Bioorganic Marine Chemistry Volume 2

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Preface

Burgeoning research into marine natural products during the past two decades has in no small measure been due to an heightened and world-wide interest in the ocean, to the development of new sophisticated computer-driven instrumentation, and to major advances in separation science. Organic chemists have been fully aware that processes in living systems occur in an aqueous medium. Nevertheless, the chemists who have specialized in the study of small molecules have found it expedient to use organic rather than aqueous solvents for the isolation and manipulation of secondary metabolites. The emergence of new chromatographic techniques, the promise of rewarding results, not to mention the relevance of polar molecules to life itself, have contributed to a new awareness of the importance of organic chemistry in an aqueous medium.

The first chapter in Volume 2 of Bioorganic Marine Chemistry reflects the growing interest and concern with water-soluble compounds. Quinn, who pioneered the separation of such molecules, has contributed a review which closely links techniques with results and is based on practical experience. The second chapter, by Stonik and Elyakov, examines the vast chemical literature of the phylum Echinodermata – over one fourth of it in difficulty accessible Russian language publications. The Soviet authors evaluate the data for their suitability as chemotaxonomic markers.

Two ecological chapters round out Volume 2. Sammarco and Coll offer a comprehensive discussion of octocorals, their coral reef environment, including food, reproduction, predation, competition for space. The final chapter by Tachibana takes up chemical defense in fishes. Although the biology of fishes has a long and distinguished natural history, our knowledge of the molecular basis of some ecological phenomena involving fishes is extremely limited for the obvious reason that this research is difficult, involving as it does highly polar molecules and intricate bioassays.

I should like to thank all contributors for their timely cooperation and, as always, I would like to hear from colleagues who have suggestions or comments for this or future topics in "Bioorganic Marine Chemistry".

February 1988 Paul J. Scheuer

Table of Contents

Chemistry of Aqueous Marine Extracts: Isolation Techniques

Ronald J. Quinn[1]

Contents

Abstract

Various techniques for isolating compounds from aqueous extracts of marine organisms are reviewed. The major isolation techniques involve separation by differences in molecular size, charge or adsorption properties. Successful strategies employed to isolate bioactive constituents from extracts which displayed activity in a variety of biological screens are discussed. The recent marine natural product literature is reviewed and the isolation procedures for compounds isolated from aqueous extracts are summarized.

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Bioorganic Marine Chemistry, Volume 2

1 Introduction

Compounds obtained from aqueous extracts of marine organisms have contributed very little to the increasing number of new organic molecules isolated from marine organisms. We have over a number of years achieved the isolation of the bioactive constituents from aqueous extracts which displayed activity in a variety of biological screens [1]. In many other investigations, where an initial aqueous extraction was performed, subsequent investigations were limited to those compounds which partitioned into an organic phase. The isolation of polar, low molecular weight natural products presents a serious challenge to the organic chemist, particularly from the marine environment with the presence of sodium chloride and other salts in all aqueous extracts. Notwithstanding the difficulties, polar water-soluble constituents can be isolated and purified by a number of different techniques which in combination provide a powerful separating capacity. The recent technique of introducing functional groups onto a number of chromatographic phases has added a new level of versatility and appears to allow the natural product chemist to make many more advances in the chemistry of polar natural products.

It is possible to use several properties of molecules to effect separation; the most useful properties are molecular size, net or potential charge and adsorption capacity. Macromolecules are easily separated from the salts and other low molecular weight contaminants, and further purification of these large molecules can be pursued by standard biochemical methods. The low molecular weight compounds, which include a large number of potentially biologically active constituents, are more interesting to the organic chemist, but have been largely avoided because of the difficulties of separation.

The various molecular properties that can be used to achieve separation will be discussed. Several examples will be given, including a series of marine extracts which displayed biological activity and which were investigated by a variety of techniques in order to determine the most appropriate isolation strategy.

