TBSO(CH$_2$)—≀

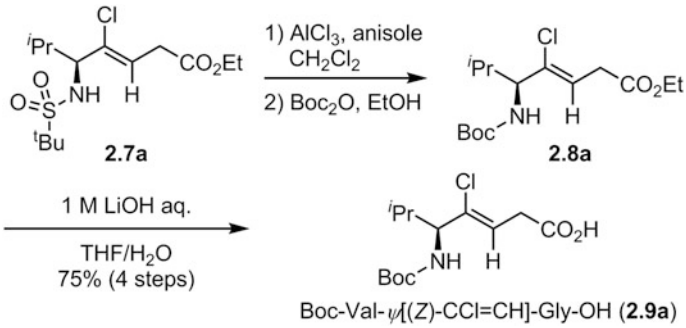
Entry	Substrate	**2.7**, *Z*/*E*[b]	**2.7**, yield (%)[c] (product)
1	(*E*)-**2.6a**	>20::1	99 (**2.7a**)
2	(*E*)-**2.6b**	>20::1	98 (**2.7b**)
3	(*E*)-**2.6c**	>20::1	97 (**2.7c**)
4	(*E*)-**2.6d**	>20::1	84 (**2.7d**)
5	(*E*)-**2.6e**	>20::1	71 (**2.7e**)

[a]All reactions were carried out at −78 °C for 30 min on a 0.3 mmol scale with 4 equiv of organocuprates in the presence of Li salts
[b]Determined by ^{1}H NMR with the unpurified reaction mixture
[c]Yields of isolated products

the reductive products **2.7a–e** (entries 1–5). In all cases, the reductive products were obtained with excellent *Z*-selectivity (>20:1). The substrates with various sterically bulky alkyl groups gave reduced compounds in excellent yields regardless of the steric factors of the alkyl side chains (entries 1–3). Other precursors of CADIs are possible to be supplied by exchanging the aldehyde of the starting material and using a similar synthetic method.

Although peptide isosteres including *N*-hetero-compounds such as Bus–Lys (Cbz)–ψ[(*Z*)–CCl=CH]–Gly–OEt can allow multiple products, the substrates including those with an aromatic group (entry 4) and an oxygen-containing group (entry 5) gave high yields of Bus–Phe–ψ[(*Z*)–CCl=CH]–Gly–OEt and Bus–Ser (TBS)–ψ[(*Z*)–CCl=CH]–Gly–OEt with no decrease in *Z*-selectivity.

The substrate scope of the reductive dechlorination utilizing Gilman cuprates was thus confirmed, and the reduced compound **2.7a** was converted into a Boc-protected carboxylic acid Bus–L–Xaa–ψ[(*Z*)–CCl=CH]–Gly–OH (**2.8a**) (Scheme 2.4), which can be used in peptide chemistry. Deprotection of the Bus group in the presence of $AlCl_3$ and anisole followed by Boc protection gave the ester Boc–L–Xaa–ψ[(*Z*)–CCl=CH]–Gly–OEt (**2.8a**). Finally, compound **2.8a** was hydrolyzed under basic condition to give the Boc-protected CADI Boc–L–Xaa–ψ[(*Z*)–CCl=CH]–Gly–OH (**2.9a**) in four steps and 75% yield without isomerization of the olefinic bond. The Bus group was easily removed without significant loss of the optical purity according to the reported procedures (Sun et al. 1997; Watanabe et al. 2011; Fujita et al. 2016).

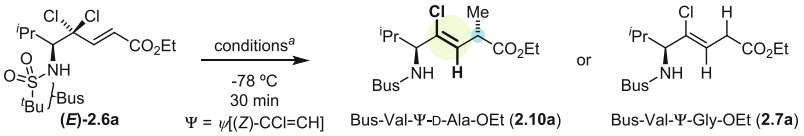


Scheme 2.4 Conversion to Boc–Val–ψ[(*Z*)–CCl=CH]–Gly–OH (**2.9a**)

2.1.5 *Remote Stereoinduction in the Organocuprate-Mediated Allylic Alkylation of Allylic* Gem*-Dichlorides: Highly Diastereoselective Synthesis of (Z)-Chloroalkene Dipeptide Isosteres*

As described in Sect. 2.1.3, we examined the allylic alkylation of (***E*)-2.6** with various organocuprates (Table 2.3). All of the reactions tested provided (*Z*)-chloroalkene products **2.10a** and **2.7a** with undetectable amounts of the (*E*)-chloroalkene isomers (*Z*/*E* = >20:1). The choice of organocuprate was found to be critical. The use of Gilman cuprate ($Me_2CuLi{\cdot}LiI{\cdot}2LiBr$) afforded only the reductive dechlorinated compound Bus–Val–ψ[(*Z*)–CCl=CH]–Gly–OEt (**2.7a**) in 97% yield possibly via a single electron transfer mechanism (entry 1) (Tang and Ellman 1999, 2002) but cyanocuprates prepared from CuCN or organometallic reagents effectively provided the desired α-methylated compound Bus–Val–ψ[(*Z*)–CCl=CH]–D–Ala–OEt (**2.10a**) (entries 2–9). The reaction with $MeCu(CN)Li{\cdot}LiBr$ afforded **2.10a** in 90% yield, and remarkably, the reaction proceeded with an excellent degree of diastereoselectivity (entry 2). The absolute stereochemistry of **2.10a** was established by X-ray analysis and the single diastereomer obtained was an L,D-type isostere (Fig. 2.1). The addition of 1 equiv of LiCl to organocuprates resulted in a slightly improved yield and product selectivity (entry 3) and the reaction with additional LiCl or $BF_3{\cdot}OEt_2$ led to similar results (entries 4 and 5). We then turned our attention toward exploration of the use of other methylmetal species for the preparation of methyl cyanocuprates (entries 6–9), because the acceptance of various organometallic reagents can contribute to the diversity of the α-substituent, which corresponds to the side chain of amino acids. In all methylmetal species tested, full conversion was obtained and the choice of organometallic reagents did not influence the stereochemical outcome of the reaction. The use of MeMgBr gave a slightly lower yield and product selectivity (entry 6). The use of MeZnCl obtained by transmetalation of MeMgBr with $ZnCl_2$ had a beneficial effect on the product selectivity and led to the exclusive formation of the desired

Table 2.3 Reactivity of **(*E*)-2.6a** with organocuprates



Entry	Reagents	Additive [equiv]	**2.10a**:**2.7a**[b]	**2.10** or **2.7** yield (%)[c]	*Z*/*E* of **2.10a**[b]	dr[b] **2.10a**
1	$Me_2CuLi{\cdot}LiI{\cdot}2LiBr$	–	1:>20	**2.7a**, 97	–	–
2	MeCu(CN)Li·LiBr	–	13:1	**2.10a**, 90	>20:1	>20:1[d]
3	MeCu(CN)Li·LiBr	LiCl [4]	>20:1	**2.10a**, 99	>20:1	>20:1[d]
4	MeCu(CN)Li·LiBr	LiCl [8]	16:1	**2.10a**, 91	>20:1	>20:1[d]
5	MeCu(CN)Li·LiBr	LiCl [4] $BF_3{\cdot}OEt_2$ [4]	19:1	**2.10a**, 91	>20:1	>20:1[d]
6	MeCu(CN)MgBr	LiCl [4]	7:1	**2.10a**, 84	>20:1	>20:1[d]
7[e]	MeCu(CN)ZnCl	LiCl [8]	>20:1	**2.10a**, 86	>20:1	>20:1[d]
8	MeCu(CN)ZnMe	LiCl [8]	>20:1	**2.10a**, 88	>20:1	>20:1[d]
9[f]	MeCu(CN)$AlMe_2$	LiCl [8]	>20:1	**2.10a**, 85	>20:1	>20:1[d]

[a]Unless otherwise noted, all reactions were carried out at −78 °C for 30 min on a 0.1 mmol scale with 4 equiv of organocuprates in the presence of metal salts
[b]Determined by ^1H NMR with the unpurified reaction mixture
[c]Yields are for the isolated products
[d]Only a single diastereomer was detected
[e]0 °C for 2 h
[f]0 °C for 4 h

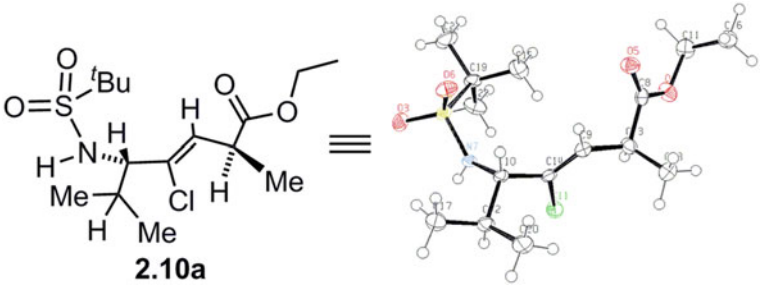
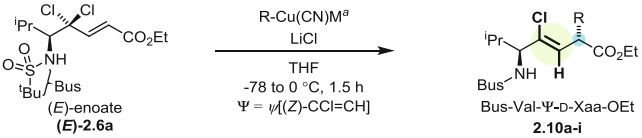


Fig. 2.1 Single-crystal X-ray analysis of **2.10a**

α-methylated compound (**2.10a**) with excellent diastereoselectivity (entry 7). Furthermore, Me_2Zn and Me_3Al were also used without any decrease of diastereoselectivity, although with slightly lower yields (entries 8 and 9).

With reliable conditions identified, the scope of this diastereoselective allylic alkylation was explored (Table 2.4). The reaction was found to tolerate various alkyl cyanocuprates, providing the regio- and diastereoselective 1,4-asymmetric

Table 2.4 Scope of diastereoselective allylic alkylation of **(*E*)-2.6a**



Entry	**R**Cu(CN)M[a]	Bus–Val–**Ψ**–D–Xaa–OEt	*Z*/*E*[b]	dr[b]	Yield (%)[c]
1	MeCu(CN) ZnCl	Bus–Val–**Ψ**–D–Ala–OEt	>20:1	>20:1	**2.10a**, 86
2	EtCu(CN) ZnCl	Bus–Val–**Ψ**–D–Abu(2)–OEt	>20:1	>20:1	**2.10b**, 99
3	^{n}BuCu(CN) ZnCl	Bus–Val–**Ψ**–D–Nle–OEt	>20:1	>20:1	**2.10c**, 94
4	^{i}BuCu(CN) ZnBr	Bus–Val–**Ψ**–D–Leu–OEt	>20:1	>20:1	**2.10d**, 99
5	BnCu(CN) ZnBr	Bus–Val–**Ψ**–D–Phe–OEt	>20:1	>20:1	**2.10e**, 98
6	(2-naphthyl) Cu(CN)ZnCl	Bus–Val–**Ψ**–D–Nal–OEt	>20:1	>20:1	**2.10f**, 94
7	$EtO_2C(CH_2)_2$ Cu(CN)ZnCl	Bus–Val–**Ψ**–D–Glu(OEt)–OEt	>20:1	>20:1	**2.10g**, 98
8	AllylCu(CN) ZnCl	Bus–Val–**Ψ**–D–(Allyl)Gly–OEt	>20:1	>20:1	**2.10h**, 54
9	2,6-diMePh Cu(CN)ZnCl	Bus–Val–**Ψ**–D–(2,6-diMePh) Gly–OEt	>20:1	>20:1	**2.10i**, 96

[a]All reactions were carried out at −78 °C for 1.5 h on a 0.3 mmol scale with 4 equiv of organocuprates in the presence of Li salts
[b]Determined by ^{1}H NMR with the unpurified reaction mixture
[c]Yields of isolated products

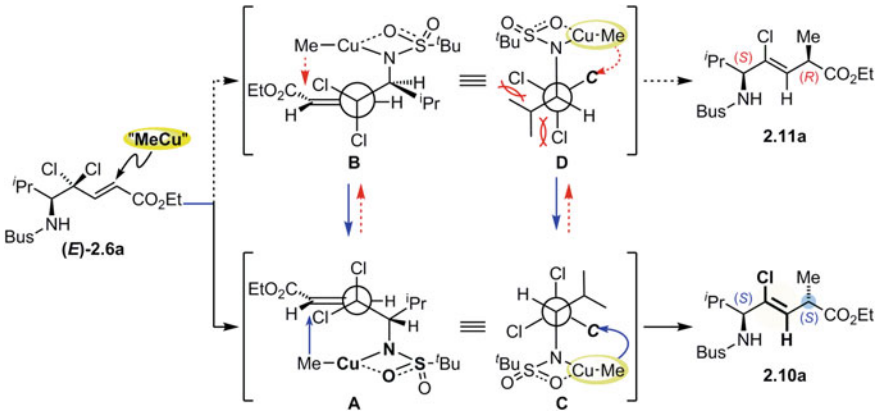
induction products (**2.10a–i**) in moderate to good yields (54–99%) (entries 1–9). In all cases, α-alkylated products were obtained with excellent *Z*-selectivity and diastereoselectivity (>20:1). When the organocuprates, prepared from organozinc reagents, were employed, the corresponding α-alkylated products (**2.10b–g**) could be isolated in excellent yields (entries 2–7). Interestingly, this new strategy is also applicable to α-allylation (allyl–allyl cross-coupling), a challenging task in organocopper chemistry (Sasaki et al. 2007; Hornillos et al. 2013; Yamanaka et al. 2004; Bartholomew et al. 2008). The allyl zinc–copper reagent, which was obtained by transmetalation from allylmagnesium chloride with $ZnCl_2$, also reacts with excellent diastereoselectivity, furnishing the synthetically useful α-allylated product (**2.10h**) in moderate yield (entry 8). However, the allyl cyanocuprate, prepared directly from allylmagnesium bromide, gave only the reduction product (**2.7a**) and no alkylated product, clearly indicating the utility of transmetalation from Grignard reagents with Zn salts in this reaction system. A similar superiority was observed with a sterically hindered 2,6-dimethylphenyl organocopper reagent: the

transmetalation from 2,6-dimethylphenylmagnesium bromide to 2,6-dimethylphenylzinc chloride produced **2.10i** with an increase in the yield from 65 to 96% (entry 9).

As a limitation of this reaction, the organocopper reagent that was prepared from *O*-alkyl compounds such as the $TBSO(CH_2)_3Li$ (Narumi et al. 2010) or the reagent that was prepared from copper salts and a β-alkyl compound reported by Sawamura group (Nagao et al. 2012) led to multiple products.

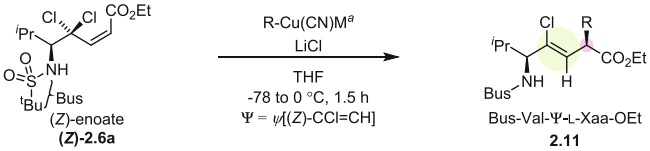
This exclusive formation of L,D-type isosteres (**2.10a–i**) with a (*Z*)-alkene structure suggests that allylic alkylation of **(*E*)-2.6a** can occur only by the stereospecific facial attack of organocuprates on allylic electrophiles. The general anti-selectivity in organocopper-mediated S_N2' reactions leads to the invoking of two conformers **A** and **B**, either of which can allow the antiparallel position of the organocuprate and the leaving chloride group (Scheme 2.5). Although steric repulsion between the isopropyl group and an olefinic proton could partly destabilize the conformer **A**, the steric repulsions could be reduced between C4, which bears two chlorines, and C5, which bears the bulky isopropyl and Bus-protected amino groups, as shown in the staggered conformation **C**. Here, the facial attack of organocopper reagents would be supported by the coordination effect of the sulfonamide group at C5, leading to the exclusive formation of **2.10a**. On the other hand, there is potential steric repulsion between the two chlorine atoms and the isopropyl group in the other staggered conformation **D**, resulting in the destabilization of the conformer **B** preventing the formation of **2.10a**.

To better rationalize the observed diastereoselectivity and stereochemical outcome, we have considered switching from an (*E*)-enoate to a (*Z*)-enoate, which would lead to the stereoselective formation of L,L-type isosteres. Thus, (2*Z*)-γ,γ-dichloro-α,β-enoate [**(*Z*)-2.6**] was prepared and applied to the allylic alkylation (Table 2.5). It was found that **(*Z*)-2.6** also works efficiently in the allylic alkylation, providing the corresponding α-alkylated products (**2.11a–i**) without a significant



Scheme 2.5 Possible mechanism of diastereoselectivity via 1,4-asymmetric induction

Table 2.5 Scope of diastereoselective allylic alkylation of **(*Z*)-2.6**



Entry	**R**Cu(CN)M[a]	Bus–Val–**Ψ**–L–Xaa–OEt	*Z*/*E*[b]	dr[b]	Yield (%)[c]
1	MeCu(CN)ZnCl	Bus–Val–**Ψ**–Ala–OEt	>20:1	>20:1	**2.11a**, 99
2	EtCu(CN)ZnCl	Bus–Val–**Ψ**–Abu(2)–OEt	>20:1	>20:1	**2.11b**, 95
3	^{n}BuCu(CN)ZnCl	Bus–Val–**Ψ**–Nle–OEt	>20:1	>20:1	**2.11c**, 92
4	^{i}BuCu(CN)ZnBr	Bus–Val–**Ψ**–Leu–OEt	>20:1	>20:1	**2.11d**, 92
5	BnCu(CN)ZnBr	Bus–Val–**Ψ**–Phe–OEt	>20:1	>20:1	**2.11e**, 99
6	(2-naphthyl) Cu(CN)ZnCl	Bus–Val–**Ψ**–Nal–OEt	>20:1	>20:1	**2.11f**, 99
7	$EtO_2C(CH_2)_2$ Cu(CN)ZnCl	Bus–Val–**Ψ**–Glu(OEt)–OEt	>20:1	>20:1	**2.11g**, 93
8	AllylCu(CN)ZnCl	Bus–Val–**Ψ**–(Allyl)Gly–OEt	>20:1	>20:1	**2.11h**, 51
9	2,6-diMePh Cu(CN)ZnCl	Bus–Val–**Ψ**–(2,6-diMePh)Gly–OEt	>20:1	>20:1	**2.11i**, 94

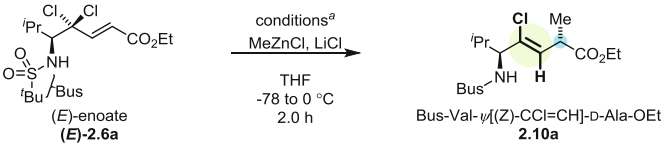
[a]All reactions were carried out at −78 °C for 1.5 h on a 0.3 mmol scale with 4 equiv of organocuprates in the presence of Li salts
[b]Determined by ^{1}H NMR with the unpurified reaction mixture
[c]Yields of isolated products

decrease of reaction yields or diastereoselectivity (entries 1–9). As expected, the stereochemistry of **2.11a–i** corresponded to L,L-type isosteres, indicating that allylic alkylation of allylic *gem*-dichlorides proceeded through the stereospecific facial attacks of organocuprates, which was a key intermediate in the stereochemical outcome.

2.1.6 Stereoselective Synthesis of Xaa-Yaa Type (Z)-Chloroalkene Dipeptide Isosteres by Treatment of Allylic Gem*-Dichlorides via Organocopper Reagents Prepared with a Quantity of Catalyst or Equivalent Copper (I) Salt and Organozinc Reagents*

According to the previous sections concerning various synthesized CADIs, we examined the possibility of minimizing the quantities of copper salts necessary for the preparation of organocopper reagent as an improvement of atom economy (Trost et al. 1991; Trost 1995) (Table 2.6). Treatment of **(*E*)-2.6a** with 50 mol% of

Table 2.6 Reactivity of **(*E*)-2.6a** with organocopper reagents by the difference of the amount of copper salts (I)



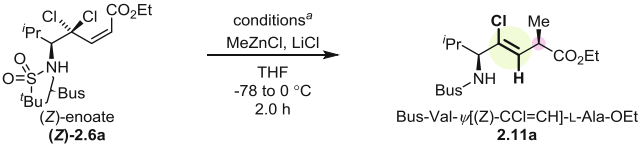
Entry	Copper salt[b]	**2.10a**, *Z*/*E*[c]	**2.10a**, dr[c]	**2.10a**, yield (%)[d]
1	50 mol% CuCN	>20:1	>20:1	83
2	50 mol% CuI	>20:1	>20:1	94
3	30 mol% CuI	>20:1	>20:1	93
4	20 mol% CuI	>20:1	>20:1	80
5	10 mol% CuI	>20:1	>20:1	63
6	20 mol% CuBr	>20:1	>20:1	73
7	20 mol% CuCl	>20:1	>20:1	88
8	20 mol% Cu(OAc)	>20:1	>20:1	78
9	20 mol% Cu(TC)	>20:1	>20:1	78
10	30 mol% CuCl	>20:1	>20:1	99
11[b]	30 mol% CuCl	>20:1	>20:1	99

[a]All reactions were carried out at −78 °C for 1.0 h on a 0.3 mmol scale with 4.0 equiv of organozinc reagents and 8.0 equiv of LiCl
[b]With 2.4 equiv of organozinc reagents
[c]Determined by ^{1}H NMR with the unpurified reaction mixture
[d]Yields were determined by ^{1}H NMR of the unpurified reaction mixture using 1,4-dinitrobenzene as an internal standard

CuCN or CuI and the organozinc reagent afforded the diastereoselective allylic alkylation (**2.10a**) in high yield and with high diastereoselectivity (entries 1–2). Decreasing the amount of copper salt to 20 mol% gave similar results (entries 3–5). The alkylated compound (**2.10a**) was also produced by other copper salts such as CuBr, CuCl, Cu(OAc), and Cu(TC) (entries 6–9). CuCl gave the best results among copper salts (entry 7). After evaluation of the amount of CuCl, the use of 30 mol% CuCl was identified as providing excellent yield and diastereoselectivity (entry 10), and decreasing the amount of organozinc reagent to 2.4 equivalents gave the same result (entry 11). Based on these results, 30 mol% CuCl (entry 11) was adopted as the best quantity for this reaction.

When using copper salts and organozinc reagents as catalysts, we considered producing L,L-type CADIs (Bus–Val–ψ[(*Z*)–CCl=CH]–L–Yaa–OEt) by switching olefin geometry in substrates from an (*E*)-enoate to a (*Z*)-enoate with a small amount of copper salt. Thus, the (*Z*)-enoate [**(*Z*)-2.6a**] was prepared and applied to the allylic alkylation (Table 2.7). Although the reaction with a catalytic amount of copper salt led to a low yield of the α-alkylated product (entries 1–4), copper salts and organozinc reagents provided compound **2.11a** in high yield (entry 5).

Table 2.7 Reactivity of **(*Z*)-2.6a** with organocopper reagents by the difference of the amount of copper salts (I)



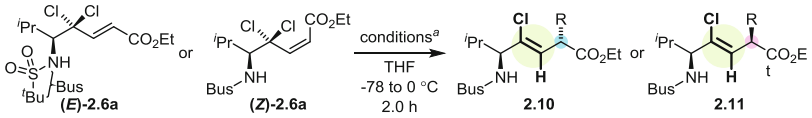
Entry	Copper salt	**2.11a**, *Z*/*E*[b]	**2.11a**, dr[b]	**2.11a**, yield (%)[c]
1	50 mol% CuCN	>20:1	>20:1	5.7
2	30 mol% CuCl	>20:1	>20:1	11
3	50 mol% CuCl	>20:1	>20:1	48
4	100 mol% CuCl	>20:1	>20:1	92
5	140 mol% CuCl	>20:1	>20:1	99

[a]All reactions were carried out at −78 °C for 2.0 h on a 0.3 mmol scale with 2.4 equiv of organozinc reagents and 8.0 equiv of LiCl
[b]Determined by ^{1}H NMR with the unpurified reaction mixture
[c]Yields were determined by ^{1}H NMR of the unpurified reaction mixture using 1,4-dinitrobenzene as an internal standard

Accordingly, we selected 140 mol% CuCl as the optimal condition. Once the optimal condition for providing L,D- or L,L-type CADIs was identified, the scope of this diastereoselective allylic alkylation was explored (Table 2.8). Treatment of the substrates, **(*E*)-2.6a** and **(*Z*)-2.6a** with the organocopper reagents, prepared from the 30 mol% of the copper catalyst and 140 mol% of the organozinc reagents, provided the corresponding α-alkylated products **2.10a**, **d**, **e**, **g**, and **h** and **2.11a**, **d**, **e**, **g**, and **h**, respectively, in high yield and diastereoselectivity (entries 1–10). Especially, the decreased quantity of copper reagent afforded α-allylation products in higher yield than the cyanocuprate (entries 5 and 10). In case, treatment of the substrate (*E*)- or (*Z*)-enoate with 4 equiv of organocuprates provided the alkylated product in 54 or 51% isolated yield, respectively (Table 2.4 entry 8 and Table 2.5 entry 8).

To investigate whether this reaction could provide various α-alkylated products while reducing the amount of copper salts used, we explored the reactivity of substrates in this diastereoselective allylic alkylation. Treatment of substrates of various (*E*)- or (*Z*)-enoates under the optimized conditions led to the production of the corresponding alkylated compounds (Table 2.9). In all cases, the diastereoselective allylic alkylation products (**2.12a–c** and **2.13a–c**) were formed with excellent *Z*-selectivity (>20:1). The substrates with sterically bulky alkyl groups or an aromatic group also produced alkylated products in excellent yields and high diastereoselectivity (>20:1) regardless of the other alkyl groups present.

Table 2.8 Scope of diastereoselective allylic alkylation of **(*E*)-** or **(*Z*)-2.6a**



Entry	Substrate	Copper salt (I)	RZnX	*Z*/*E*[b]	dr[b]	**2.10** or **2.11**, yield[c] (%)
1	**(*E*)-2.6a**	30 mol% CuCl	MeZnCl	>20:1	>20:1	**2.10a**, 99
2	**(*E*)-2.6a**	30 mol% CuCl	^{i}BuZnBr	>20:1	>20:1	**2.10d**, 98
3	**(*E*)-2.6a**	30 mol% CuCl	BnZnBr	>20:1	>20:1	**2.10e**, 97
4	**(*E*)-2.6a**	30 mol% CuCl	$EtO_2(CH_2)_2ZnBr$	>20:1	>20:1	**2.10g**, 98
5	**(*E*)-2.6a**	30 mol% CuCl	AllylZnCl	>20:1	>20:1	**2.10h**, 70
6	**(*Z*)-2.6a**	140 mol % CuCl	MeZnCl	>20:1	>20:1	**2.11a**, 97
7	**(*Z*)-2.6a**	140 mol % CuCl	^{i}BuZnBr	>20:1	>20:1	**2.11d**, 95
8	**(*Z*)-2.6a**	140 mol % CuCl	BnZnBr	>20:1	>20:1	**2.11e**, 94
9	**(*Z*)-2.6a**	140 mol % CuCl	$EtO_2(CH_2)_2ZnBr$	>20:1	>20:1	**2.11g**, 96
10	**(*Z*)-2.6a**	140 mol % CuCl	AllylZnCl	>20:1	>20:1	**2.11g**, 66

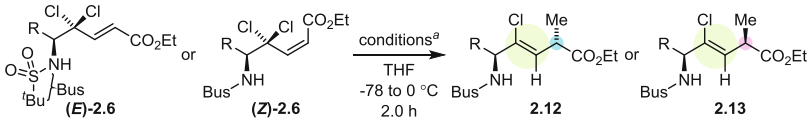
[a]All reactions were carried out at −78 °C for 30 min on a 0.3 mmol scale with 30 or 140 mol% of CuCl and 2.4 equiv of organozinc reagents in the presence of Li salts
[b]Determined by ^{1}H NMR with the unpurified reaction mixture
[c]Yields of isolated products

2.1.7 Conclusion

In conclusion, a method has been developed for the stereoselective synthesis of isosteres such as Bus–L–Xaa–ψ[(*Z*)–CCl=CH]–Gly–OEt using organocuprate-mediated reduction. In spite of the limitations posed by nitrogen-containing substrates, this reaction produces the *Z*-chloroalkene in high yields with excellent *Z*-selectivity. In addition, transformation of the protecting group of the amine and hydrolysis of the ester can be performed. These findings have shown the feasibility of the synthesis of Xaa-Gly type isosteres.

In addition, a highly diastereoselective allylic alkylation of allylic *gem*-dichlorides with organocuprates via 1,4-asymmetric induction has been performed. Although the limitation of prepared organocopper reagents exists, the diastereoselective synthesis of both L,L-type and L,D-type (*Z*)-chloroalkene dipeptide isosteres

Table 2.9 Tolerance of substrates **(*E*)-** and **(*Z*)-2.6** in diastereoselective allylic alkylation



Entry	Substrate	R	Copper salt (I)	*Z*/*E*[b]	dr[b]	**2.12** or **2.13**, yield[c] (%)
1	**(*E*)-2.6a**	^{i}Pr	30 mol% CuCl	>20:1	>20:1	**2.12a**, 99
2	**(*E*)-2.6b**	^{i}Bu	30 mol% CuCl	>20:1	>20:1	**2.12b**, 93
3	**(*E*)-2.6c**	^{n}Bu	30 mol% CuCl	>20:1	>20:1	**2.12c**, 98
4	**(*E*)-2.6d**	Bn	30 mol% CuCl	>20:1	>20:1	**2.12d**, 95
5	**(*Z*)-2.6a**	^{i}Pr	140 mol% CuCl	>20:1	>20:1	**2.13a**, 97
6	**(*Z*)-2.6b**	^{i}Bu	140 mol% CuCl	>20:1	>20:1	**2.13b**, 96
7	**(*Z*)-2.6c**	^{n}Bu	140 mol% CuCl	>20:1	>20:1	**2.13c**, 97
8	**(*Z*)-2.6d**	Bn	140 mol% CuCl	>20:1	>20:1	**2.13d**, 96

[a]All reactions were carried out at −78 °C for 30 min on a 0.3 mmol scale with 30 or 140 mol% of CuCl and 2.4 equiv of MeZnCl in the presence of Li salts
[b]Determined by ^{1}H NMR with the unpurified reaction mixture
[c]Yields of isolated products

can be achieved by this strategy in high yield and with excellent (*Z*)-selectivity and diastereoselectivity. The use of zinc–copper reagents was found to increase the chemical yields and product selectivity of the reaction.

We have also established an efficient synthetic method of L,D- or L,L-type CADIs utilizing diastereoselective allylic alkylation. Treatment of the substrates by switching olefin geometry of allylic *gem*-dichlorides with 30 or 140 mol% CuCl and organozinc reagents can provide various L,D- or L,L-type CADIs in high yield and with excellent diastereoselectivity.

2.2 Application of a Chloroalkene Dipeptide Isostere: Synthesis of a CADI-Containing a Cyclic Arg–Gly–Asp Peptide

2.2.1 Introduction to $\alpha_V\beta_3$ Integrin and Cyclic Arg–Gly–Asp Peptides

The integrin family constitutes cell surface glycoproteins, which consist of α and β subunits, and plays a role in cell–cell and cell–matrix adhesive interaction (Hynes 1987; 1992; Humphries 2000). The integrin family can be divided into four groups

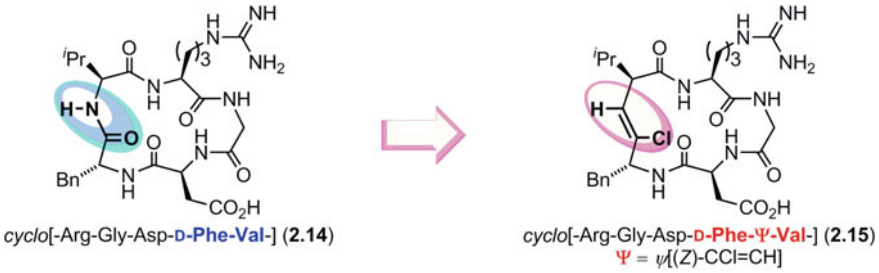


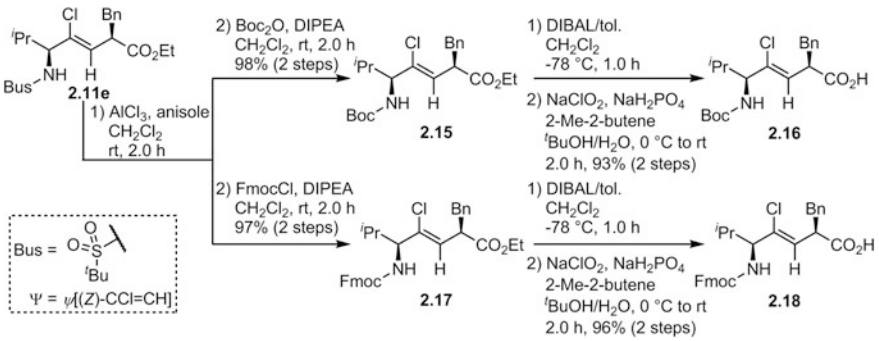
Fig. 2.2 Modification for a CADI-containing cyclic RGD peptide

of receptors, which are characterized by ligands involving leucocytes, collagen, laminin, and Arg–Gly–Asp (RGD). In the large family of integrin receptors, RGD receptors have been a focus in drug discovery research. The $\alpha_V\beta_3$ integrin complex is a vitronectin receptor. It is expressed in various cells and is involved in tumor-induced angiogenesis (Brooks et al. 1994; Varner and Cheresh 1996; Haier et al. 2002; Shattil et al. 2010; Danhier 2012) and adhesion of osteoclasts to bone matrix (Ross et al. 1993; Schaffner and Dard 2003). Consequently, $\alpha_V\beta_3$ integrin antagonists have been developed as potentially important cancer therapeutics against tumor metastasis and candidate compounds have been designed based on the role of the RGD motif in the recognition sequence of integrin receptors (Stilz et al. 2001; Manzoni et al. 2009; Mas-Moruno et al. 2016). In 1991, Kessler and coworkers reported that highly potent and selective inhibitors based on the Arg–Gly–Asp (RGD) motif led to the discovery of a cyclic pentapeptide, *cyclo*[–Arg–Gly–Asp–D–Phe–Val–] (**4.1**), which is a highly bioactive $\alpha_V\beta_3$ integrin antagonist (Aumailley et al. 1991). The cyclic RGD peptide (**4.1**) possesses the turn motif of βII′/γ, and was proposed to adopt two distinctive secondary structures in DMSO solution; a type II′ β-turn with D–Phe at the $i + 1$ position and a γ-turn with Gly at the $i + 1$ position (Haubner et al. 1996, 1997; Nikiforovich et al. 2000; Xiong et al. 2002).

In this section, an application for peptide synthesis utilizing the synthetic method of chloroalkene dipeptide isosteres (CADIs) and synthesis of a CADI-containing cyclic RGD peptide **4.2** is described. In this peptide, the peptide bond between D-Phe and Val was replaced by a chloroolefin bond in order to enforce an ideal βII′/γ structure, and compound **4.2** has been evaluated as a peptidomimetic (Fig. 2.2).

2.2.2 *Preparation of Bor- or Fmoc-Protected Carboxylic Acids of CADIs for Peptide Synthesis*

Synthetic methods for various L,D- or L,L-type CADIs were established and discussed in the previous section. A Boc-protected carboxylic acid Boc–Val–ψ[(*Z*)–CCl=CH]–Phe–OH (**2.16**) or an Fmoc-protected compound, Fmoc–Val–ψ[(*Z*)–

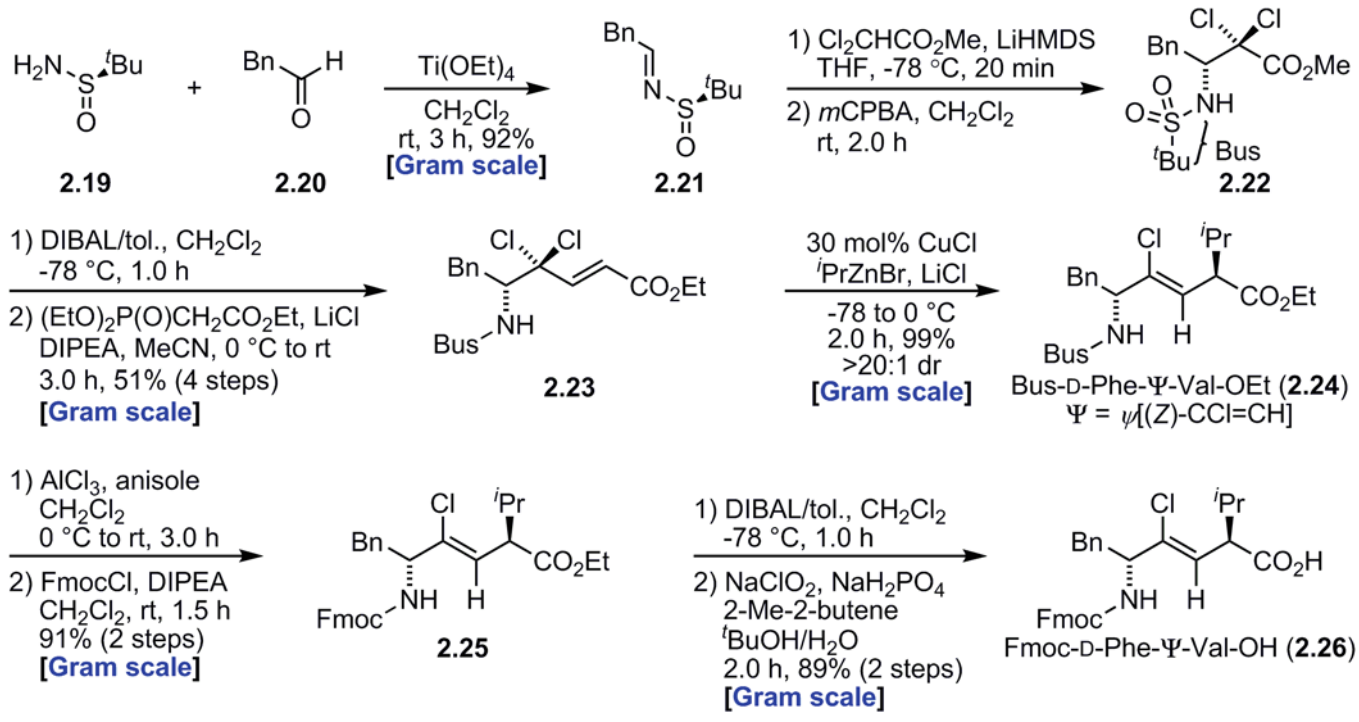


Scheme 2.6 Conversion into Boc- or Fmoc–Val–[(*Z*)–CCl=CH]–Phe–OH (**2.16**, **2.18**)

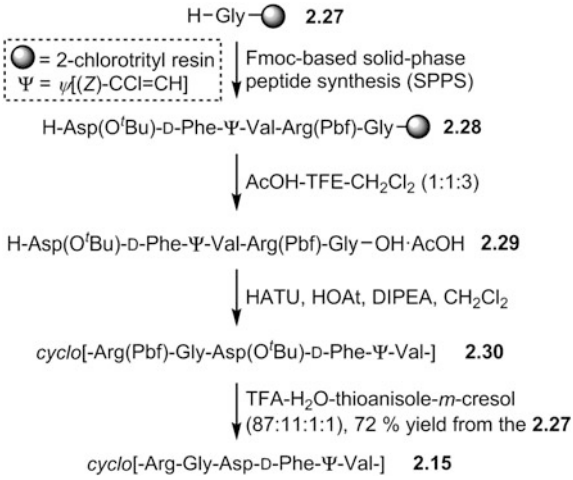
CCl=CH]–Phe–OH (**2.18**) were prepared from a CADI (**2.11e**) for introduction into peptides (Scheme 2.6). Deprotection of the Bus group with $AlCl_3$ and anisole (Sun and Weinreb 1997; Enders et al. 2010; Mita et al. 2013) followed by Boc or Fmoc protection produced Boc-ester (**2.15**) or Fmoc ester (**2.17**), respectively. The ester group of **2.15** or **2.17** was reduced to the corresponding aldehyde at −78 °C and was followed by Pinnick oxidation to provide the desired Boc-protected carboxylic acid Boc–Val–ψ[(*Z*)–CCl=CH]–Phe–OH (**2.16**) or the Fmoc-protected compound Fmoc–Val–ψ[(*Z*)–CCl=CH]–Phe–OH (**2.18**), respectively, in high yield overall from **2.11e** with no epimerization or olefin isomerization. Although the ester group of **2.15** can be hydrolyzed under several basic conditions to provide the corresponding carboxylic acid, these reactions require long reaction time for completion or resulted in low to moderate yields. Easy access to Boc- or Fmoc-protected carboxylic acids from these common intermediates is possible using known methods for the transformation.

2.2.3 *Synthesis of Fmoc–D–Phe–ψ[(Z)–CCl=CH]–Val–OH and Introduction to an RGD Peptide Utilizing Solid-Phase Technique*

Fmoc–D–Phe–ψ[(*Z*)–CCl=CH]–Val–OH (**2.26**) was produced by established synthetic methods. As shown in Scheme 2.7, nucleophilic addition of the lithium enolate of methyl dichloroacetate to the chiral *N*-sulfinylaldimine (**2.21**), prepared from (*R*)-*tert*-butylsulfinamide (**2.19.**) (Robak et al. 2010) and phenylacetaldehyde (**2.20**), was followed by oxidation of the *N*-sulfinyl group with *m*-CPBA to provide the *N*-Bus-protected α,α-dichloro-β-amino ester (**2.22**). Reduction of **2.22** with DIBAL followed by the Horner–Wadsworth–Emmons reaction afforded the desired



Scheme 2.7 Synthesis of Fmoc–D–Phe–ψ[(*Z*)–CCl=CH]–Val–OH



Scheme 2.8 Synthesis of the CADI-containing cyclic RGD peptide utilizing Fmoc-based solid-phase peptide synthesis

(*E*)-enoate (**2.23**). Diastereoselective allylic alkylation utilizing organocopper reagents prepared from 30 mol% CuCl and 2-propylzinc bromide, afforded the desired chloroalkene product (**2.24**) in high yield and with excellent diastereoselectivity. Deprotection of the Bus group with $AlCl_3$ and anisole was followed by Fmoc protection to give the ester **2.25**. The ester group of **2.25** was reduced to the corresponding aldehyde at −78 °C, and this was followed by Pinnick oxidation to provide the desired Fmoc-protected carboxylic acid (**2.26**) in 81% yield from the Bus-protected ester (**2.24**) with no decrease in diastereoselectivity or olefin isomerization. These 10 steps proceeded smoothly to provide the desired compound from starting materials and in this way, the Fmoc-protected carboxylic acid (**2.26**) became available through a gram-scale synthesis in 38% overall yield.