Following discussion of the molecular properties, isolation of the biologically active constituents of the sponge *Tedania digitata* [2,3], the red alga *Gracilaria edulis (lichenoides)* [4], the holothurian *Pentacter crassa* [5], the soft coral *Nephthea* sp. [6], the sponges *Haliclona* and *Chalinopsilla* spp. [7], and the octopus *Hapalochlaena maculosa* [8], will be used to illustrate successful strategies for the isolation of bioactive constituents from aqueous marine extracts. This will be followed by an evaluation of the marine natural products literature to highlight constituents isolated from aqueous marine extracts and a discussion of the role of reversed phase adsorbents.

There is little doubt that any biological screening program which screens both organic and aqueous extracts will discover many active aqueous extracts. The broadly based pharmacological screening program carried out on crude extracts at the former Roche Research Institute of Marine Pharmacology in Australia revealed that approximately half of the observed pharmacological activities occurred in the organic extracts and half in the aqueous extracts. While the full details of the pharmacological screening have not been published, a review of the pharmacological and microbiological screening of 159 algae [9] and the results of

the antimicrobial screening of algae have been published [10]. This chapter attempts to provide information that will facilitate the chemical evaluation of aqueous marine extracts.

The isolation of polar low molecular weight constituents should be pursued by mild methods which will not degrade the constituents. Aqueous extracts are a nutrient-rich medium in which bacterial and fungal growth may occur and suitable precautions must be taken.

2 Useful Properties Allowing Separations

2.1 Size

Diafiltration can be used to separate compounds by their effective size. Ultrafilters consist of thin semipermeable membranes bound to a porous sustrate. They are manufactured with a variety of pore sizes and are characterized by nominal molecular weight limits above which most species are retained. It is possible to use ultrafilters in series in order to obtain a fractionation based on the size of the molecules.

Diafiltration in which a constant volume is maintained in the sample solution by continuous addition of wash solution results in the retention of large species in their initial concentration, while the permeable solutes are diluted in the ultrafiltrate. Table 1 gives the results for diafiltration of crude extracts using an ultrafilter of 10 000 dalton cut-off in series with an ultrafilter which is rated at 500 dalton.

Diafiltration offers a coarse fractionation by molecular size, whereas gel permeation chromatography offers the ability to obtain greater resolution. Molecules are retarded as they pass through the porous gel medium depending on the time spent within the pores and are eluted in order of decreasing size, molecules larger than the pore size are excluded and elute first. It is possible to use gel permeation columns connected in series in a similar fashion to ultrafilters in series. If a gel with a lower exclusion volume is connected in tandem to the eluant of an initial gel column, material excluded by the second gel will retain its relative position as eluted from the first gel, while material that enters the pores of the second gel will be further separated. Figure 1 shows the separation achieved on a Pharmacia Sephadex G-25 column connected in series with a Pharmacia Sephadex G-10 column. A crude aqueous ethanolic extract of a sponge of the family Verongidae displayed antimicrobial activity against both *Staphylococcus aureus* and *Escherichia coli* [10]. The active constituent was shown to be the known compound 2,6-dibromo-4-acetamido-4-hydroxycyclohexa-2,5-dienone (3,5-dibromoverangiaquinol) (**1**) [11], which constituted 1.3% of the crude extract or 0.2% of the dry sponge.

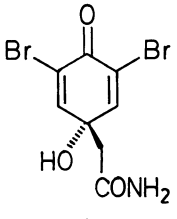

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Table 1. Diafiltration of a series of crude marine extracts: Distribution by size