Finally, the synthesis of the CADI containing the RGD peptide was performed by established protocols (Scheme 2.8) (Barlos et al. 1989a, b, 1991). A protected peptide resin (**2.28**) was constructed by Fmoc-based solid-phase peptide synthesis (SPPS) on a glycinyl 2-chlorotrityl (Clt) resin (**2.27**), which provides side chain-protected peptides by subsequent mild acidic treatment (Barlos et al. 1989a, b, 1991). Exposing the resin **2.28** to AcOH–TFE–CH_2Cl_2 (1:1:3) provided the protected peptide (**2.29**), which was cyclized using HATU and HOAt as condensation agents (Belvisi et al. 2001; Manzoni et al. 2009) to give the protected CADI-containing cyclic pentapeptide (**2.30**). In the final step, deprotection of the cyclic peptide (**2.30**) with 87% TFA was carried out, and the crude peptide formed was then purified by HPLC to provide the desired cyclic peptide (**2.15**) with a D–Phe–ψ[(*Z*)–CCl=CH]–Val-type isostere, in 72% yield from the resin **2.27**.

2.2.4 *Evaluation of the Synthesized Cyclic CADI-Containing Peptide,* Cyclo*[–Arg–Gly–Asp–D–Phe–ψ[(Z)–CCl=CH]–Val–]*

The CADI-containing cyclic RGD peptide (**2.15**) was evaluated for its inhibitory effect against integrin-mediated cell attachment and the results are shown in Fig. 2.3. The CADI-containing peptide (**2.15**) showed approximately 20-fold higher inhibitory activity (IC_{50} = 0.497 nM) compared with the Kessler's peptide (**2.14**) (IC_{50} = 10.9 nM). This result shows that the CADI-containing cyclic RGD peptide has 6–10-fold higher inhibitory activity than other pseudopeptides such as an ADI-containing peptide (**2.31**) or a TADI-containing peptide (**2.32**) (Fig. 2.4) (Oishi et al. 2002, 2006).

According to this result, the stabilized whole structure of the CADI-containing cyclic RGD peptide (**2.15**) strongly interacts with $\alpha_V\beta_3$ integrin compared to the parent RGD peptide. This is due to the more highly rigid structure of the chloroalkene (Fig. 2.5a) and the 1,3-allylic strain exerted by the chlorine atom, which is higher than that caused by the parent amide bond (Fig. 2.5b).

2.2.5 *Conclusion*

In this section, successful formation of Boc- or Fmoc-protected carboxylic acids of CADIs from the common intermediate for peptide synthesis was described and led to a gram-scale synthesis of Fmoc–D–Phy–[(*Z*)–CCl=CH]–Val–OH (**2.26**). The CADI developed in this way was also applicable to Fmoc-based solid-phase peptide synthesis. In addition, introduction of CADI to a bioactive cyclic RGD peptide was achieved, and the synthesized CADI-containing cyclic RGD peptide as a peptidomimetic was shown to be an inhibitor superior to the parent peptide.

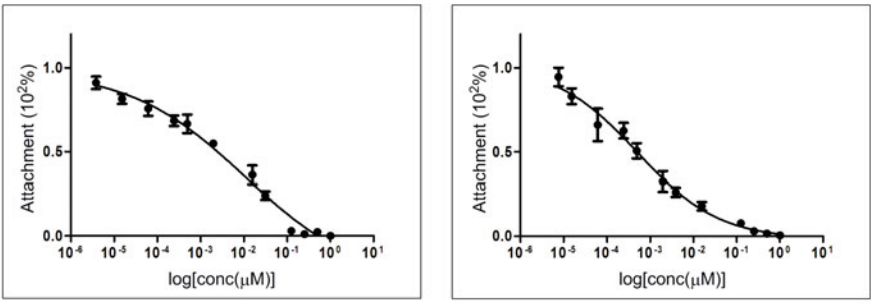


Fig. 2.3 The inhibitory effect of cyclic RGD peptides against HDF attachment to vitronectin

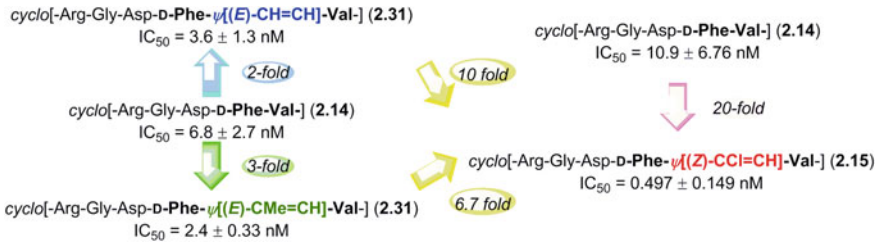


Fig. 2.4 Comparison of inhibitory activity of other peptidomimetics against HDF attachment to vitronectin

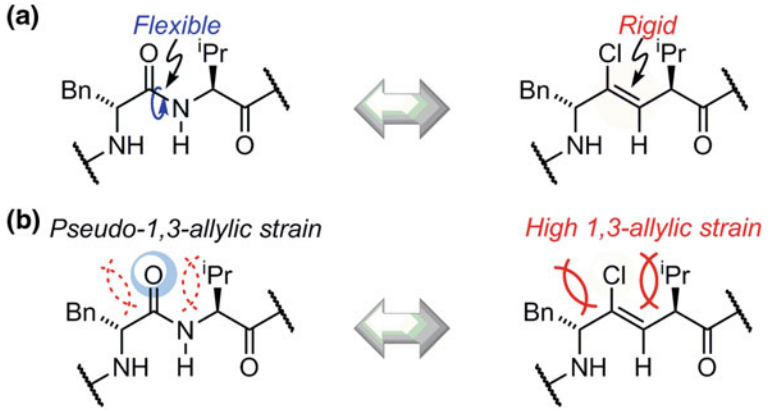


Fig. 2.5 The whole structure stabilized by the chloroalkene structure. **a** Highly rigid structure of the chloroalkene; **b** the 1,3-allylic strain exerted by the chlorine atom, which is higher than that caused by the parent amide bond

2.3 Summary and Future Perspectives of CADI

2.3.1 Summary

Treatment of γ,γ-dichloro-α,β-enoyls as substrates with organocopper reagents has led to chloroalkene structures via a stereoselective induction manner in a one-pot organocuprate-mediated reduction/asymmetric alkylation. In addition, stereoselective S_N2' type allylic alkylation also has been shown to proceed via 1,4-asymmetric induction (Fig. 2.6).

As mentioned above, as an extension of these reactions, the synthesis of various chloroalkene dipeptide isosteres has been developed (Fig. 2.7). Treatment of allylic *gem*-dichlorides with higher order organocuprates has led to L-Xaa-Gly type isosteres. In addition, using diastereoselective allylic alkylation, treatment of the allylic *gem*-dichlorides as substrates has provided the corresponding L,D- or L,L-type CADI by switching olefin geometry with 30 or 140 mol% CuCl and organozinc reagents.

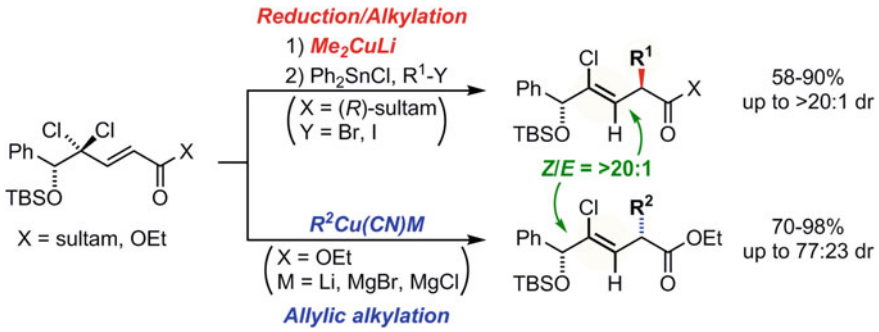


Fig. 2.6 Stereoselective formation of trisubstituted (*Z*)-chloroalkenes adjacent to a tertiary carbon stereogenic center by organocopper reagents

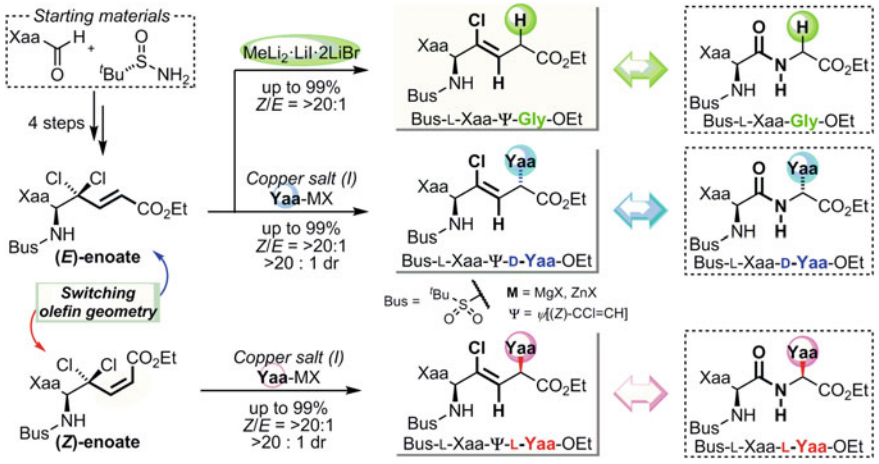


Fig. 2.7 Synthesis of Xaa-Yaa type (*Z*)-chloroalkene dipeptide isosteres utilizing organocopper reagents with allylic *gem*-dichlorides

Conversion of compounds obtained in the previous section to Boc- or Fmoc-protected carboxylic acids via common intermediates has succeeded in the utilization of peptide synthesis in a gram-scale synthesis. The prepared CADIs are also applicable to Fmoc-based solid-phase peptide synthesis and, in addition, introduction of CADI to a bioactive cyclic RGD peptide was applied, and the synthesized CADI-containing cyclic RGD peptidomimetic was identified as a better inhibitor than the parent peptide (Fig. 2.8).

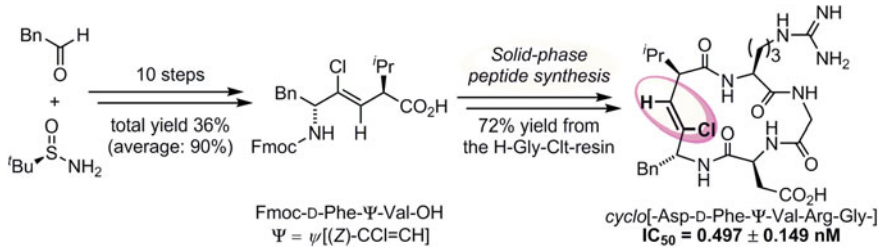


Fig. 2.8 Application of a chloroalkene dipeptide isostere

2.3.2 *Future Perspectives*

Chloroalkene structures with stereogenic centers are expected to work as platforms in many synthetic processes. Such processes can be performed by functional transformation (Nemoto et al. 1988; Bhattacharya et al. 2006; Jastrzebska et al. 2007; Ma and Herzon 2016; Clough et al. 2005), cross-coupling reactions (Chow et al. 2012; Fleming and Pearce 1980; Hudrlik et al. 1983; Lambert and Wang 1988; Córsico and Rossi 2004), or formation of carbon–carbon bonds (Fig. 2.9) (Tan and Negishi 2006; Guinchard et al. 2009). In this way, this study is considered to be a contribution to synthetic organic chemistry.

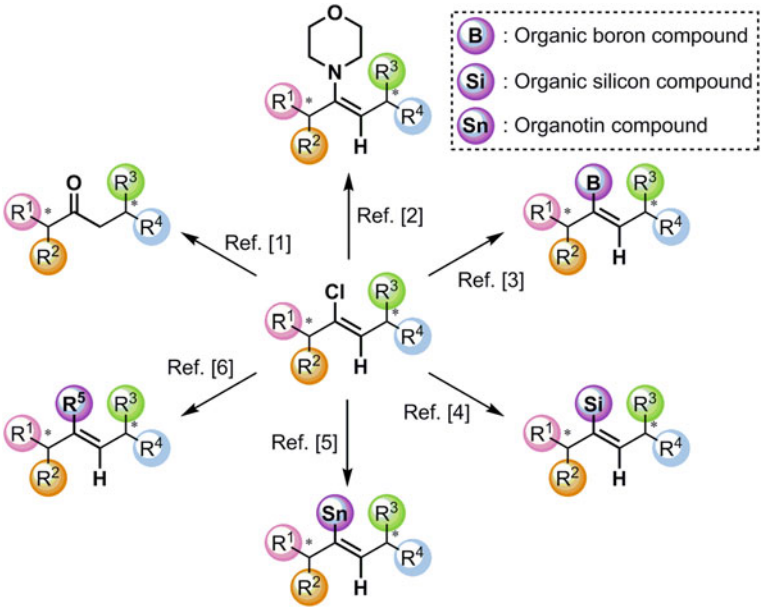


Fig. 2.9 Transformation of chloroalkene structures including stereogenic centers

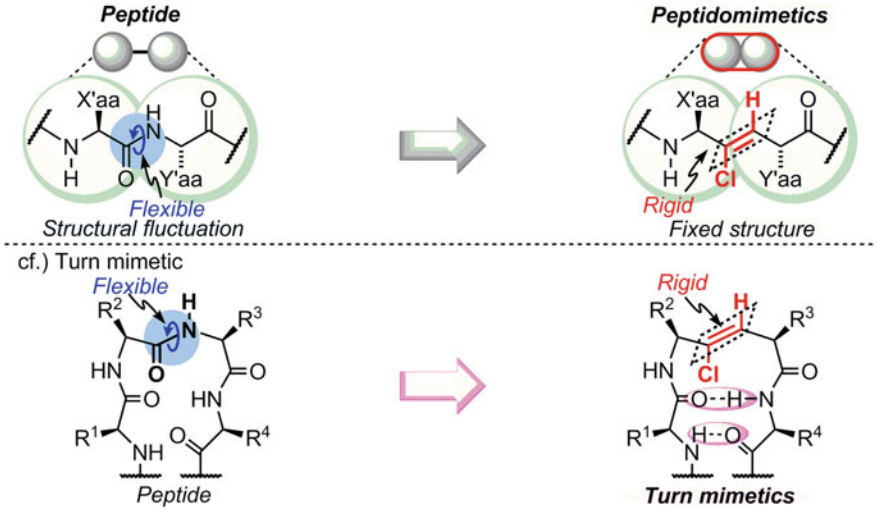


Fig. 2.10 Utilization of regulated configuration around chloroalkene

Through the development of the synthetic strategies of CADIs, easy access to Fmoc-protected carboxylic acid derivatives of CADIs has become possible, more than access to other alkene-type dipeptide isostere derivatives (Oishi et al. 2002, 2006). Although the direct alkylation failed, transformation to hydrophilic functional groups in side chains could be expected by synthesis from a CADI-containing allyl group (Narumi et al. 2008). These results are a contribution to medicinal chemistry based on peptidomimetics. For example, the conversion of natural peptide bonds to CADIs might confer higher resistance to enzymatic degradation (Misu et al. 2014), and have structural effects involving promotion of secondary structures such as β- and/or γ-turns on other bioactive peptides due to regulated configuration around chloroolefin conformations (Fig. 2.10) (Oishi et al. 2008). In addition, exchange from an appropriate dipeptide sequence to a CADI leads to the possibility of functional modifications such as transformation from an agonist to an antagonist (Fig. 2.11) (Fujimoto et al. 1996).

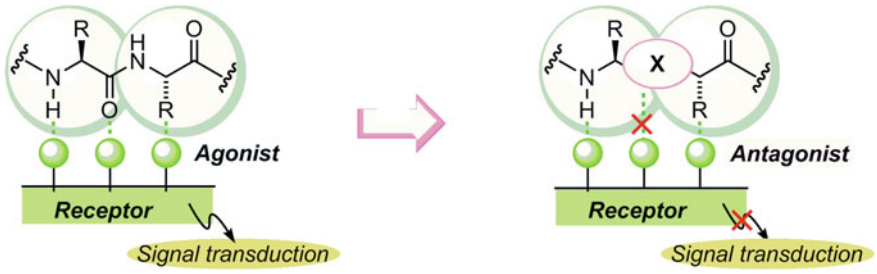


Fig. 2.11 Transformation of mechanism from an agonist to an antagonist

The development of chloroalkene structures and CADIs might lead to new peptidomimetics. Since research into innovative drug development based on peptides is now increasing (Craik et al. 2013; Albada and Metzler-Nolte 2016), this study is expected to be a contribution to organic chemistry, peptide chemistry, and medicinal chemistry.

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Chapter 3
Conformational-Restricted Cyclic Peptides

Abstract Peptides are important biological molecules and have various physiological actions. Thus, these might be great candidates for drugs. Cyclic peptides are useful to find biologically active molecules because structural conformation of these compounds might be determined relatively easily, and especially cyclic pentapeptides are applicable as conformationally restricted templates. To date, several antihuman immunodeficiency virus (HIV) drugs such as reverse transcriptase inhibitors, protease inhibitors, and integrase inhibitors have been developed, and the use in combination of these drugs has brought great success in the treatment of HIV-infected and acquired immunodeficiency syndrome (AIDS) patients. We have developed several anti-HIV agents including CXCR4 antagonists, allosteric type integrase inhibitors, fusion inhibitors, vaccines, CD4 mimics, and matrix/capsid fragment peptides. These have been developed based on the corresponding peptides and proteins, and might be useful for an expansion of the drug repertoire. This chapter describes the development of CXCR4 antagonists based on conformational-restricted cyclic peptides. Bivalent CXCR4 ligands linked with two molecules of an FC131 derivative, [*cyclo*(–D–Tyr–Arg–Arg–Nal–D–Cys–)], connected by poly (L-proline) or PEGylated poly(L-proline) linkers having 5.5–6.5 nm lengths show maximum binding affinity for CXCR4, suggesting that the native state of the CXCR4 dimer has the distance between the ligand binding sites (5.5–6.5 nm). Fluorescent-labeled bivalent ligands are useful tools for the detection of cancer cells that overexpress CXCR4 on the surface. In addition, bivalent CXCR4 ligands linked with two molecules of a T140 derivative are expected as the therapeutic potential for cancer/leukemia.

Keywords HIV · Chemokine receptor · CXCR4 antagonist
Conformational-restricted cyclic peptide · T140 · Bivalent CXCR4 ligand

H. Tamamura et al., *Mid-size Drugs Based on Peptides and Peptidomimetics*,
SpringerBriefs in Pharmaceutical Science & Drug Development,
https://doi.org/10.1007/978-981-10-7691-6_3

3.1 Introduction to HIV

We have developed several peptidomimetics, which target human immunodeficiency virus (HIV). Thus, introduction concerning HIV is described in this section. HIV, the causative virus of acquired immunodeficiency syndrome (AIDS), was discovered by Montagnier et al. in 1983. HIV infects human cells to destroy their immune systems and cause immunodeficiency. The number of people with HIV infection in the world has certainly been beyond 30 million and several anti-HIV drugs have been developed in the last 30 years (Fig. 3.1). HIV is classified into retroviruses. DNA is produced from its RNA genome via the enzyme reverse transcriptase and is then incorporated into the host genome by an integrase enzyme. The first generation of anti-HIV drugs that were initially used for clinical treatment was reverse transcriptase inhibitors such as azidothymidine (AZT) (Mitsuya and Erickson 1999), which suppress the enzyme action and block reverse transcription. The second generation of drugs that were clinically used consisted of protease inhibitors, which prevent the cleavage of HIV precursor proteins into active proteins. These drugs are usually administered in two- or three-drug cocktails in highly active antiretroviral therapy (HAART), which has brought great success and hope in the clinical treatment of HIV infection and AIDS (Mitsuya and Erickson 1999). HAART enables to lower the HIV level in the blood to below the detection level, but has considerable side effects, the emergence of multidrug-resistant (MDR) HIV-1 strains, and high costs. By these serious drawbacks, we have been encouraged to develop novel drugs with different action mechanisms.