Species	Extract	Biological activity	Amicon UM10 retentate[a] wt%	Amicon UM05 retentate[b] wt%	Dia-filtrate[c] wt%
Sponge *Ircinia* sp	Water	Atrial stimulant anti-inflam-matory	14.5	11.0	73.5
	Methanol	Atrial stimulant	4.3	8.1	75.9
Soft coral *Sinularia flexibilis*	Methanol	Hypotensive	14.6	13.5	53.2
Gorgonian *Rumphella* sp	Water	Anti-yeast[i]	12.7	2.1	52.3
Sponge	Water	Hypotensive	9.8	12.8	59.5
Sponge	Water	Atrial stimulant	4.4	7.8	70.5
Chondrilla sp	Methanol	Hypotensive	16.7	9.5	70.2
Brown alga *Acrocarpia paniculata*	Water	Anti-bacterial[d]	10.3	3.8	63.5
Red alga *Laurencia elata*	Water	Anti-bacterial[g]	13.4	9.7	67.1
Gorgonian *Briacium* sp	Water	Hypotensive	5.0	10.2	90.1
Soft coral *Sinularia* sp	Water	Hypotensive atrial stimulant	2.3	9.8	84.2
Sponge *Pericharax* sp	Water	Anti-yeast[h]	8.9	19.4	70.1
Soft coral *Sinularia* sp	Water		11.2	8.6	81.0
Sponge	Water	Anti-amphetamine barbiturate potentiation	22.6	6.2	
Brown alga *Scytothaminas australis*	Water	Hypotensive	16.9	6.9	65.9
Sponge *Dysidea fragilis*	Water	Atrial stimulant	1.0	20.4	78.1
Sponge *Pachychalina* sp	Water	Hypertensive anti-fungal[j]	1.2	25.5	60.0
Soft coral *Sinularia* sp	Water	Hypotensive anti-bacterial[e]	5.2	25.4	48.3
Sponge *Ancorina* sp	Water	Anti-bacterial[f, g]	4.7	23.2	49.6
Sea pen *Scytalium sersii*	Water	Hypotensive	10.0	7.9	80.5
Brown alga *Hormosira banksii*	Water	Anti-fungal[k]	25.7	6.7	57.6
Holothurian *Thelenota ananas*	Water	Bronchodilator anti-fungal[j, k]	5.1	11.3	56.6
Soft coral *Sarcophyton* sp	Water	Nicotine blocker	2.6	12.5	80.7

Table 1 (continued)

Species	Extract	Biological activity	Amicon UM10 retentate[a] wt%	Amicon UM05 retentate[b] wt%	Dia-filtrate[c] wt%
Soft coral *Sinularia* sp	Water	Bronchodilator	21.2	36.5	33.5
Sponge *Ianthella filiformis*	Water	EMG	9.8	4.0	51.5
Brown alga	Water	Anti-convulsant	29.4	10.4	63.3
Xiphophora chondrophylla	Methanol	Anti-convulsant	18.7	10.8	70.3

[a] Approximate M.W. > 10,000 dalton.
[b] Approximate M.W. 10,000–500 dalton. UM05 retentate refers to material which passed through the UM10 ultrafilter and was retained by the UM05 ultrafilter.
[c] Approximate M.W. < 500 dalton.
[d] Gram-positive bacteria *Sa Staphylococcus aureus.*
[e] Gram-positive bacteria *Spy Streptococcus pyogenes.*
[f] Gram-negative bacteria *Ec Esherichia coli.*
[g] Gram-negative bacteria *Pm Proteus mirabilis.*
[h] Yeast *Ca Candida albicans.*
[i] Yeast *Cn Cryptococcus neoformans.*
[j] Fungi *Tm Trichophyton mentagrophytes.*
[k] Fungi *Ma Microsporum audouini.*

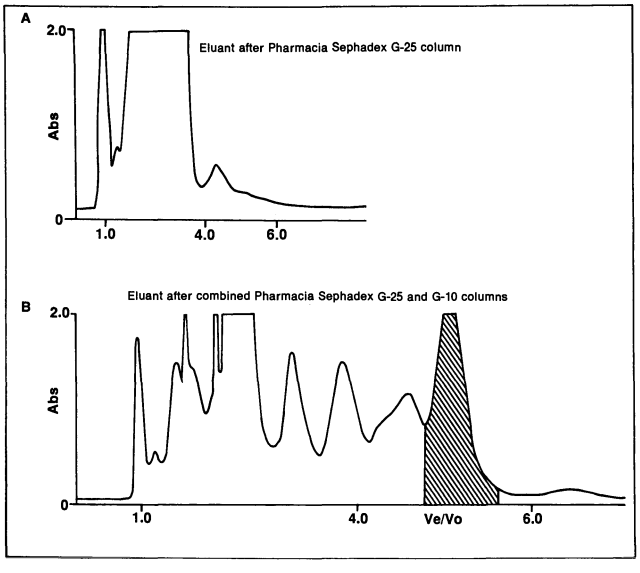


Fig. 1. Chromatography of crude extract (1.2 g) of a sponge of the family *Verongidae* on Pharmacia Sephadex G-25 fine (38.8 × 2.5 cm) in series with Pharmacia Sephadex G-10 (41.4 × 2.5 cm). Eluant: water, UV monitor: 254 nm, 2.0 A full scale

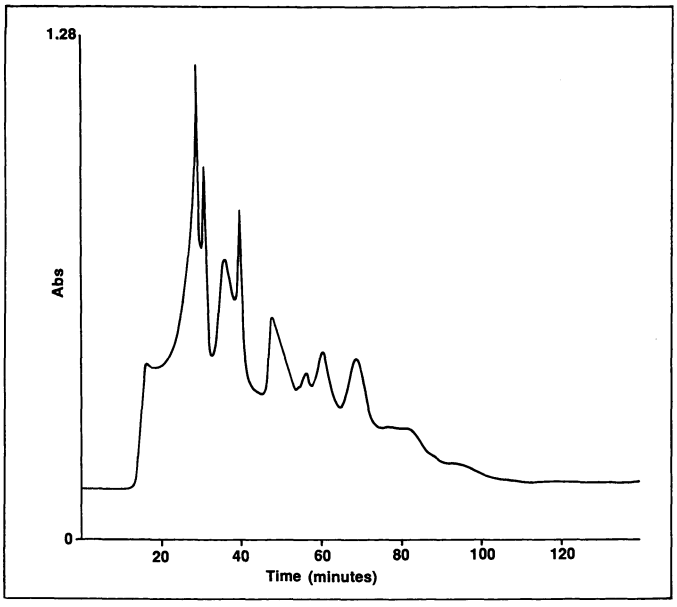


Fig. 2. Chromatography of crude extract (1.0 g) of *Stelleta conulosa* on Merck TSK-HW-40(S). (27 × 3.8 cm). Eluant: water, UV monitor: 254 nm, 1.28 A full scale, flow rate: 5 ml/min, pressure: 100 psi

Gel permeation chromatography with soft compressible gels is a slow process. The example in Fig. 1, where 1.2 g of crude extract yielded 15.7 mg of the biologically active fraction took 20.5 hours. The introduction of more rigid supports for gel permeation chromatography has allowed this process to be speeded up considerably. Figure 2 shows a separation on Merck Fractogel TSK HW-40 (S).

Diafiltration offers a ready answer to the question of molecular size. Stirred cells connected in series can be an effective choice for small scale separations. A pressurized reservoir placed between the nitrogen supply and the stirred cells will automatically maintain the fluid level in the cells and provide diafiltration under conditions of constant volume. The resolving power of diafiltration is not high, as each filter provides only retentate (containing compounds excluded by the membrane) and diafiltrate (containing compounds which pass through the membrane); in addition, there is no distinct cut-off point. This means that the compound of interest, if it is of a molecular size close to the nominal cut-off value, may be distributed over both fractions. Small organic molecules are separated with the fraction that contains the salts; hence the degree of purification is small: however, the technique has decided advantages for purification of excluded larger molecules. Diafiltration is a particularly mild method, as it can be run in a cold room at 4° C. Furthermore, the process can be run unattended if a pressurized reservoir is used; as water is the solvent, the fractions can be directly freeze-dried. Resolution of a mixture is much greater with gel permeation chromatography. Sodium chloride travels as a discrete band and can be separated from many small organic molecules. The ability to use rigid supports in conjunction with HPLC

equipment found in most organic chemistry laboratories may mean that this will be the favored approach for most workers.