The HIV-1 replication mechanism involving the dynamic supramolecular mechanism at the HIV entry and fusion steps has been elucidated and is shown in Fig. 3.1. At first, an HIV envelope protein, gp120, interacts with a cellular surface protein, CD4. This phenomenon causes a conformational change in gp120 and its subsequent binding to the cellular receptors, chemokine receptors such as CCR5 (Alkhatib et al. 1996; Choe et al. 1996; Deng et al. 1996; Dragic et al. 1996) and CXCR4 (Feng et al. 1996). CCR5 and CXCR4 are the main coreceptors used for the entry of macrophage-tropic (R5-) and T cell line-tropic (X4-) HIV-1, respectively. This binding triggers the exposure of another envelope protein, gp41, and the penetration of its N-terminus into the cell membrane, followed by the formation of the trimer-of-hairpins structure of gp41, which causes fusion of HIV to the cell membrane, completing the infection process (Chan and Kim 1998). The identification of this dynamic molecular machinery has encouraged us to develop new inhibitors which suppress HIVentry and/or fusion targeting the receptors, CD4, CCR5, and CXCR4, and the viral proteins, gp120 and gp41.

The first “fusion inhibitor”, enfuvirtide (fuzeon/T-20) (Roche/Trimeris), was approved by the Food and Drug Administration (FDA) in 2003 to treat patients with advanced HIV in combination with other anti-HIV drugs (Wild et al. 1993). A CCR5 coreceptor antagonist, maraviroc (Pfizer), was approved by the FDA in 2007 for use in combination with other anti-HIV drugs as an entry inhibitor for the treatment of patients infected with R5-HIV-1 (Walker et al. 2005). In 2007, the

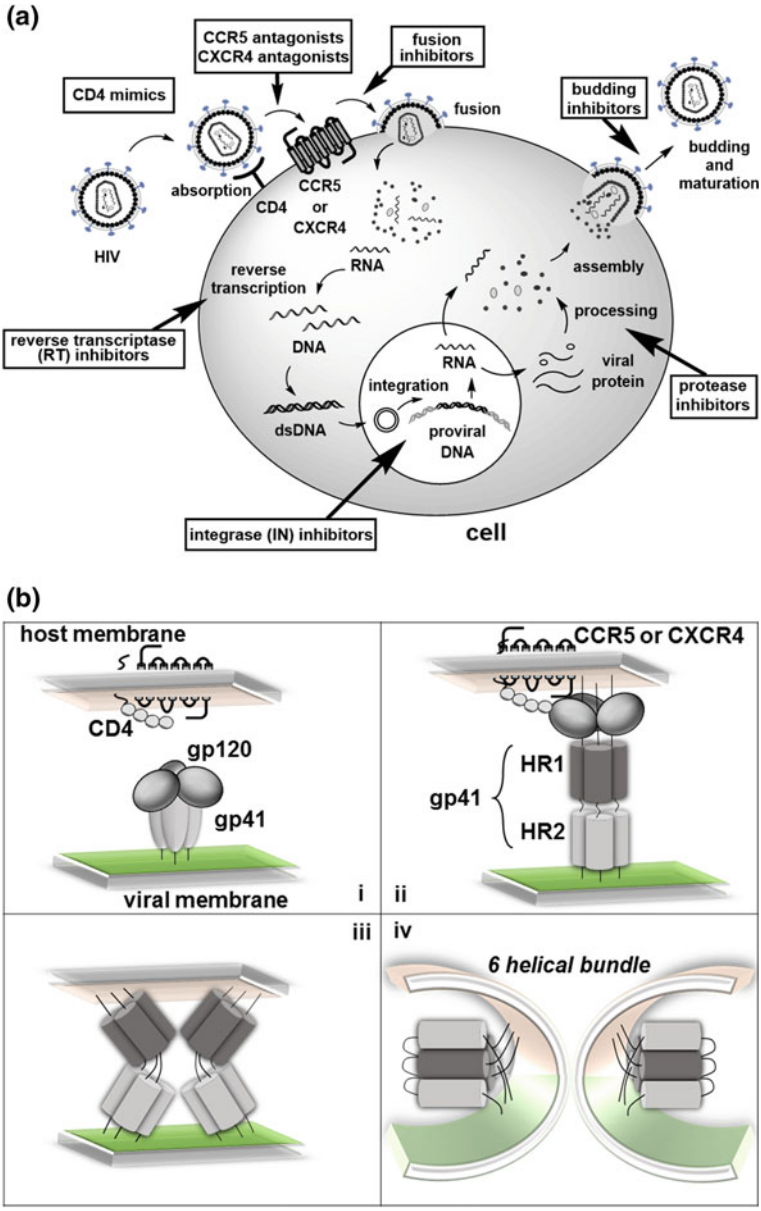

Fig. 3.1 **a** HIV-1 replication cycle and anti-HIV drugs that are effective at its various steps; **b** mechanisms of HIV-1 entry and fusion

FDA approved the first "integrase inhibitor", raltegravir (Isentress) (Merck Sharp & Dohme Corp.) (Cahn and Sued 2007; Grinsztejn et al. 2007). Subsequently, elvitegravir (Gilead Sciences, Inc./JT) was approved by the FDA in 2012 as an integrase inhibitor for use in patients starting treatment of HIV infection for the first time (Shimura et al. 2008; Sax et al. 2012). In 2013, dolutegravir (Shionogi/GSK) was also approved by the FDA as an integrase inhibitor, which has been marketed as Tivicay (Raffi et al. 2013). This book adopts CXCR4 antagonists, integrase inhibitors, vaccines/fusion inhibitors, CD4 mimics, and matrix/capsid fragment peptides, which include our research.

3.2 Chemokine Receptor CXCR4 Antagonists Based on Conformational-Restricted Cyclic Peptides

Interaction of CD4 with an HIV envelope protein gp120 leads to a conformational change, which causes its subsequent binding to the cellular coreceptors, CCR5 and CXCR4 as described in the previous section. CXCR4 is the main coreceptor for the entry of T cell line-tropic (X4-) HIV-1 strains, which are the major species in the late stage of HIV infection and AIDS. CXCR4 antagonists are considered to block entry of X4-HIV-1 into cells. On the other hand, the interaction between CXCR4 and its endogenous ligand, stromal cell-derived factor 1 (SDF-1)/CXCL12 (Nagasawa et al. 1994; Bleul et al. 1996; Oberlin et al. 1996), plays physiological roles in embryogenesis of cardiovascular, hematopoietic, and central nervous systems. This interaction has also been relevant to various pathological conditions such as cancer (Müller et al. 2001; Tamamura et al. 2003a; Takenaga et al. 2004), leukemia (Tsukada et al. 2002; Juarez et al. 2003), and rheumatoid arthritis (Nanki et al. 2000; Tamamura et al. 2004). Therefore, the CXCL12/CXCR4 axis is an excellent drug target, and CXCR4 antagonists might be useful for the treatment of these diseases. Several peptidic and non-peptidic CXCR4 antagonists have been developed to date.

A polyphemusin II analog, 14-mer peptide T140 (**3.1**), has been found by Fujii and Tamamura to be a potent CXCR4 antagonist (Fig. 3.2a) (Tamamura et al. 1998). Tachyplesins and polyphemusins are 17-mer and 18-mer self-defense peptides, which naturally exist in the hemocyte debris of the Japanese horseshoe crab (*Tachypleus tridentatus*) and the American horseshoe crab (*Limulus polyphemus*), respectively, and show broad spectrum antimicrobial activity against several strains of bacteria and viruses (Nakamura et al. 1988; Miyata et al. 1989). Our diligent structure–activity relationship studies on these peptides have produced a polyphemusin analog, T22 ([$Tyr^{5,\ 12}$, Lys^{7}]-polyphemusin II) (Masuda et al. 1992; Nakashima et al. 1992) and a shortened 14-mer peptide, T140, as anti-HIV peptides (Tamamura et al. 1998). T22 and T140 significantly block the X4-HIV-1 entry owing to their competitive binding to CXCR4 (Murakami et al. 1997, 1999; Xu et al. 1999). Four amino acid residues contained in T140, Arg^{2}, L-3-(2-naphthyl)

alanine (Nal)^3, Tyr^5 and Arg^{14}, are indispensable for the expression of high activity (Tamamura et al. 2000). T140 is not sufficiently stable in mouse/feline serum or in rat liver homogenate (Tamamura et al. 2001, 2003b). Thus, it was modified at the N-/C-terminus to suppress the biodegradation. This led to the development of more effective compounds, which have high CXCR4-antagonistic activity and increased biostability. The biostable version of T140 derivatives (Tamamura et al. 2003b, c) has significant inhibitory activity not only against HIV infection but also against cancer/leukemia (Tamamura et al. 2003a; Takenaga et al. 2004): 4F-benzoyl-TN14003/BL-8040/BKT-140 (BioLineRx Ltd.) is a Phase II drug candidate for the treatment of acute myeloid leukemia (AML), and other types of hematological cancer (http://www.biolinerx.com). Furthermore, 4F-benzoyl-TN14003 mobilizes hematopoietic stem cells from the bone marrow into peripheral blood, and induces the mobilization of cancer cells from the bone marrow and other sites thereby exposing these cells to anticancer therapy inducing apoptosis. According to its preclinical studies, 4F-benzoyl-TN14003 is effective, both alone and in combination with the anticancer drug rituximab, in reducing bone marrow metastasis of lymphoma cells and stimulating lymphoma cell death (http://www.biolinerx.com). To develop smaller CXCR4 antagonists, a pharmacophore-guided approach has been performed based on four critical amino acid residues of T140 (Tamamura et al. 2011), Arg^2, Nal^3, Tyr^5, and Arg^{14}, and adopting cyclic pentapeptides as conformationally restricted templates with functional groups, which are used in the efficient discovery of bioactive lead compounds in medicinal chemistry (Fukami et al. 1995; Haubner et al. 1996, 1997; Porcelli et al. 1999; Spatola and Crozet 1996; Wermuth et al. 1997). Screening the library of cyclic pentapeptides using two L/D-Arg, L/D-Nal, L/D-Tyr, and Gly brought the finding of FC131 (**3.2**), which was a potent CXCR4 antagonist comparable to T140 (Fujii et al. 2003) (Fig. 3.2a). The pharmacophore-guided approach based on cyclic pentapeptide templates led to downsizing of T140 (**1**) into FC131 (**3.2**) (Fujii et al. 2003). FC131 derivatives such as compound **3.3**, which contains amidine-type dipeptide isosteres, have been developed. Replacement of amide bonds in FC131, except for the D-Tyr-Arg position, by an amidine group enhanced inhibitory activity against CXCL-12 binding as well as against HIV-1 infection of X4-HIV-1 strains. In addition, these derivatives showed selectivity for CXCR4 (Inokuchi et al. 2011). Based on the β-hairpin structure of polyphemusin II, several protein epitope mimetic (PEM) (Robinson et al. 2008) molecules such as POL3026 (**3.4**) (DeMarco et al. 2006) and POL6326 (**3.5**) (Obrecht et al. 2012), which have potent antagonistic activity against CXCR4, have been designed and developed. POL6326 (**3.5**) has progressed into a Phase II clinical trial for autologous stem cell transplantation in newly diagnosed multiple myeloma patients. FPI-X4 (**3.6**), which corresponds to amino acid residues 408-423 of human serum albumin (HSA), has moderate inhibitory activity against HIV-1 infection by an X4-HIV-1 strain (IC_{50} = 15.8 μM) and also moderate binding affinity (IC_{50} = 8.6 μM) for CXCR4. FPI-X4 (**3.6**) acts as an inverse agonist for CXCR4, therefore it does not induce Ca^{2+} mobilization or receptor internalization (Zirafi et al. 2015). LY2510924 (**3.7**), which was identified based on a rational design approach, specifically blocks CXCL-12

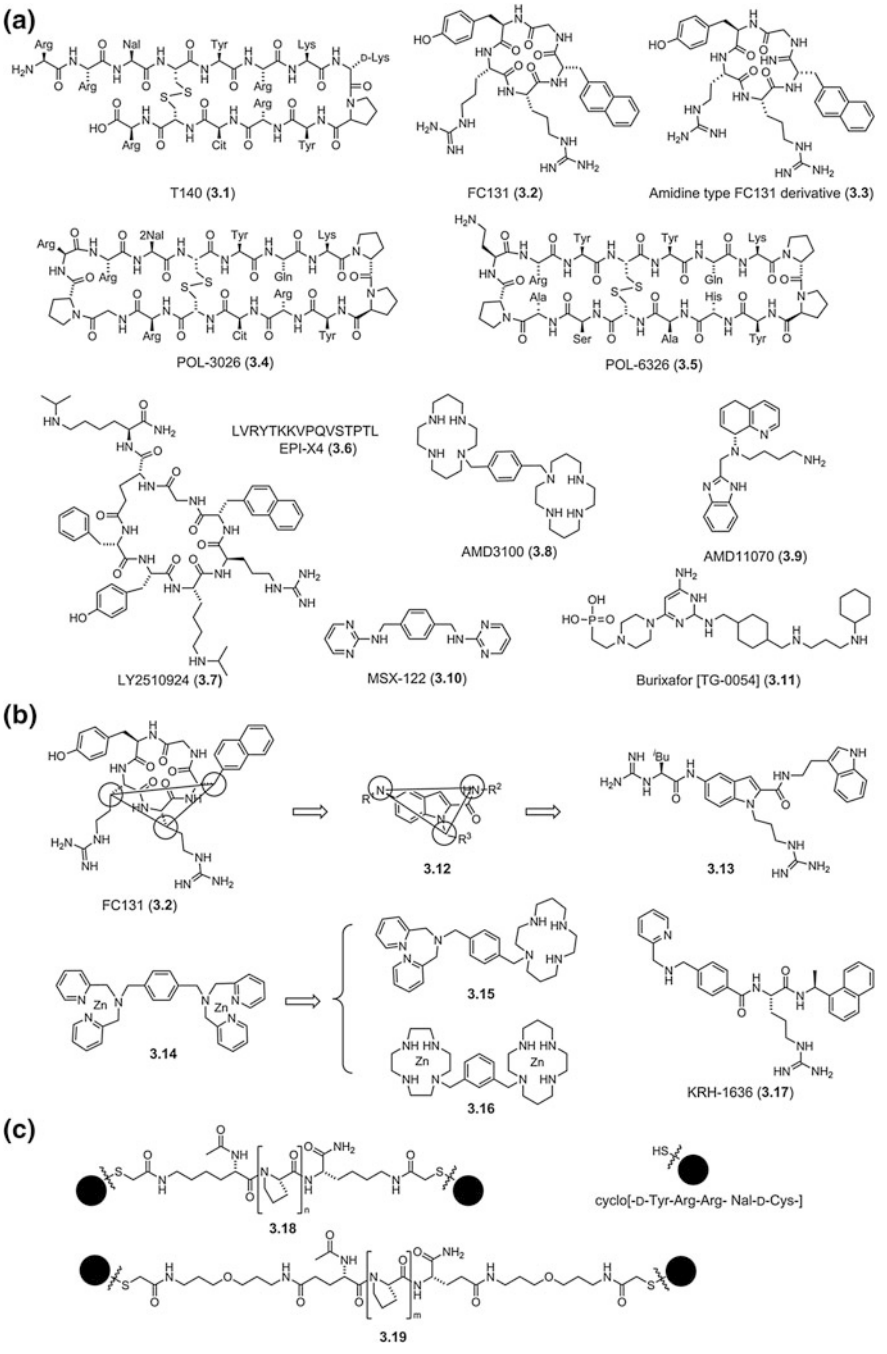

Fig. 3.2 **a** Structures of peptidic CXCR4 antagonists; **b** development of non-peptidic CXCR4 antagonists; **c** structures of bivalent CXCR4 ligands. A maximum increase in binding affinity for CXCR4 was observed in (**3.18**) (n = 20) and (**3.19**) (m = 12)

binding to CXCR4 (IC_{50} = 0.079 nM) (Peng et al. 2015). LY2510924 has now been transferred into Phase II clinical studies for cancer.

A low molecular weight non-peptide, bicyclam AMD3100 (**3.8**) (Genzyme Corp.), was previously reported as the first CXCR4 antagonist to progress to clinical trials for the treatment of HIV-1-infected patients (Fig. 3.2a) (Schols et al. 1997; De Clercq 2003), however, its application as an anti-HIV drug dropped out due to its cardiovascular effects. Furthermore, several small CXCR4 antagonists with potent anti-HIV activity have been reported based on the structure of AMD3100 (**3.8**), which contains at least two nitrogen atoms (e.g., pyridine groups) on each side of the *p*-xylene template (Pettersson et al. 2010; Zhan et al. 2007). However, these compounds depend on rapid oxidative metabolism, involving poor biostability. In succession to AMD3100 (**3.8**), several non-cyclam CXCR4 antagonists have been developed (Bridger et al. 2010; Skerlj et al. 2010; De Clercq 2002). AMD3100 (**3.8**), which was designated as plerixafor/mozibil (Genzyme Corp.), was used as an immunostimulant to mobilize hemopoietic stem cells into the blood in patients with cancer. The stem cells can be extracted from the blood in patients for transplantation by granulocyte colony-stimulating factor (G-CSF). Combination of G-CSF with plerixafor can enhance stem cell transplantation in cancer patients (Wagstaff 2009). A tetrahydroquinoline-containing compound, AMD11070 (AMD070) (**3.9**) (Genzyme Corp.), has been found as an effective CXCR4 antagonist (Stone et al. 2007; Gudmundsson et al. 2009). Antiviral evaluation, pharmacokinetic analysis, and Phase I/II studies of AMD11070 have been performed to assess its effect against X4-HIV-1 infection. MSX-122 (**3.10**), which has been identified by rational design and structural and pharmacologic analysis, is a partial CXCR4 antagonist that fails to mobilize stem cells, which leads to reduction of the risk of long-term blocking of metastasis caused by other CXCR4 antagonists (Liang et al. 2012). TG-0054 (burixafor) (**3.11**) (TaiGen Biotechnology Co., Ltd., Taipei, Taiwan) is a selective CXCR4 antagonist, which is currently in Phase II clinical trials to assess the therapeutic effect of HSC mobilization alone or in combination with G-CSF in patients with multiple myeloma, non-Hodgkin's lymphoma, and Hodgkin's disease (Hsu et al. 2015). Chemical modification of the cyclic pentapeptide FC131 (**3.2**) has produced several non-peptidic CXCR4 antagonists through the use of an indole template modified from the peptide backbone of FC131 (**3.2**) and the disposition of the original pharmacophore moieties (Fig. 3.2b). Compounds linked with three pharmacophore moieties such as compound **3.13** have been found to have CXCR4 antagonistic activity at micromolar levels (Ueda et al. 2008). Nonpeptides with the dipicolylamine (Dpa)-zinc(II) complex structure have been developed as potent CXCR4 antagonists (Tamamura et al. 2006). A Dpa-Zn complex containing a xylene scaffold (**3.14**) binds to CXCR4 at the 50 nM level. Combination of alkylamino and pyridiyl moieties as structural features, which are contained in both the Dpa-Zn complex (**3.14**) and AMD3100 (**3.8**), has led to the development of compounds **3.15** and **3.16** with 30 nM at 10 nM activity levels for binding to CXCR4, respectively (Tanaka et al. 2011). KRH-1636 (**3.17**) (Kureha Chemical & Daiichi Sankyo Co. Ltd.), which was obtained by the intensive modification of the N-terminal tripeptide of T140,

Arg–Arg–Nal, was found to be an orally bioavailable CXCR4 antagonist (Ichiyama et al. 2003). Subsequently, several derivatives such as KRH-2731, which may be promising as novel anticancer drugs, have been developed (Iwasaki et al. 2009). These small CXCR4 antagonists are attractive and useful leads for the chemotherapy against cancer.

3.3 Bivalent CXCR4 Ligands Connected by Rigid Polyproline Linkers

Seven transmembrane G protein-coupled receptors (GPCRs) normally exist as dimers and/or higher order oligomers to express physiological functions. The chemokine receptor CXCR4 belongs to the GPCR family (Percherancier et al. 2005; Berchiche et al. 2007). Thus, we designed and synthesized several bivalent ligands of CXCR4 linked with two molecules of an FC131 derivative, [*cyclo*(–D–Tyr–Arg–Arg–Nal–D–Cys–)], connected by poly(L-proline) or PEGylated poly(L-proline) linkers of various lengths (**3.18**, **3.19**) (Tanaka et al. 2010) (Fig. 3.2c). Poly-L-prolines have been utilized as rigid linkers between two functional units, which form poly(L-proline) helices maintaining a length of 0.9 nm per turn (Arora et al. 2002; Sato et al. 2007; Kuemin et al. 2009; Schuler et al. 2005). A maximum binding affinity for CXCR4 was observed for bivalent ligands with two types of the linkers having 5.5–6.5 nm lengths. This result has shown that the native state of the CXCR4 dimer has the distance between the ligand binding sites (5.5–6.5 nm), and that fluorescent-labeled bivalent ligands are useful tools for cancer detection that can assess the density of CXCR4 on the surface of cancer cells. Thus, we synthesized bivalent CXCR4 ligand s with near-infrared (NIR) dyes at the terminus or the center of the poly-L-proline linker. These are valuable probes which are useful in studies on the behavior of cancer cells with overexpression of CXCR4 (Fig. 3.3) (Nomura et al. 2015a).