2.2 Charge

Ion exchange chromatography can offer a highly selective process. Many polar organic constituents will have ionizable functional groups. Such compounds can be adsorbed to ion-exchange resins bearing a suitable functional group of opposite charge.

Table 2 provides data for the behavior of a series of extracts of marine organisms with strong cation and anion exchangers. The goal of this experiment was to determine if a biologically active constituent could be adsorbed on an ion-exchange resin, for which strong exchangers offer the best choice. For compounds to be adsorbed they must be charged at the pH at which the chromatography is conducted. They can then be eluted by changing the pH to make the adsorbed molecules neutral, or to make the ion exchange resin neutral. Elution of bound constituents from strong ion exchange resins will often require extreme conditions, under which decomposition may occur. In that case a purification scheme must be developed in which a suitable weak ion exchange resin is employed together with a volatile buffer so that the eluants can be directly freeze-dried. Pharmacia SP-Sephadex and BioRad AG 50 are both sulfonic acid cation exchange resins and were used as free sulfonic acids. In one case, extracts were dissolved in 3% acetic acid in order to protonate any basic groups, while in the other the extract was dissolved in water. Pharmacia QAE-Sephadex and Bio-Rad AG 1 are quaternary ammonium anion exchange resins. In one case, extracts were dissolved in 1% ammonia in order to ionize any acids, while in the other the extract was dissolved in water. The recovered weights are often greater than the amount added, as the counter ions are eluted and compounds may be eluted as salts. Regarding the behavior of a biologically active constituent, it must be borne in mind that initial adsorption to the resin that results in loss of biological activity indicates that the biologically active constituent has a potentially ionizable functional group and that isolation by ion exchange chromatography should be explored further. While in many cases biological activity may not be observed in the fractions eluted under the rather harsh conditions of this initial screening, it is possible to modify the conditions of the ion exchange chromatography to allow its use in a separation scheme. These further investigations will take into account the stability of the constituents, the counter-ion selectivity and elution of the constituent of interest by neutralizing the charge on the constituent, by neutralizing the charge on the resin, by increasing the ionic strength of the buffer, or by using a buffer containing highly selective ions.

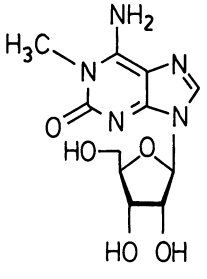
The selectivity of ion-exchange chromatography is well illustrated by the isolation of the pharmacologically active nucleoside 1-methylisoguanosine (**2**) from the sponge *Tedania digitata* [3]. By buffering the extract at pH 3.5, the active constituent could be absorbed on a sulfonic acid cation exchange resin. Elution with a buffer at pH 5.3 resulted in elution of the bioactive material. Rechromatography of this active fraction under the same conditions, followed by recrystalli-