T140-related peptides have significant inhibitory activity against cancer/leukemia (Tamamura et al. 2003a; Takenaga et al. 2004). 4F-benzoyl-TN14003/BL-8040/BKT-140 (BioLineRx Ltd.) (Tamamura et al. 2003b, c) is a Phase II drug candidate for the treatment of acute myeloid leukemia (AML), and other types of hematological cancer (http://www.biolinerx.com). Thus, bivalent ligands based on a 14-mer peptide T140 derivative with polyproline linkers have been designed and synthesized (Tanaka et al. 2017). The binding affinity of these bivalent ligands is increased as the linker length increases up to the 12-mer proline linker. The inhibitory activity against migration on Jurkat cells is also correlated to their linker lengths. The T140-derived bivalent ligands with the 9- and 12-mer proline linkers showed the most effective inhibition against migration at 1 μM, which is remarkably higher than that of known CXCR4 antagonists in the monomer structure. As a ligand of bivalent ligands, a 14-mer peptide T140 derivative might be more suitable

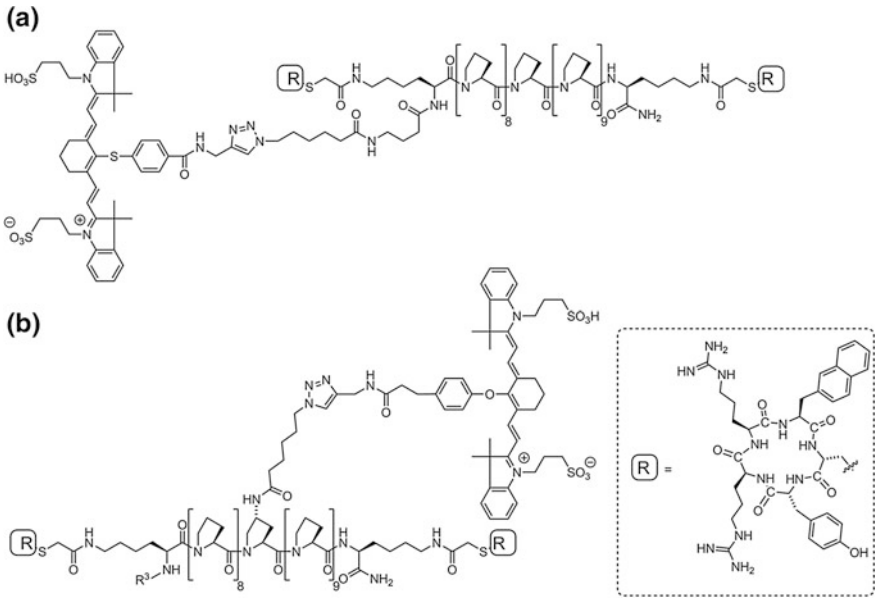

Fig. 3.3 Bivalent CXCR4 ligands labeled with near-infrared (NIR) dyes at the terminus (**a**) or at the center (**b**) of the poly-L-proline linker

than a cyclic pentapeptide FC131 derivative because the former can cover broadly an extensive surface of CXCR4 than the latter. Thus, bivalent T140 derivatives are expected as the therapeutic potential.

3.4 Exploratory Studies on Trivalent CXCR4 Ligands to Identify Their Recognition for the CXCR4 Dimer

To investigate whether CXCR4 might possibly form multimeric oligomers after dimerization, trivalent ligands having rigid poly-L-proline linkers were designed and synthesized. According to our experimental results in biological assays for the CXCR4 binding affinity, the trivalent ligands recognize the dimeric form of CXCR4 on the cellular surface. The highest affinity ligand with 9-L-proline linkers binds to CXCR4 with remarkable specificity, which was confirmed by the fluorescent imaging and flow cytometry analysis. The bivalent and trivalent ligands showed 17- and 47-fold increases in binding activity, respectively, compared to the corresponding monomeric ligand, possibly because of a synergistic effect in the binding of the ligand units. However, the binding activity of the trivalent ligand is approximately threefold higher than that of the bivalent ligand, because three patterns might exist for the dimer recognition in the trivalent ligand (Fig. 3.4) (Nomura et al. 2015b). As a result, it has been proven that CXCR4 does not exist as

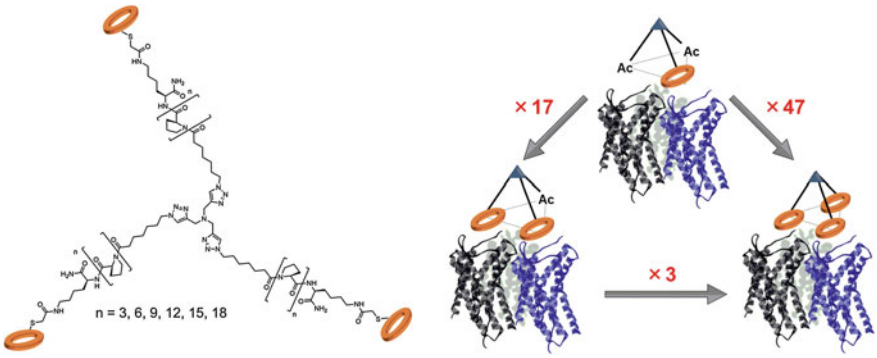

Fig. 3.4 The trivalent ligand designed for exploration of GPCR multimerization shows specific recognition for the CXCR4 dimer. The structure of an orange circle is shown as "R" in Fig. 3.3

the trimer, therefore it is suspected that the dimer units are oligomerized. The multimeric form of CXCR4 will be investigated by the design of oligomeric ligands with rigid linkers in future.

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Chapter 4
Peptidomimetics That Mimic the Secondary Structures of Peptides

Abstract Cyclic peptides are useful to find biologically active molecules because of restricted conformation, as described in the previous chapter. Generally, peptidomimetics point peptide bond isosteres that mimic primary structures of peptides, in the broad sense, include mimetics of secondary and tertiary structures of peptides, which are useful for the development of inhibitors of protein–protein interactions. Peptidomimetics of secondary structures sometimes involve conformational restriction. In this chapter, an example of stapled peptides is introduced as mimetics of α-helical structures. Stapled peptides have a covalent hydrocarbon link formed by ring-closing metathesis reaction between sequential turn pitches of α-helical peptides. This staple strategy was applied to allosteric type integrase inhibitors, which were found through screening an overlapping peptide library derived from HIV-1 gene products. A stapling strategy was adopted in place of octa-arginine conjugation to increase the cell membrane permeability of the above peptides. The stapled peptides showed potent anti-HIV activity in cells comparable to that of the original octa-arginine-conjugated peptide and lower cytotoxicity.

Keywords Stapled peptide HIV integrase inhibitor · Cell membrane permeability

4.1 Introduction to HIV Integrase Inhibitors

HIV-1 integrase (IN) is an enzyme required for stable infection of viral DNA into chromosomal genome of human host cells through 3'-end processing and strand transfer reactions. HIV-IN, a 32 kDa protein, contains 288 amino acid residues, and is composed of an N-terminal, catalytic core, and C-terminal domains (Asante-Appiah and Skalka 1997; Hindmarsh and Leis 1999). The catalytic core domain has three carboxylate residues, Asp^{64}, Asp^{116}, and Glu^{152}, which are critical for coordination with two magnesium ions and catalyzes the 3'-end processing and strand transfer reactions that lead to cleavage and formation of phosphodiester bonds, respectively (Fig. 4.1a) (Ellison and Brown 1994; Vink et al. 1994; Wolfe

H. Tamamura et al., *Mid-size Drugs Based on Peptides and Peptidomimetics*,
SpringerBriefs in Pharmaceutical Science & Drug Development,
https://doi.org/10.1007/978-981-10-7691-6_4

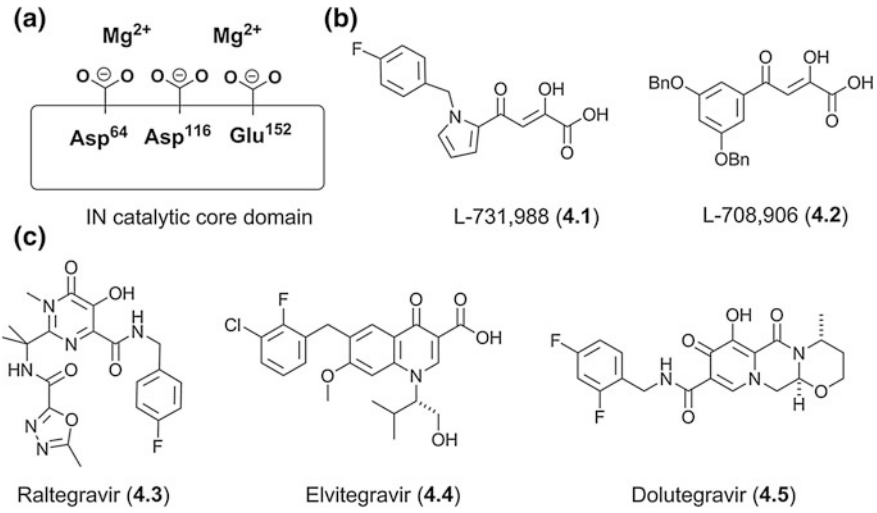

Fig. 4.1 **a** The IN catalytic core domain having the triad carboxy groups of the side chains at Asp^{64}, Asp^{116}, and Glu^{152}, which are critical for coordination of two magnesium ions; **b** diketoacid (DKA) type and DKA mimic IN inhibitors; **c** quinolone- and pyrimidinone-related and other IN inhibitors

et al. 1996). Thus, several IN strand transfer inhibitors possessing two-magnesium-binding pharmacophores, which interact with the three carboxylate residues, have been developed. Initially, diketoacids (DKAs), which contain a two-magnesium-binding pharmacophore, have been developed as first generation IN inhibitors (**4.1**, **4.2**) (Fig. 4.1b). These inhibitors were designed based on an interactive model of the binding to IN through coordination with two magnesium ions (Grobler et al. 2002). A pyrimidinone derivative, raltegravir (Isentress) (**4.3**) (Merck Sharp & Dohme Corp.), was the first IN inhibitor that was approved by the FDA (Fig. 4.1c) (Cahn and Sued 2007; Grinsztejn et al. 2007). Initially, raltegravir was approved in 2007 only for patients who showed resistance against other HAART drugs, but later the FDA expanded in 2009 its approval to its use in combination of raltegravir with other anti-HIV agents. Elvitegravir (**4.4**) (Gilead Sciences, Inc./JT), which had a quinolone template, was the second IN inhibitor that was approved by the FDA (Sato et al. 2006). This inhibitor shows properties of IN inhibitory and anti-HIV activities at nanomolar levels as well as moderate bioavailability and low clearance. A CYP3A inhibitor, cobicistat, is capable of protecting elvitegravir from metabolism, and these drugs are used as a combinational regimen. The combined drug Stribild is composed of elvitegravir, cobicistat and two reverse transcriptase inhibitors, tenofovir and emtricitabine (Sax et al. 2012). Taking this tablet once per day is effective and laborless for patients. Furthermore, elvitegravir shows cross-resistance with raltegravir-resistant strains (Marinello et al. 2008). Dolutegravir (Tivicay) (**4.5**) (Shionogi/GSK) was approved as the third IN inhibitor by the FDA in 2013, which was proven to be a potent anti-HIV agent with a low clearance and

sufficient oral bioavailability (Raffi et al. 2013; Min et al. 2010; Kobayashi et al. 2011). Even monotherapy of patients using dolutegravir once per day effectively reduces RNA levels with high retention of blood concentrations as well as suitable pharmacokinetic profiles. Development of these three HIV-1 IN inhibitors has remarkably advanced chemotherapy against HIV-1 infection and AIDS with combinational regimens.

4.2 Helix Mimetics: HIV Integrase Inhibitors

To date, we have developed different types of HIV-1 IN inhibitors (Suzuki et al. 2010a, b). Screening an overlapping peptide library derived from HIV-1 gene products has found three Vpr-derived fragment peptides having significant IN inhibitory activity (Fig. 4.2). These three inhibitory peptides are overlapping and sequentially successive in order. Twelve- and eighteen-mer peptides based on the above original Vpr sequence conjugated by an octa-arginyl group that has cell membrane permeability (Suzuki et al. 2002) at the C-terminus showed IN inhibitory activity and anti-HIV activity in cell-based replication assays. The action mechanism of these peptides is unclear in details although they possibly bind to the cleft

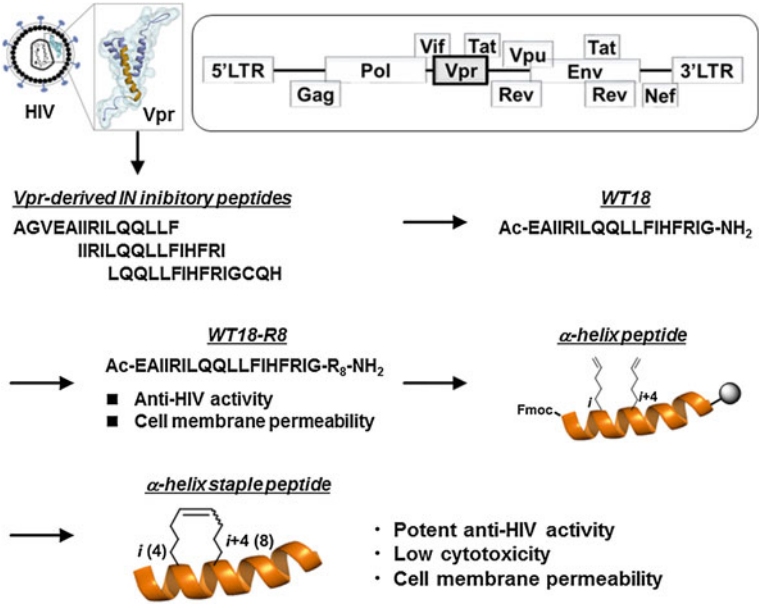

Fig. 4.2 Vpr fragment peptides with an allosteric IN inhibitory mechanism and synthetic methodologies for cell penetration: conjugation of an octa-arginyl group and a stapling technique

between the amino-terminal domain and the core domain of HIV-1 IN and inhibit HIV-1 integration. This cleft region is apart from the integrase active site which is interacted by nucleic acids. It is suggested that the Vpr-derived peptides inhibit the integration in an allosteric manner. The original sequences of the above Vpr fragments are originally located in an α-helical region of the parent Vpr protein. Conjugation of the Vpr fragments with an octa-arginyl group caused not only significant inhibition of HIV replication associated with cell membrane permeability but also relatively high cytotoxicity. To develop new generation inhibitors, a stapling strategy involving stabilization of α-helixes was adopted in place of octa-arginine conjugation to increase the cell permeability of the above peptides. This strategy is an alternative means to boost cell penetration (Fig. 4.2) (Schafmeister et al. 2000). A series of stapled peptides, which have a covalent hydrocarbon link formed by ring-closing metathesis reaction between sequential turn pitches of α-helical peptides, were designed and synthesized (Fig. 4.2) (Blackwell and Grubbs 1998). CD spectra showed that stapled peptides form α-helical structures while the corresponding linear peptides form β-sheet-like structures. In cell-based assays, some of the stapled peptides showed potent anti-HIV activity comparable to that of the original octa-arginine-conjugated peptide and lower cytotoxicity (Nomura et al. 2013). Fluorescent imaging found that these stapled peptides were significantly cell permeable. The application of this stapling strategy to Vpr fragments caused a remarkable increase in their potency of IN inhibition in cells and a significant reduction in their cytotoxicity. Subsequently, we found that the functional role of the octa-arginine sequence might also be correlated to binding affinity for the target DNA and thus the IN inhibitory activities in vitro as well as cell penetration. Since arginine guanidino groups can bind to phosphate groups of DNA, oligo-arginine sequences play a critical role in DNA binding. According to effects of the length of oligo-arginine sequences on DNA binding, the addition of tetra-/hepta-arginine is sufficient to increase IN inhibitory activity without significant cytotoxicity. Compounds (**4.6**) and (**4.7**) with tetra- and penta-arginine sequences have potent IN inhibitory activity, DNA binding affinity, and relatively low cytotoxicities (Fig. 4.3). Thus, stapled peptides are useful as leads of IN inhibitors.

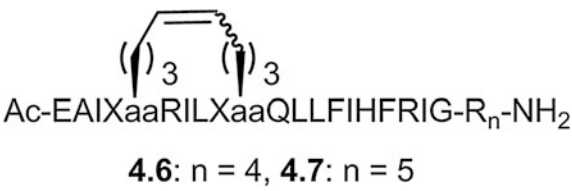

Fig. 4.3 Structures of stapled peptides **4.6** and **4.7** with tetra- and penta-arginine sequences, respectively. Xaa: an L-amino acid without a side chain

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Chapter 5
Peptidomimetics That Mimic the Tertiary Structures of Peptides

Abstract In the previous chapter, peptidomimetics of secondary structures involving stapled peptides are described. This chapter introduces peptidomimetics of tertiary structures of peptides, which are also useful for the development of inhibitors of protein–protein interactions. The HIV-1 gp41 ectodomain contains N-terminal heptad repeat (NHR) (heptad repeat 1; HR1) and C-terminal heptad repeat (CHR) (heptad repeat 2; HR2) domains, both of which have helical structures. N36 or C34 is an NHR- or CHR-derived helical peptide, respectively. A three-helical bundle mimetic corresponding to the equivalent trimeric form of N36 was designed and synthesized. As a result, mice immunized with this N36 trimer mimetic induced neutralizing antibodies with higher binding affinity for the N36 trimer than that for the corresponding monomer. Furthermore, a three-helical bundle mimicking the equivalent trimeric form of C34 and the C34 dimer mimetic was designed and synthesized. As a result, the HIV-1 inhibitory potencies of the C34 trimer and dimer mimetics are one hundred times higher than that of the corresponding monomer. The NHR region is more suitable as a vaccine target than the CHR region while the CHR region is more suitable as an inhibitor target. In each case, the assembly of triple (or double)-helical peptides using a C_3-symmetric template is effective to remodel and mimic the natural tertiary structures of the proteins.

Keywords Three-helical bundle mimetic · HIV vaccine · Fusion inhibitor
C_3-Symmetric template

5.1 Introduction to HIV Vaccine and Fusion Inhibitors Based on an Envelope Protein gp41

Use of antibodies and vaccines has been an effective treatment for infectious diseases. In the case of HIV infection, immunization (Cabezas et al. 2000) and de novo engineering of monoclonal antibodies (Abs) using molecular evolution methods have caused the development of some HIV antibodies (Burton et al. 1991). However, it is difficult to show broad HIV-neutralizing activity. Few Abs with broad

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HIV-neutralizing activity include gp120 Abs, 2G12 (Trkola et al. 1996) and b12 (Pantophlet et al. 2003) and gp41 Abs, 2F5 (Conley et al. 1994; Ofek et al. 2004; Alam et al. 2007; Nelson et al. 2007) and 4E10 (Cardoso et al. 2005; Alam et al. 2007; Nelson et al. 2007). The endo- and ectodomains of gp41 are separated by the transmembrane region. The gp41 ectodomain contains a hydrophobic amino-terminal fusion peptide, followed by N-terminal heptad repeat (NHR) (heptad repeat 1; HR1) and C-terminal heptad repeat (CHR) (heptad repeat 2; HR2) domains, both of which have helical structures. At a membrane fusion step, NHR and CHR form a "six-helical bundle" conformation, which involves a central parallel trimer of NHR surrounded by three strands of CHR arranged in an antiparallel manner. This mechanism might lead to a useful strategy to design artificial antigens that elicit broadly neutralizing antibodies to produce chemical molecules that mimic the natural trimer on the viral surface so that induced antibodies might recognize the NHR trimer and suppress formation of a natural "six-helical bundle" structure. Previously, some molecules, which are recombinantly produced or expressed on the surface of particles such as pseudovirions or proteoliposomes (Yang et al. 2001; Sanders et al. 2002; Grundner et al. 2002), were reported. To date, several synthetic antigens and inhibitors have also been developed based on peptidomimetics connected with various templates corresponding to the native structure of gp41 (De Rosny et al. 2001; Tam and Yu 2002; Louis et al. 2003; Xu and Taylor 2007). However, the templates, which were used for assembly of these helical peptides, had branched linkers with different lengths (Tam and Yu 2002). X-ray crystallographic analysis of the "six-helical bundle" structure involved by NHR and CHR showed that the distance between any two residues at the N-terminus of the N-region of gp41 might be approximately 10 Å (Chan et al. 1997). Ideal mimetics of NHR might efficiently induce neutralizing monoclonal Abs (mAb).

5.2 HIV Vaccine Based on a gp41 Fragment N36

N36 or C34 is an NHR- or CHR-derived helical peptide, respectively. A three-helical bundle mimetic corresponding to the equivalent trimeric form of N36 was designed and synthesized to check whether mice immunized with this N36 trimer mimetic can induce antibodies with high binding affinity for the N36 trimer (Nakahara et al. 2010). N36 has relatively high hydrophobicity and low aqueous solubility. Thus, the triplet repeat of arginine and glutamic acid was fused to the N-terminus of an N36-derived peptide to increase the aqueous solubility, which was designated as N36RE (Fig. 5.1). To form a triple helix, a C_3-symmetric template, which precisely mimics the gp41 pre-fusion form, was designed (Fig. 5.1). The linker tethered to this template (Grundner et al. 2002) has three same branches of equal length with a hydrophilic structure and a ligation site required for coupling with N36RE. A C_3-symmetric template with its three-armed aldehyde scaffold was conjugated with Cys-containing unprotected N36RE (N36REGC) by thiazolidine ligation (Liu and Tam 1994; Tam and Miao 1999; Tam et al. 2001; Eom et al. 2003;

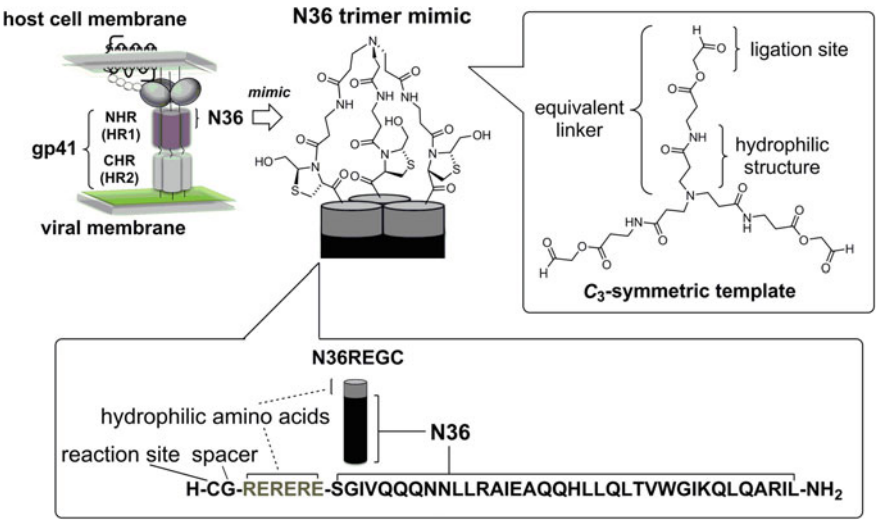

Fig. 5.1 Schematic representation of gp41 and the sequence of an NHR (HR1) region peptide N36 involving introduction of an Arg-Glu motif, a spacer residue Gly and a reaction site Cys with an aldehyde group of a three-branched linker

Sadler et al. 2008) to produce the trimer triN36e (Fig. 5.1). The CD analysis of the synthetic peptides showed that the helical content of the trimer triN36e was higher than that of the monomer N36RE, and that the mixture of triN36e and a C34-derived monomer peptide, C34RE, had higher helicity compared with triN36e alone (Nakahara et al. 2010). Thus, the interaction of C34RE with tri36e induces a higher helical form (Chan et al. 1998). To investigate whether antibodies might be induced, mice were immunized with the trimer triN36e. Antibody induction was then evaluated by serum titer ELISA against coated synthetic monomer N36RE and trimer triN36e antigens. The antisera induced by triN36e immunization showed approximately 30 times higher affinity for the triN36e antigen on the ELISA plate than for the N36RE antigen. It suggests that the triN36e-induced antisera have a structural preference for binding with triN36. In anti-HIV assays, the triN36e-induced antisera showed an approximately fourfold higher neutralizing activity than the N36RE-induced antisera. Taken together, the N36 trimeric form can induce antibodies with higher neutralization activity than the monomer form.

5.3 HIV Fusion Inhibitors Based on a gp41 Fragment C34

The gp41 ectodomain contains a hydrophobic amino-terminal fusion peptide, followed by two peptides, 51 mer from the NHR region and 43 mer from the CHR region, designated as N51 and C43, respectively (Lu et al. 1995). Several CHR

region peptides have been reported to prevent the formation of a natural "six-helical bundle" structure by binding to the central parallel trimer of NHR thereby suppressing membrane fusion (Shimura et al. 2010). A CHR region 34 mer peptide C34, which is shorter than C43, has potent inhibitory activity against HIV-1 fusion (Chan et al. 1997). A 36-residue peptide, Enfuvirtide (fuzeon/T-20, Roche/Trimeris), which slightly slides from the helical region of CHR toward the C-terminal side of C34 with 24 residues in common with C34, was approved as the first fusion inhibitor for its clinical use in HIV/AIDS treatment by the FDA in 2003 (Wild et al. 1993). These peptides bind to the NHR region to suppress formation of the six-helical bundle structure (Kilby et al. 1998). C34 contains hydrophobic residues required for binding into the hydrophobic pocket of the central parallel trimer of NHR (Chan et al. 1997). Therefore, we previously designed and synthesized a three-helical bundle mimicking the equivalent trimeric form of C34.

In the synthesis of the three-helical bundle mimic, the triplet repeat of arginine and glutamic acid was fused to the C-terminus of the C34-derived peptide to increase its aqueous solubility, and a glycine thioester was added to the C-terminus. This product was designated as C34REG-thioester (Fig. 5.2). To form a

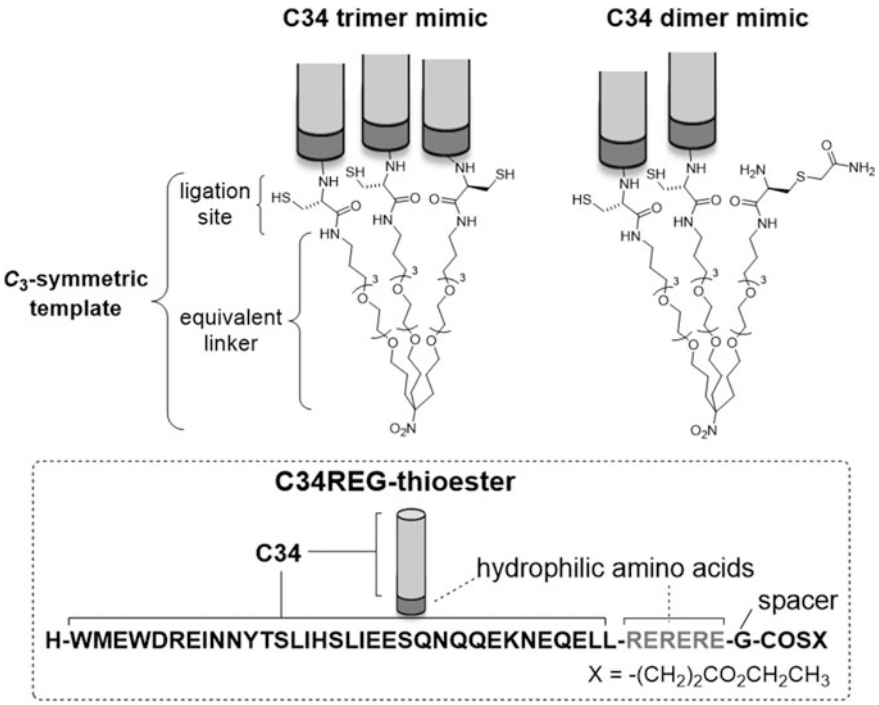

Fig. 5.2 The sequence of a CHR (HR2) region peptide C34 involving introduction of an Arg-Glu motif, a spacer residue Gly and a reaction site thioester with a thiol group of a three-branched linker. Remodeling of dynamic structures of CHR regions based on a three-branched linker to C34 trimer and dimer mimics leads to synthetic antigen molecules and fusion inhibitors

Table 5.1 Anti-HIV activity (IC_{50}) and cytotoxicity (CC_{50}) of CHR-derived peptides determined in anti-HIV and cytotoxic assays, respectively

	C34[a]	C34 REG	triC34e	diC34e
IC_{50} (nM)[b]	44	120	1.3	0.73[d]
CC_{50} (μM)[c]	>15	>15	>5	>5

[a]HIV-1 IIIB C34 peptide
[b]IC_{50} values were determined by luciferase signals in TZM-bl cells infected with HIV-1 (NL4-3 strain)
[c]CC_{50} values were determined by the reduction of the viability of TZM-bl cells. All data are the mean values from at least three experiments
[d]The value was calculated using the IC_{50} value of triC34 as the standard (Nomura et al. 2012)

triple-helical structure to mimic the gp41 pre-fusion form, the C_3-symmetric template with a linker having three branches of equal length, a hydrophilic structure, and a ligation site for coupling with the C34REG-thioester was designed (Fig. 5.2). An unprotected C34REG-thioester was coupled with three cysteine residues on a three-armed template to yield the trimer triC34e (Fig. 5.2) (Nomura et al. 2012). According to CD analysis, the C34-derived monomer C34REG and the trimer triC34e form random structures, which are different from N36-derived peptides (Nomura et al. 2012), whereas the mixture of C34REG and N36RE and that of triC34e and N36RE form α-helix structures although the helical content of the latter mixture is significantly lower than that of the former mixture. As a result, the assembly of three peptide strands in triC34e by covalent bonds might produce some difficulty in formation of a six-helical bundle structure by the trimer triC34e with three N36 peptides. Anti-HIV assays showed that the HIV-1 inhibitory potency of triC34e is one hundred times higher than that of C34REG (Table 5.1), suggesting that a trimeric form is critical to an inhibitory structure. It is an additional advantage that triC34e has no significant cytotoxicity. As a reference, the HIV-1 inhibitory activity of the N36 trimer mimetic triN36e is three times higher than that of the monomer N36RE, indicating the peptide content of N36 is critical, but both have modest inhibitory activity (Nakahara et al. 2010). Antibody induction of the C34 trimer mimetic triC34e was also investigated (Hashimoto et al. 2012) as was that of the N36 trimer mimetic triN36et. Antibody induction was evaluated by serum titer ELISA against coated synthetic monomer C34REG and trimer triC34e antigens. The antisera induced by triC34e immunization showed approximately 23-fold higher affinity for the triC34e antigen on the ELISA plate than for the C34REG antigen. It suggests that the triC34e-induced antisera have a structural preference for binding with triC34e. This result is consistent with that shown in immunization of the N36 trimer mimetic triN36e. However, the neutralization activity of the triC34e-induced antibodies is not sufficiently high, which is nearly equal to that of the monomer C34REG-induced antibodies. Therefore, the NHR region is more suitable as a vaccine target than the CHR region while the CHR region is more suitable as an inhibitor target.

The C34 dimer mimetic was chemically synthesized to identify a key structure required for the inhibitory activity of the CHR-derived trimer mimetic triC34e

against HIV-1 fusion. The same unprotected C34REG-thioester was stoichiometrically condensed with the same C_3-symmetric template with three cysteine residues, and the resulting free thiol groups was then carboxymethylated with iodoacetamide to produce the C34 dimer mimetic diC34e (Fig. 5.2) (Nomura et al. 2013). The anti-HIV-1 activity of diC34e was proven to be nearly equal to that of the trimer mimetic triC34e (Table 5.1). According to these results, two units of the C34 peptide in the dimer form might bind to the NHR region in a cooperative manner. In the C34 dimer or trimer mimetics, dimerization or trimerization of the C34 peptide does not increase the α-helicity of the peptides judging by CD analysis (Nomura et al. 2013). However, in the mixture with the N36 monomer N36RE, all the C34-derived peptides, monomer C34REG, dimer diC34e, and trimer triC34e show similar α-helicity.

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Chapter 6
Conjugated Compounds Involving Peptides

Abstract Conjugated compounds, based on linking of some peptides and other peptides or small molecules, are important candidates for mid-size drugs. In this chapter, hybrid molecules of small CD4 mimics and peptidic coreceptor antagonists were designed and synthesized, and then evaluated as HIV entry inhibitors. In addition, overlapping libraries of fragment peptides of matrix (MA) and capsid (CA) proteins, which were conjugated with a cell membrane permeable signal, were prepared to discover potent lead compounds which express HIV inhibitory activity inside infectious cells.

Keywords Conjugated compound · CD4 mimic · Coreceptor antagonist Matrix · Capsid · Cell membrane permeable signal

6.1 HIV Entry Inhibitors Based on the Conjugation of CD4 Mimics and Coreceptor Antagonists

As described in Sect. 3.1, the binding of gp120 to CD4 causes a conformational change in gp120 and then leads to the binding to the coreceptor CCR5 or CXCR4. To date, many unsuccessful challenges to develop soluble CD4 molecules as anti-HIV drugs have been reported. However, CD4-related molecules have been known to suppress the interaction of gp120 with CD4 and thus might be HIV entry inhibitors. Several small-sized CD4 mimics have already been developed in our and other laboratories, including NBD-556 (**6.1**) (Zhao et al. 2005; Schön et al. 2006), YYA-021 (**6.2**) (Yamada et al. 2010; Narumi et al. 2010; Yoshimura et al. 2010), JRC-II-191 (**6.3**) (Lalonde et al. 2011) and BMS806 (**6.4**) (Lu et al. 2007) (Fig. 6.1). NBD-556, YYA-021, and JRC-II-191 bind to gp120 to inhibit binding of gp120 to CCR5 or CXCR4, and thereby block HIV entry. BMS806 binds to gp120 without significant effect on CD4 binding to suppress the CD4-induced exposure of gp41. Our laboratory has developed several CD4 mimics (Narumi et al. 2011, 2013; Hashimoto et al. 2013; Mizuguchi et al. 2016; Ohashi et al. 2016) based on NBD-556 and YYA-021. These compounds bind to the conserved pocket in gp120,

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https://doi.org/10.1007/978-981-10-7691-6_6

the "Phe43 cavity", and cause a conformational change in gp120, opening the envelope (Schön et al. 2006, 2011), as it is observed in the binding of soluble CD4 to gp120. As a result, neutralizing antibodies such as an anti-V3 monoclonal antibody KD-247 can bind to gp120 (Fig. 6.2) (Yoshimura et al. 2010). YIR-819 (**6.5**) and YIR-821 (**6.6**), both of which have a monocyclohexyl group and a guanidino group, have been developed and were found to interact effectively with Val430 and either Asp368 or Asp474 on the surface of the Phe43 cavity. These compounds show a remarkable synergistic anti-HIV activity with KD-247, and thus might be useful envelope protein openers. These are desirable drug candidates for the combinational use with neutralizing antibodies. In addition, these CD4 mimics show remarkable synergistic anti-HIV activity with CXCR4 antagonists such as T140 as well as with KD-247 (Yamada et al. 2010), indicating that the interaction of CD4 mimics with gp120 might facilitate exposure of the coreceptor binding site of gp120. In our speculation, CD4 mimics conjugated with CXCR4 antagonists might show a higher synergistic effect to cause enhancement of anti-HIV activity. Thus, hybrid molecules based on conjugation of a CD4 mimic molecule with a CXCR4 antagonist were designed and synthesized.

According to SAR studies of CD4 mimic molecules on the binding affinity with gp120, the anti-HIV activity, and the CD4 mimicry on conformational changes of gp120 (Zhao et al. 2005; Schön et al. 2006; Yamada et al. 2010; Yoshimura et al.

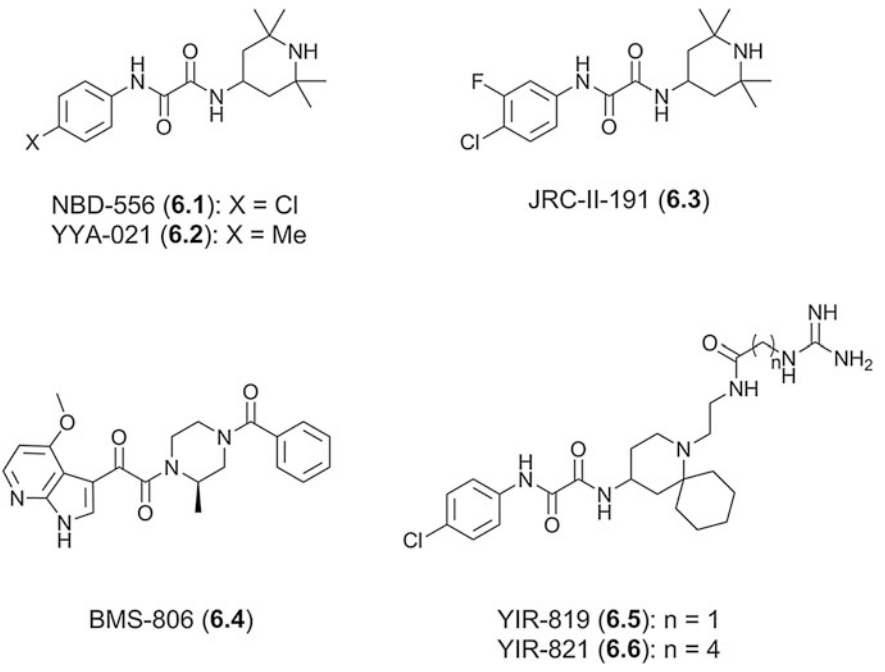


Fig. 6.1 Structures of small CD4 mimics

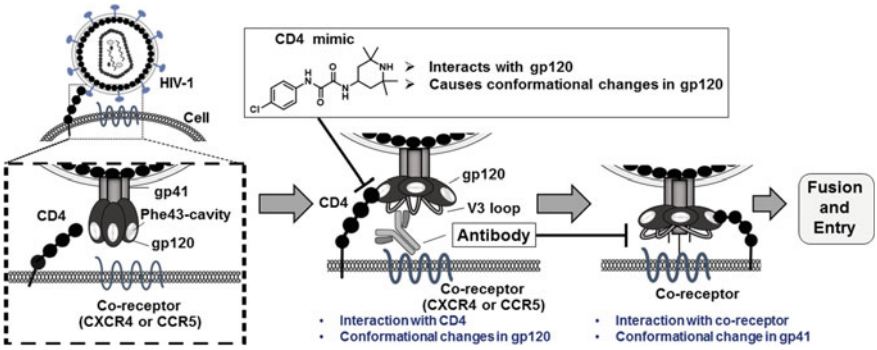
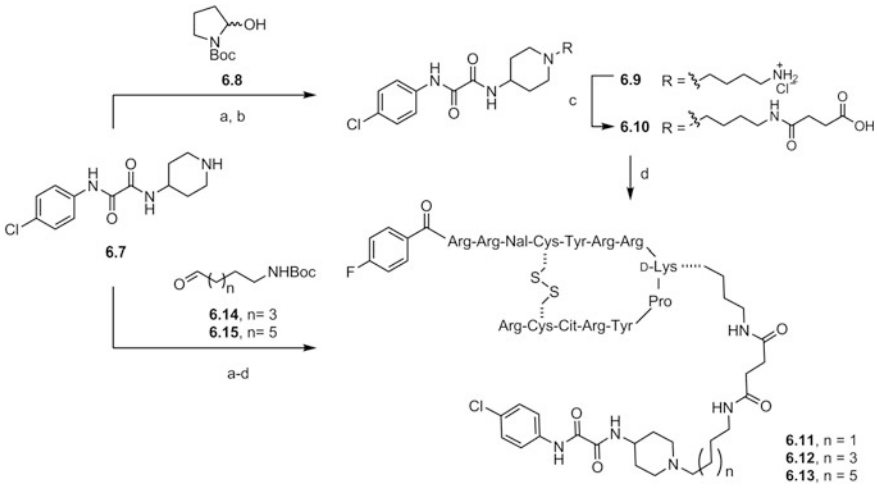


Fig. 6.2 HIV-1 entry mechanism and strategies for inhibition

2010; Madani et al. 2008; Haim et al. 2009), the piperidine ring was adopted as a connection site with a T140 derivative. An NBD556 derivative (**6.7**), in which the four methyl groups of NBD556 were deleted, showed anti-HIV activity comparable to that of NBD556. Since alkylation of the piperidine nitrogen of compound **6.7** had no significant effect on anti-HIV activity, the alkylamine moiety was attached to the piperidine nitrogen to link with T140 analogs: a linker moiety was attached to the nitrogen atom on the piperidine ring of compound **6.7**. A T140 analog, 4F-benzoyl-TZ14011 (Hanaoka et al. 2006), was conjugated with the carboxy group of the linker to produce hybrid molecules **6.11**–**6.13** having linkers of different lengths (Scheme 6.1). Reductive alkylation of **6.7** was performed by



Scheme 6.1 Synthetic scheme of hybrid molecules **6.11**–**6.13**. *Reagents and conditions*: **a** $NaBH(OAc)_3$, AcOH, CH_2Cl_2; **b** TFA, then 4 M HCl/1,4-dioxane; **c** succinic anhydride, pyridine, DMF, then 4 M HCl/1,4-dioxane; **d** 4F-benzoyl-TZ14011, EDC.HCl, $HOBt.H_2O$, Et_3N, DMF

Table 6.1 CXCR4-binding activity (EC_{50}), anti-HIV activity (IC_{50}), and cytotoxicity (CC_{50}) of hybrid molecules **6.11–6.13**[a]

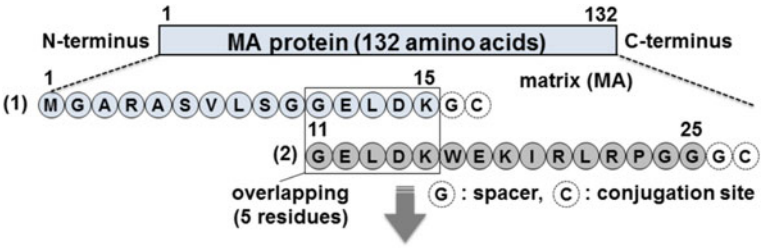
Compound	EC_{50}[b] (μM)	IC_{50}[c] (μM)	CC_{50}[d] (μM)	CC_{50}/IC_{50}
4F-benzoyl-TZ14011	0.0059	0.0131	ND	ND
NBD-556	ND	0.210	ND	19.2
6.11	0.0044	0.0509	8.60	169
6.12	0.0187	0.0365	8.00	219
6.13	0.0071	0.0353	8.60	244
AZT	ND	0.0493	ND	ND

[a]All data are the mean values for at least three independent experiments
[b]EC_{50} values are based on the inhibition of [^{125}I]-SDF-1 α binding to CXCR4 transfectants on CHO cells
[c]IC_{50} values are based on the protection from HIV-1-induced cytopathogenicity in MT-2 cells
[d]CC_{50} values are based on the reduction of the viability of mock-infected MT-2 cells
ND not determined

treatment with N^{α}-Boc-pyrrolidin-2-ol **6.8** followed by deprotection of the Boc group with TFA to provide the amine **6.9**. The amine **6.9** was treated with succinic anhydride to give the acid **6.10**, which was attached to the side chain amino group of D-Lys[8] of 4F-benzoyl-TZ14011 with EDC-HOBt to yield a hybrid molecule containing a tetramethylene linker (**6.11**). In addition, other hybrid molecules (**6.12**, **6.13**) having hexa- and octamethylene linkers were prepared using aldehydes **6.14** and **6.15**, respectively (Narumi et al. 2010). Three hybrid molecules (**6.11–6.13**) inhibited the binding of CXCL12 to CXCR4 at levels of EC_{50} values of 0.0044, 0.0187, and 0.0071 μM, respectively, when the EC_{50} value of 4F-benzoyl-TZ14011 was shown to be 0.0059 μM (Table 6.1). These results suggest that the introduction of the CD4 mimic analog into the D-Lys[8] residue of 4F-benzoyl-TZ14011 has no significant effect on CXCR4 binding affinity. In the anti-HIV MTT assay with a IIIB(X4) strain, the IC_{50} values of the hybrid molecules **6.11–6.13** were 0.0509, 0.0365, and 0.0353 μM, respectively, while the IC_{50} value of 4F-benzoyl-TZ14011 was shown to be 0.0131 μM: the potency of **6.11–6.13** was thus two to fourfold lower than that of the parent compound 4F-benzoyl-TZ14011. According to the results, conjugation of CD4 mimics with T140 analogs shows no significant synergistic effect. Since the combinational use of NBD556 with T140 shows a remarkable synergistic effect, the lower potency of hybrid molecules might be attributed to deficiencies in linking a CD4 mimic with a CXCR4 antagonist or in the characters of the linkers. However, conjugation of NBD556 with 4F-benzoyl-TZ14011, at a minimum, improved the selectivity index of CD4 mimic. Although these hybrid molecules have no significant synergistic effect, it could be speculated that conjugation of a CD4 mimic with a selective CXCR4 antagonist might lead to the development of novel type of CD4 mimic-based HIV-1 entry inhibitors through optimization of the linker.

6.2 HIV Inhibitors Based on the Conjugation of Matrix Peptides with Cell Penetration Peptides

The matrix (MA) is contained in the Gag precursor protein, Pr55Gag, and in the virion MA proteins are located close to the viral membrane (Freed 1998; Bukrinskaya 2007). MA proteins play an important role in assembly of the virion shell of HIV-1. Several reports have shown that MA-derived peptides such as MA (47–59) suppress HIV infection (Niedrig et al. 1994), and that MA-derived peptides such as MA(31–45) and MA(41–55) show anti-HIV activity (Cannon et al. 1997). Morikawa et al. have reported that MA(61–75) and MA(71–85) inhibit MA dimerization, which is an indispensable step for the formation of the virion shell (Morikawa et al. 1995). However, there has been no discussion on the mechanism in which the above MA peptides penetrate cell membranes. Thus, we previously designed and prepared an overlapping library of MA fragment peptides having a cell membrane permeable signal to discover potent lead compounds which express HIV inhibitory activity inside infectious cells (Narumi et al. 2012). An overlapping peptide library spanning the whole sequence of the MA protein, p17, of Pr55Gag of HIV-1 NL4-3 was constructed: the MA 132 amino acid residue sequence was divided into 15-residue segments from the N-terminus with an overlap of 5 residues (Fig. 6.3). The Cys residues contained in the original MA sequence were replaced by Ser residues to simplify the synthesis. In the C-terminus of all of the MA fragment peptides (1–13), Gly and Cys residues were added as a spacer and a conjugation site, respectively. The N-terminal chloroacetyl group of an octa-arginyl peptide (Suzuki et al. 2002) was conjugated to the side chain thiol group of the Cys residue of the above MA fragment peptides to add cell membrane permeability. These conjugated peptides were designated as MA peptides 1L-13L. In control peptides that might be short of cell membrane permeability, iodoacetamide was conjugated to the thiol group of the Cys residue to yield the control MA peptides 1C-12C (Fig. 6.4). The anti-HIV activity and cytotoxicity of the above MA peptides were evaluated to show the results in Table 6.2. All of the MA peptides containing an octa-argininyl sequence showed moderate to potent anti-HIV activity against X4-HIV-1 as well as R5-HIV-1 strains with the exception of 3L and 4L. In particular, the MA peptides 8L and 9L containing an overlapping sequence TIAVL showed the most potent anti-HIV activity. The control MA peptides 6C and 9C slightly showed anti-HIV activity against both strains, and 2C showed high anti-HIV activity, but the other control MA peptides showed no significant anti-HIV activity (data not shown) (Narumi et al. 2012). The 2C region of the MA domain is correlated to the localization of Gag to the plasma membrane (PM) (Ono 2004) and thus 2C might competitively inhibit the interaction between MA and PM. According to the above results, the MA peptides reach inside host cells due to the addition of the octa-arginyl sequence to inhibit viral replication. However, these octa-arginine containing peptides have relatively high cytotoxicity whereas the control MA peptides are less cytotoxic. The control MA peptides 8C and 9C exhibited no significant cytotoxicity, on the other hand through the addition of the



fragment number	Sequence
1	H-MGARASVLSGGELDKGC-NH_2
2	Ac-GELDKWEKIRLRPGGGC-NH_2
3	Ac-LRPGGKKQYKLKHIVGC-NH_2
4	Ac-LKHIVWASRELERFAGC-NH_2
5	Ac-LERFAVNPGLLETSEGC-NH_2
6	Ac-LETSEGSRQILGQLQGC-NH_2
7	Ac-LGQLQPSLQTGSEELGC-NH_2
8	Ac-GSEELRSLYNTIAVLGC-NH_2
9	Ac-TIAVLYSVHQRIDVKGC-NH_2
10	Ac-RIDVKDTKEALDKIEGC-NH_2
11	Ac-LDKIEEEQNKSKKKAGC-NH_2
12	Ac-SKKKAQQAAADTGNNGC-NH_2
13	Ac-DTGNNSQVSQNYGC-NH_2

Fig. 6.3 An overlapping peptide library spanning the whole sequence of 132 amino acid residues of the MA protein

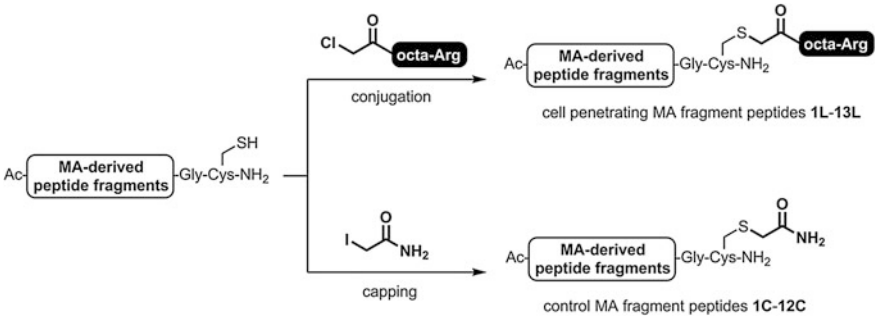


Fig. 6.4 Synthesis of cell-penetrating MA fragment peptides with an octa-arginyl group (1L-13L) (upper) and their control peptides (1C-12C) (lower)

Table 6.2 Anti-HIV activity (IC_{50}) and cytotoxicity (CC_{50}) of some MA peptides containing an octa-argininyl sequence

MA-derived peptide	MT-4 cell	PM1/CCR5 cell		MT-4 cell
	NL4-3 (MTT assay)	NL(AD8) (MTT assay)	JR-CSF (p24 ELISA)	(MTT assay)
	IC_{50} (μM)[a]	IC_{50} (μM)[b]	IC_{50} (μM)[c]	CC_{50} (μM)[d]
MA-1L	30	30	40	>50
MA-2L	21	>31	ND	32
MA-7L	35	37% inhibition at 50 μM	35% inhibition at 50 μM	>50
MA-8L	2.3	5.8	7.8	9
MA-9L	2.1	0.43	0.58	5.7
MA-10L	43	42% inhibition at 50 μM	27	>50
MA-11L	18	17% inhibition at 25 μM	23	>50
MA-13L	20	0.43	11	>50
AZT	0.020	0.459	0.17	>100
SCH-D	ND	0.026	0.0014	ND

[a]IC_{50} values are based on the protection from X4-HIV-1 (NL4-3)-induced cytopathogenicity in MT-4 cells, which were evaluated by the MTT assay
[b]IC_{50} values are based on the protection from R5-HIV-1 [NL(AD8)]-induced cytopathogenicity in PM1/CCR5 cells, which were evaluated by the MTT assay
[c]IC_{50} values are based on the protection from R5-HIV-1 (JR-CSF strain)-induced cytopathogenicity in PM1/CCR5 cells, which were evaluated by the p24 ELISA assay
[d]CC_{50} values are based on the reduction of the viability of MT-4 cells (the MTT assay)
All data are the mean values from at least three independent experiments

octa-arginyl sequence, MA-8L and MA-9L showed significant cytotoxicity. Since the octa-arginyl sequence might be contributed to the expression of cytotoxicity, adoption of different effective strategies for cell penetration such as the staple strategy, which is described in Chap. 4, would be desirable. To perform fluorescent imaging of living cells, the fluorophore-labeled MA peptides containing the octa-arginyl sequence were incubated with HeLa, A549, and CHO-K1 cells, and the imaging was then analyzed by a fluorescence microscope (Narumi et al. 2012). As a result (not shown), the octa-arginine containing MA peptides can penetrate cell membranes, indicating that MA peptides such as 8L and 9L might inhibit HIV replication inside cells. According to these results, two possible explanations on the inhibitory mechanism of these MA fragment peptides can be envisaged: (i) The fragment peptides might competitively attack an MA protein to suppress the assembly and/or shelling of MA proteins, and (ii) These peptides might attack a cellular protein to block its interaction with MA proteins. In future, further studies are required to elucidate detailed action mechanisms. In general, the construction of an overlapping peptide library of fragment peptides derived from a parent protein conjugated with a cell membrane permeable moiety such as the octa-arginyl

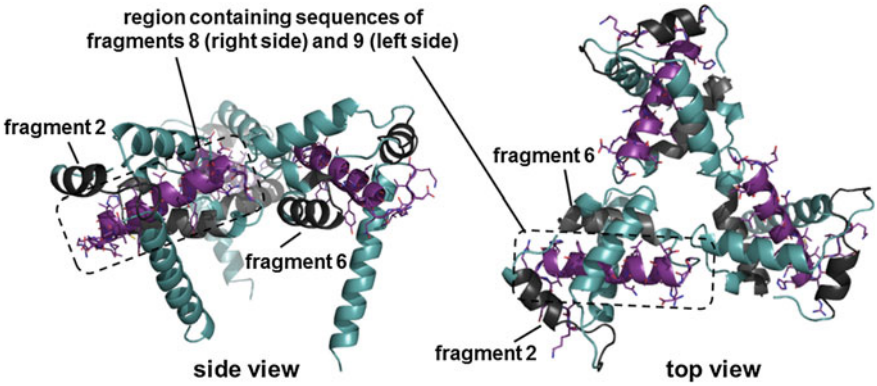


Fig. 6.5 Structures of the interface between two MA trimers. The α-helixes containing the amino acid sequence corresponding to the MA peptide fragments 8 and 9 are indicated in a purple color

sequence is a useful strategy to find lead compounds that express biological activity inside cells. The region corresponding to MA peptides 8L and 9L is located in the exterior surface of an MA monomer, and in the interface between two MA trimers (Fig. 6.5) (Rao et al. 1995; Hill et al. 1996; Kelly et al. 2006), which through mutual interaction lead to the formation of an MA hexamer followed by MA oligomerization. Thus, this amino acid sequence is therefore indispensable for the MA assembly, and the MA peptides 8L and 9L might suppress the MA hexamer formation/oligomerization and/or degradation through competitive binding to the parent MA.

Peptides attached by an octa-argininyl sequence can penetrate cells and intracellularly express biological activity. However, most of the peptides might be captured inside endosomes. Thus, it is difficult to evaluate precise biological activity, and some peptides might be evaluated with activity lower than the actual activity. Lysosomotropic agents involving chloroquine are very useful because these agents can enter inside cells and accumulate in endosomes to cause accumulation of counter anions of protons followed by endosomal swelling and then bursting (Fig. 6.6) (El-Sayed et al. 2009; Erbacher et al. 1996; Ciftci and Levy 2001). Thus, after cell membrane penetration of the MA-derived fragment peptides conjugated with an octa-argininyl sequence, the chloroquine might efficiently release the MA-derived peptides from endosomes. We attempted to establish an efficient methodology of cell-based anti-HIV assays involving the addition of chloroquine to find anti-HIV compounds among several peptides that are conjugated with an octa-argininyl sequence. The effects of the addition of chloroquine at several concentrations on viability of HIV-1-infected and mock-infected MT-4 cells and on the cytotoxicity and the anti-HIV activity of some agents were investigated. As a result, the addition of 5 μM chloroquine was proven to be suitable for release of octa-arginine-attached peptides from endosomes with no significant effect on cells. It is possible that more potent peptides, which are related to MA-8L and MA-9L, might exist between these two sequences. Individual peptides MA-8-9-1L

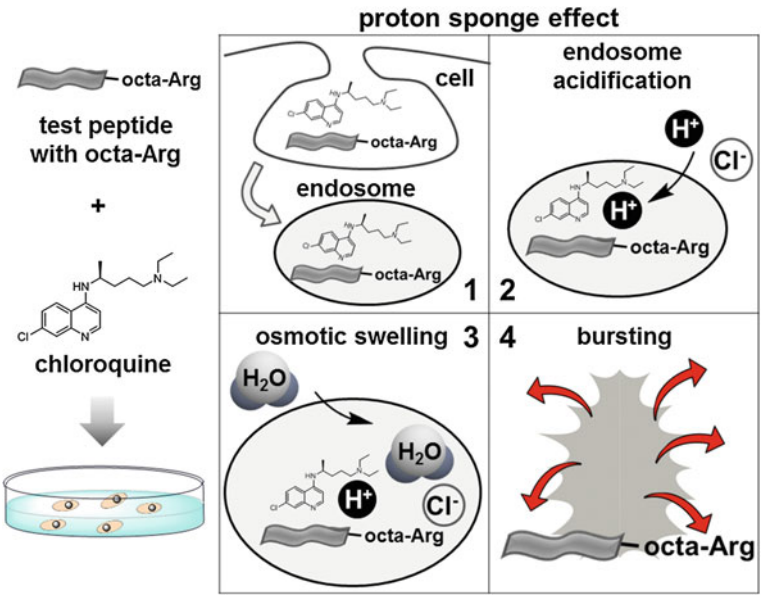


Fig. 6.6 Presentation of endosome bursting induced by treatment with chloroquine

to MA-8-9-9L having the sequences shifted by an amino acid residue and a random peptide with a shuffled sequence of MA-9L (MA-9RL) were synthesized. The anti-HIV activity of MA peptides MA-8-9-1L to MA-8-9-9L and MA-9RL in the absence of the addition of chloroquine was evaluated in the anti-HIV assay using an X4-HIV-1 strain (Table 6.3). MA-8-9-1L to MA-8-9-9L failed to show significant anti-HIV activity below the corresponding CC_{50} values. MA-9RL (shuffled sequence) or MA-9C (no octa-arginine) did not show significant anti-HIV activity. MA-8-9-5L and MA-8-9-7L showed anti-HIV activity at a moderate level similar to MA-9L with relatively high cytotoxicity. In the absence of the addition of chloroquine, high anti-HIV activity was not observed. Therefore, 5 μM chloroquine was added in the anti-HIV assay. As a result, most of the MA peptides showed moderate to high anti-HIV activity. MA-8-9-5L to MA-8-9-9L showed remarkably higher activity in the presence of chloroquine than in the absence. In the presence of 5 μM chloroquine, MA-8-9-5L, MA-8-9-7L and MA-9L showed higher anti-HIV activity at submicromolar levels. MA-8-9-3L and MA-8-9-4L showed 4–5 μM levels of anti-HIV activity in the presence of 5 μM chloroquine whereas significant anti-HIV activity was not observed in the absence of chloroquine. These results have demonstrated that the addition of chloroquine is useful for the release of the peptides from endosomes. MA-8-9-1L, MA-8-9-2L, or MA-9RL (shuffled sequence) did not show significant anti-HIV activity below approximately 10 μM, which might be consistent with their CC_{50} values: the order of the composed amino acids is important for anti-HIV activity. In all of the individual peptides, from MA-8-9-1L to MA-8-9-9L, cytotoxicity was almost similar in the absence or

Table 6.3 Anti-HIV activity (IC_{50}) and cytotoxicity (CC_{50}) of some MA peptides containing an octa-argininyl sequence between MA-8L and MA-9L in the presence or absence of chloroquine

MA peptide	Chloroquine (–)		Chloroquine (5 μM)		Chloroquine (5 μM)	
	MT-4 cell		MT-4 cell		PM1/CCR5 cell	
	NL4-3 EC_{50} (μM)[a]	CC_{50} (μM)[b]	NL4-3 EC_{50} (μM)[a]	CC_{50} (μM)[b]	NL(AD8) EC_{50} (μM)[c]	CC_{50} (μM)[d]
MA-8-9-3L	>7.7	7.7	4.5	8.1	2.3	8.4
MA-8-9-4L	>8.5	8.5	4.6	10	>5.2	5.2
MA-8-9-5L	5.2	9.2	0.85	9.2	1.3	8.2
MA-8-9-6L	>4.8	4.8	1.0	4.4	>4.6	4.6
MA-8-9-7L	6.3	11	0.64	12	2.8	8.7
MA-8-9-8L	>7.0	7.0	1.7	7.7	1.4	7.6
MA-8-9-9L	>18	18	2.0	18	5.6	17
MA-9L	6.3	10	0.62	11	1.1	7.2
MA-9RL	>8.2	8.2	>8.2	8.2	>8.7	8.7
MA-9C	>50	>50	22	47	>50	>50
AZT	0.12	>100	0.013	>100	0.51	>100
AMD3100	0.032	>50	0.025	>50	>50	>50
SCH-D	>5.0	>5.0	>5.0	>5.0	0.0049	>5.0

[a–d]Shown in Table 6.2

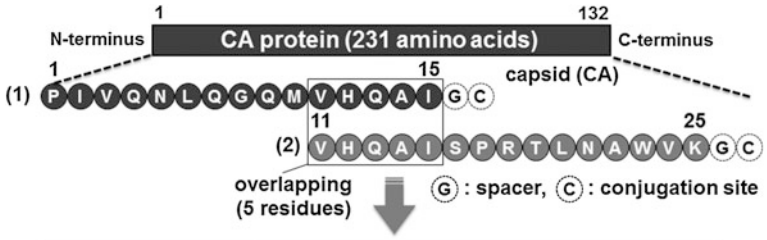
presence of chloroquine. It is suggested that the peptides of the C-terminal side of the 25-mer fragment sequence that covers MA-8 and MA-9 have more potent inhibitory activity against X4-HIV-1 compared to the peptides from the N-terminal side. Next, the anti-HIV assay using an R5-HIV-1 strain was performed in the presence of chloroquine to evaluate the anti-HIV activity of MA peptides MA-8-9-1L to MA-8-9-9L and MA-9RL (Table 6.3). At approximately 1 μM, MA-8-9-5L, MA-8-9-8L, and MA-9L showed anti-HIV activity. MA-8-9-7L showed 3 μM levels of anti-HIV activity against R5-HIV-1, which is slightly weaker than that against X4-HIV-1. MA-8-9-3L showed higher inhibitory activity against R5-HIV-1 when compared to that against X4-HIV-1. On the other hand, MA-8-9-4L or MA-8-9-6L did not show significant anti-HIV activity against R5-HIV-1 whereas they showed moderate or high anti-HIV activity against X4-HIV-1. MA-8-9-1L, MA-8-9-2L, or MA-9RL (shuffled sequence) failed to show significant anti-HIV activity below approximately 10 μM, which corresponds to their CC_{50} values. This is common with anti-HIV activity against both strains of R5- and X4-HIV-1: these peptides do not have genuinely significant anti-HIV activity, and the order of the composed amino acids is important for anti-HIV activity. The cytotoxicity of MA peptides MA-8-9-1L to MA-8-9-9L was similar in PM1/CCR5 cells or in MT-4 cells with the exception of MA8-9-4L. As seen with anti-HIV activity against X4-HIV-1, the peptides on the C-terminal side have more potent inhibitory activity against R5-HIV-1 than those on the N-terminal side.

We have found a practically efficient way for cell-based assays through this study. The combination of the attachment of an octa-arginyl group to individual

peptides contained in whole proteins and the addition of chloroquine to cells was proven to be useful to find biologically active peptides. Practically, even in several peptides, which, in the absence of chloroquine, showed no significant anti-HIV activity, potent anti-HIV activity was observed by the above assay methodology. Since the amino acid sequence covering the MA region corresponding to MA-8L and MA-9L is located in the interface between two MA trimers (Rao et al. 1995; Hill et al.1996; Kelly et al. 2006), the MA peptides MA-8-9-5L, MA-8-9-7L, and MA-8-9-8L might inhibit MA oligomerization and/or degradation by competitive binding to the parent MA. In the future, identification of the interaction sites of these MA peptides and elucidation of their mechanism of action would be performed to find more potent inhibitors.

6.3 HIV Inhibitors Based on the Conjugation of Capsid Peptides with Cell Penetration Peptides

Capsid (CA) proteins of HIV-1 (Sarngadharan et al. 1985; Mervis et al. 1988) are important for the assembly of a core having a conical structure (Ganser et al. 1999; Pornillos et al. 2011) to shield the viral RNA genome. The CA (p24) protein is contained by the Gag precursor protein, Pr55Gag, which is assembled by oligomerization involving hexamers (Pornillos et al. 2009). Proteins MA and CA might be important targets for HIV inhibition. Several reports have mentioned that MA- and CA-derived peptides have anti-HIV activity (Niedrig et al. 1994; Cannon et al. 1997; Morikawa et al. 1995; Zentner et al. 2013). Since HIV replication involving viral uncoating and assembly is intracellularly performed, these inhibitors need to have cell membrane permeability. However, the above papers have not addressed the function of these peptides to penetrate cell membranes. According to the previous section, MA-derived peptides with significant anti-HIV activity have been found among overlapping MA peptide libraries involving the addition of an octa-arginyl group as a cell membrane permeable unit (Suzuki et al. 2002; Narumi et al. 2012). Therefore, an overlapping library of fragment peptides derived from the CA protein based on the conjugation of an octa-arginyl group was prepared as the MA peptide library was to find potent lead compounds in an anti-HIV assay using chloroquine. An overlapping peptide library spanning the whole sequence of the CA domain of Pr55Gag of HIV-1 NL4-3 was prepared (Narumi et al. 2012). This peptide library possesses 15-residue peptide segments starting at the N-terminus with overlapping 5 residues (Fig. 6.7). The CA protein was divided into 23 segments without any correlation to the helix regions H1–H12, which existed in the X-ray crystal structure (Fig. 6.8) (Pornillos et al. 2009; Gres et al. 2015). To add the cell membrane permeability to these peptides, the N-terminal chloroacetyl group of an octa-arginyl peptide (Suzuki et al. 2002) was conjugated to the side chain thiol group of the Cys residue that was introduced at the C-terminus of each segment to obtain the CA peptides containing an octa-argininyl sequence 1L-23L (Fig. 6.4). As



fragment number	sequence
S1	H-PIVQNLQGQMVHQAIGC-NH_2
S2	Ac-VHQAISPRTLNAWVKGC-NH_2
S3	Ac-NAWVKVVEEKAFSPEGC-NH_2
S4	Ac-AFSPEVIPMFSALSEGC-NH_2
S5	Ac-SALSEGATPQDLNTMGC-NH_2
S6	Ac-DLNTMLNTVGGHQAAGC-NH_2
S7	Ac-GHQAAMQMLKETINEGC-NH_2
S8	Ac-ETINEEAAEWDRLHPGC-NH_2
S9	Ac-DRLHPVHAGPIAPGQGC-NH_2
S10	Ac-IAPGQMREPRGSDIAGC-NH_2
S11	Ac-GSDIAGTTSTLQEQIGC-NH_2
S12	Ac-LQEQIGWMTHNPPIPGC-NH_2
S13	Ac-NPPIPVGEIYKRWIIGC-NH_2
S14	Ac-KRWIILGLNKIVRMYGC-NH_2
S15	Ac-IVRMYSPTSILDIRQGC-NH_2
S16	Ac-LDIRQGPKEPFRDYVGC-NH_2
S17	Ac-FRDYVDRFYKTLRAEGC-NH_2
S18	Ac-TLRAEQASQEVKNWMGC-NH_2
S19	Ac-VKNWMTETLLVQNANGC-NH_2
S20	Ac-VQNANPDSKTILKALGC-NH_2
S21	Ac-ILKALGPGATLEEMMGC-NH_2
S22	Ac-LEEMMTASQGVGGPGGC-NH_2
S23	Ac-VGGPGHKARVLGC-NH_2

Fig. 6.7 An overlapping peptide library spanning the whole sequence of 231 amino acid residues of the CA protein

controls, iodoacetamide was conjugated to the thiol group of the Cys residue to obtain the CA peptides 1C-23C (Fig. 6.4). The anti-HIV activity of the CA peptides was evaluated using X4- and R5-HIV-1 strains initially in the anti-HIV assay in the absence of the addition of chloroquine (Table 6.4). Without chloroquine, some CA peptides showed significant anti-HIV activity against both strains. The CA peptides CA-1L, CA-2L, CA-6L, CA-8L, and CA-15L showed potent anti-HIV activity against X4-HIV-1. CA-15L showed potent anti-HIV activity against R5-HIV-1, whereas CA-1L and CA-6L showed moderate activity and CA-2L or CA-8L showed no significant activity against R5-HIV-1 below 50 μM. CA-19L showed moderate activity against X4-HIV-1 and R5-HIV-1. With the exception of CA-6C,

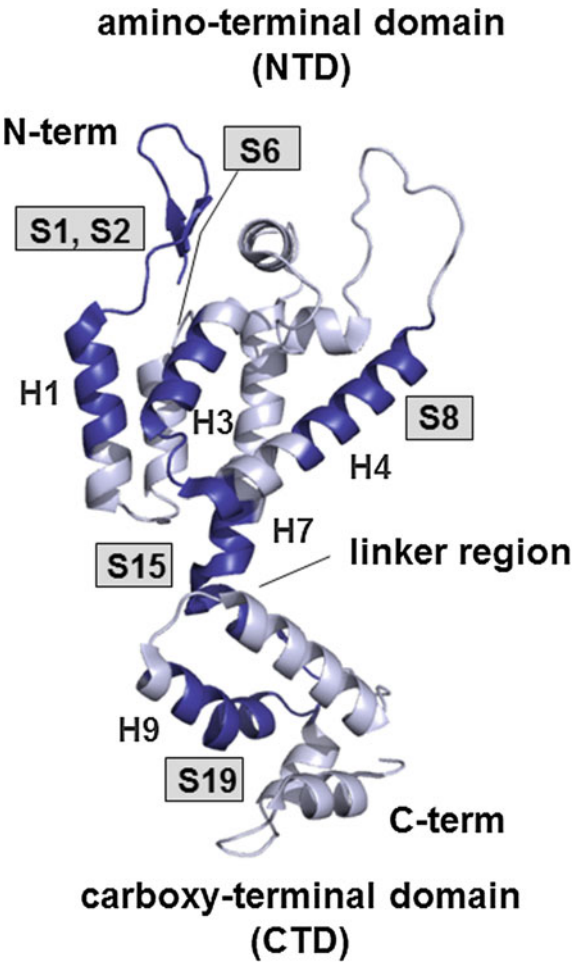


Fig. 6.8 The X-ray crystal structure of the CA protein (PDB: 3H47)

CA-8C, and CA-19C, the control CA peptides showed no significant anti-HIV activity against either strain (data not shown) (Mizuguchi et al. 2015). The sequence that covers CA-1L is located in a β-sheet/turn region in the N-terminus of the CA protein, and the sequences covering CA-2L, CA-6L, and CA-8L are located in the helical regions H1, H3, and H4, respectively (Fig. 6.8). These regions are located in the exterior surface of the CA protein molecule. Thus, CA-1L, CA-2L, CA-6L, and CA-8L have potent anti-HIV activity, but the reason why CA-2L or CA-8L has no significant activity against R5-HIV-1 is not clear. The sequence that covers CA-15L is located in the helix region H7 and the linker region connecting the N- and C-terminal domains (Fig. 6.8) (Pornillos et al. 2009; Gres et al. 2015). Since CA-15L showed potent anti-HIV activity, these regions might be critical for conformational orientation of the CA protein (Pornillos et al. 2011). The sequence that covers CA-19L is located in the helix region H9, which corresponds to the dimer

Table 6.4 Anti-HIV activity (IC_{50}) and cytotoxicity (CC_{50}) of some CA peptides containing an octa-argininyl sequence

CA peptide	MT-4 cell		PM1/CCR5 cell	
	EC_{50} (μM)[a]	CC_{50} (μM)[b]	EC_{50} (μM)[c]	CC_{50} (μM)[d]
CA-1L	5.2	>50	25	>50
CA-2L	7.8	>50	>50	>50
CA-3L	45	>50	>50	>50
CA-4L	38	>50	>50	>50
CA-5L	35	>50	>50	>50
CA-6L	9.8	>50	27	>50
CA-7L	>50	>50	36	>50
CA-8L	15	>50	>50	>50
CA-10L	34	>50	>50	>50
CA-13L	>33	33	>25	25
CA-14L	>7.8	7.8	>11	11
CA-15L	11.2	18.6	2.8	25
CA-16L	33	>50	>50	>50
CA-17L	>20	20	>18	18
CA-19L	>50	>50	25	>50
CA-20L	35	>50	>50	>50
AZT	0.063	>100	0.64	>100
AMD3100	0.033	>50	>50	>50
SCH-D	>5.0	>5.0	0.0062	>5.0

[a–d]Shown in Table 6.2

interface of the C-terminal domain of CA (Zentner et al. 2013), although 19L showed only moderate anti-HIV activity without chloroquine. With the exception of CA-13L, CA-14L, CA-15L, and CA-14C, the CA peptides did not show significant cytotoxicity below 50 μM. In general, peptides containing an octa-argininyl sequence show relatively high cytotoxicity presumably due to their high positive charges.

Selected CA peptides containing an octa-argininyl sequence were assessed in the presence of 5 μM chloroquine and compared to the corresponding assays without chloroquine (Mizuguchi et al. 2015) (Table 6.5). All of the evaluated CA peptides containing an octa-argininyl sequence, CA-1L, CA-3L, CA-5L, CA-6L, CA-7L, CA-15L, and CA-19L showed more potent anti-HIV activity against X4-HIV-1 in the presence of chloroquine than that in the absence of chloroquine. Considering the MA peptides, it is common sense that CA peptides containing an octa-argininyl sequence might be captured in endosomes after cell penetration but efficiently released from endosomes by the action of chloroquine.

The inhibitory mechanism of the above CA peptides might be speculated as follows. The CA fragment peptides might competitively attack CA protein molecules and/or cellular proteins, which are correlated to the natural interaction with the

Table 6.5 Anti-HIV activity of the CA peptides containing an octa-argininyl sequence (1L, 3L, 5L, 6L, 7L, 15L, and 19L) in the presence or absence of chloroquine

CA peptide	MT-4 cell	
	Chloroquine (-)	Chloroquine (5 μM)
	EC_{50} (μM)[a]	EC_{50} (μM)[a]
CA-1L	2.7	1.6
CA-3L	34	16
CA-5L	23	17
CA-6L	5.7	3.4
CA-7L	29	15
CA-15L	31	13
CA-19L	43	23
AZT	0.060	0.024
AMD3100	0.036	0.035
SCH-D	>5	>5

[a]Shown in Table 6.2

CA protein inside HIV-1-infected cells. The CA peptides might block the viral uncoating process at an early stage of the replication cycle or the CA oligomerization/assembly process at a later stage through the above competitive attack. In future, further studies will be required to elucidate details of the mechanisms of action of these CA peptides. These peptides are useful leads for the development of more potent inhibitory peptides or peptidomimetics, and can be classified as mid-size drugs.

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Chapter 7
Summary and Future Perspectives of Researches on Mid-size Drugs

Abstract As described up to here, there are many drug candidates in the mid-size region between low and high molecular weight. Mid-size drugs, which designate middle-size molecules, might maintain some advantages and reduce drawbacks involved by small and macromolecules.

As described up to here, there are many drug candidates in the mid-size region between low and high molecular weight. Mid-size drugs, which designate middle-size molecules, might maintain some advantages and reduce drawbacks involved by small and macromolecules. Peptide compounds, which are categorized in these mid-size drugs, are being focused for development of next generation drugs. However, therapeutical use of peptides has some limitation in several factors, which involve low metabolic stability toward proteolysis and undesired activity resulting from interactions of flexible peptides with several receptors. A medicinal research into the discovery of peptide-lead drugs has developed novel modifications of their structures for maintenance of biological activity, which leads to peptidomimetics. Peptidomimetics point not only peptide isosteres that mimic primary structures of peptides, but also mimetics that mimic secondary and tertiary structures of peptides. Peptide derivatives and peptidomimetics, which are directed to high biological activity and structural stability, can aim protein–protein interactions as well as active centers of enzymes and pockets of receptors, because mid-size drugs can cover broadly target molecules. Cyclic peptides are useful to find biologically active molecules because structural conformation of these compounds might be determined relatively easily, and especially cyclic pentapeptides are conformationally restricted templates: some examples are described in this book.

We have developed several peptidomimetics, which target HIV. Thus, as examples of mid-size drugs, several anti-HIV agents are introduced in this book. During the last several years of the twentieth century, a combinational use of reverse transcriptase inhibitors and protease inhibitors, which was designated HAART, provided a great success in clinical treatments. Furthermore, novel drugs, which belong to different categories including entry inhibitors and integrase inhibitors, have been developed and approved by the FDA for clinical use. However,

H. Tamamura et al., *Mid-size Drugs Based on Peptides and Peptidomimetics*,
SpringerBriefs in Pharmaceutical Science & Drug Development,
https://doi.org/10.1007/978-981-10-7691-6_7

new useful drugs with novel mechanisms of action are still required because of serious clinical problems including side effects, the emergence of MDR strains, and high costs. Herein, newly developed coreceptor antagonists, integrase inhibitors, vaccines, fusion inhibitors, CD4 mimics, and matrix/capsid fragment peptides are introduced.

The CXCL12/CXCR4 system is an important medicinal target, and several peptidic and non-peptidic CXCR4 antagonists have been developed to date. Based on antimicrobial peptides contained in horseshoe crabs, a potent CXCR4 antagonist, 14-mer peptide T140, has been found. T140 derivatives inhibit X4-HIV-1 entry through their competitive binding to CXCR4 and have significant inhibitory activity against HIV infection. These peptide derivatives have inhibitory activity against cancer/leukemia. A biologically stable derivative of T140, 4F-benzoyl-TN14003/BL-8040/BKT-140 (BioLineRx Ltd.), is now in Phase II clinical trials for the treatment of acute myeloid leukemia (AML). To develop smaller CXCR4 antagonists, a pharmacophore-guided approach has been performed adopting critical amino acid residues of T140 and utilizing cyclic pentapeptides as conformationally restricted templates to find a cyclic pentapeptide FC131, which was a potent CXCR4 antagonist comparable to T140. Furthermore, CXCR4 bivalent ligands containing two molecules of an FC131 derivative, [*cyclo*(–D–Tyr–Arg–Arg–Nal–D–Cys–)], which are connected by poly(L-proline) or PEGylated poly(L-proline) linkers, have been synthesized. Bivalent ligands having linkers with suitable lengths (5.5–6.5 nm) can accurately recognize the native state of the CXCR4 dimer, suggesting that two ligand binding sites are separated by 5.5–6.5 nm lengths. Fluorescent-labeled bivalent ligands can detect cancer cells with overexpression of CXCR4, and thus, might be useful for cancer diagnosis. To date, a CCR5 antagonist, maraviroc (Pfizer Inc.), has been approved by the FDA although clinical trials of several other CCR5 antagonists have been performed.

Three small HIV-1 IN inhibitors, raltegravir (Isentress) (Merck Sharp & Dohme Corp.), elvitegravir (Gilead Sciences, Inc./JT) and dolutegravir (Tivicay) (Shionogi/GSK), have been developed in AIDS chemotherapy although such inhibitors require combinational regimens. Allosteric type HIV-1 IN inhibitors, which are different from the above drugs and Vpr-derived fragment peptides, have been developed by our groups, followed by the subsequent application of a stapling strategy to obtain Vpr-derived IN inhibitory peptides with a remarkable increase in their intracellular potency.

The design of HIV vaccines and fusion inhibitors mimics on the native structure of proteins involved in the dynamic supramolecular mechanisms of HIV entry/fusion. This mimicking is proven to be an effective strategy. The N36 trimer mimic antigen with complete equivalency induces neutralizing antibodies with structural preference for the antigen. As fusion inhibitors, trimer and dimer mimics of C34 with complete equivalency have 100-fold higher anti-HIV-1 activity compared to the corresponding monomers. Effective inhibitors, which target protein–protein interactions such as six-helical bundle formation in the gp41 assembly, have attracted broad attention as mid-size drugs.

CD4 is the first and classical target for anti-AIDS chemotherapy, and thus several small-sized CD4 mimics have been reported to date. These compounds cause a conformational change in gp120 as envelope openers in a similar way with soluble CD4. Thus, neutralizing antibodies such as an anti-V3 monoclonal antibody KD-247 easily access the V3 region of gp120. Thereby, these CD4 mimics show highly synergistic anti-HIV activity with KD-247. Thus, these compounds are useful leads not only for entry inhibition but also for the use in combination with neutralizing antibodies. Furthermore, CD4 mimics also show a remarkable synergistic anti-HIV activity with CXCR4 antagonists such as T140. Thus, several hybrid molecules of a CD4 mimic molecule and a T140 derivative were also synthesized. As a result, the selectivity index of the CD4 mimics was increased. In future, novel type of CD4 mimic-based HIV-1 entry inhibitors might be developed through optimization of the linkers.

To find anti-HIV peptides, overlapping peptide libraries covering the whole sequence of MA have been prepared with the conjugation of an octa-arginyl sequence to increase cell membrane permeability. The MA peptides 8L and 9L, which might suppress the MA hexamer formation/oligomerization and/or degradation through competitive binding to the parent MA, showed potent anti-HIV activity. The combination of the conjugation of an octa-arginyl sequence as a cell penetration unit to individual peptides contained in whole proteins with the addition of chloroquine to cells used in assays is a useful strategy to search for practically active peptides.

Owing to CA, overlapping fragment peptide libraries covering the whole sequence of an HIV-1 CA protein with the addition of an octa-arginyl moiety have been constructed. Among these peptides, anti-HIV activity was detected in cell-based anti-HIV assays with the addition of chloroquine to find several anti-HIV peptides. The competitive binding of the CA peptides to CA protein molecules or cellular proteins might block the viral uncoating process or the CA oligomerization/assembly process. However, detailed action mechanisms will be elucidated in the future. Mid-size drugs such as peptide derivatives would be desirable because the above MA and CA peptides might aim protein–protein interactions and the interfaces might be extensively broad.

During anti-HIV treatment, HIV-infected patients might face the loss of efficacy of HAART due to the emergence of MDR viruses. In this case, a change of regimens of the drug combination in HAART might be effective if the amounts of the virus and CD4 in blood can be monitored. Therefore, the number of available potent drugs becomes a key in treatment of AIDS and HIV-infected patients. To increase available drug repertoires entry inhibitors such as CCR5/CXCR4 antagonists and CD4 mimics including hybrid molecules, fusion inhibitors, IN inhibitors, and MA and CA peptides might become important options.

The HIV replication cycle contains numerous protein–protein interactions. For an example, it has clearly been disclosed that the dynamic supramolecular mechanisms of entry/fusion steps involve some protein–protein interactions. Thus, the mid-size drugs described above might become effective drug leads because these compounds can recognize and cover broadly extensive interfaces.

As described in this book, several peptidomimetics that mimic primary, secondary, and tertiary structures of peptides, suitable mimetics for suitable aims, can be applied to maintain and improve biological activities and actions of peptides. In future, peptide and peptidomimetics-derived mid-size drugs will bring a comprehensive expansion of drug discovery.