Table 2. Ion exchange of a series of crude extracts

Species	Extract	MB-3	SP-Sephadex		AG-50W-X8 (H form)			QAE-Sephadex		AGI-X8 (acetate form)	
			3% HOAC	1% NH_3	H_2O	2N HCl	5N HCl	1% NH3	3% HOAC	H_2O	2N HCl
Sponge	Water	14.8	71.3	13.7	20.3	74.0	–	84.2	21.1	91.2	11.5
Ircinia sp	Methanol	6.1.	73.1	31.2	9.5	86.4	–	112.1	36.4	90.3	7.8
Soft coral *Sinularia flexibilis*	Methanol	16.9	58	27.2	14.8	64.5	8.5	50.0	44.6	64.6	3.4
Gorgonian *Rumphella*	Water	5.4	75.5	17.9	27.4	74.7	–	75.1	26.2	98.4	15.2
Sponge	Water	1.2	54.5	7.1	16.8	148.9	0.8	84	40	104	1.1
Sponge	Water	11.9	92.3	19.2	14.9	71.7	4.0	111.0	59.0	111.9	17.2
Chondrilla sp	Methanol	5.7	86.6	27.2	21.6	60.8	4.1	78.0	45.9	82.5	4.2
Brown alga *Acrocarpia paniculata*	Water	18.4	66.0	8.4	35.1	62.1	–	91.5	4.8	101.5	4.7
Red alga *Laurencia elata*	Water	22.6	97.0	0.6	41.0	66.7	–	134.5	11.8	111.8	11.0
Gorgonian *Briacium* sp	Water	3.8	86.0	10.2	19.7	81.3	6.1	117.0	23.3	87.1	13.5
Soft coral *Sinularia* sp	Water	3.9	61.3	13.4	13.6	83.5	1.6	85.2	13.5	107.2	11.9

Sponge *Pericharax* sp	Water	13.3	71.8	2.6	25.4	74.0	4.4	90.7	18.1	97.2	11.7
Soft coral *Sinularia* sp	Water	11.5	72.8	5.9	18.6	90.5	–	107.1	44.8	105.4	10.0
Sponge *Dysidea fragilis*	Water	14.5	93.1	52.3				84.4	9.7		
Sponge *Pachychalina* sp	Water	1.2	81.2	2.0	20.0	63.6	10.3	112.4	28.0	64.0	19.2
Soft coral *Sinularia* sp	Water	21.8	56.8	12.6	37.1	103.9	10.1	77.2	20.4	76.9	6.0
Sponge *Ancorina* sp	Water	5.5	56.5	19.7	21.5	61.7	10.6	80.4	16.3	79	9.6
Sea pen *Scytalium sersii*	Water	3.0	59.7	19.1	55.7	141.3	1.9	123	15.0	88.2	8.9
Brown alga *Hormosira banksii*	Water	21.8	51.5	28.9	25.1	62.0	1.0	72.3	20.9	91.1	8.5
Holothurian *Thelenata ananas*	Water	4.0	82.9	5.5	14.7	101.6	0.4	93.8	14.8	89.8	5.0
Soft coral *Sarcophyton* sp	Water	9.6	70.5	8.6	86.8	74.5	1.0	77.5	20.8	86.8	7.9
Soft coral *Sinularia* sp	Water	17.2	41.2	50.5	40.1	41.8	1.0	54.0	37.4	81.2	4.1

zation from water yielded 1-methylisoguanosine. This procedure was highly selective since pH was controlled for adsorption and elution. By this procedure 385 g of crude extract yielded 2.75 g (0.71%) of 1-methylisoguanosine.



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The crude extract was processed in two batches on a 40 × 5 cm column and the combined active fraction was rechromatographed on a column of the same size. Ion-exchange adsorbents may have a high capacity dependent on the number of functional groups present on the polymeric matrix. The resin used for this work (Bio Rad AG50W-X8, ammonium form, 200–400 mesh) has a capacity of 1.7 meq/mL. In this case, purification of an extract from 2.41 kg of freeze-dried sponge was achieved by three chromatographies on a regular size laboratory column. This may be compared with the use of gel permeation chromatography, where a column of about the same dimensions (52 × 5 cm) could only separate 4 g of a diafiltrate (500 dalton nominal exclusion limit) from 8 g of the crude extract; if one extrapolates, one finds that it would have taken 48 consecutive chromatographies to achieve the same result.

Another operational point must be noted for the isolation of low molecular weight components. A volatile buffer must be used so that the buffer salts can be removed by lyophilization. Ammonium formate at pH 3.5 and 5.3, which was 0.1 M with respect to formate, was used for the above described separation.

2.3 Adsorption

Adsorption chromatography is the most widely used method for the isolation and purification of small organic molecules. Normal adsorption chromatography has played a key role in the isolation of marine natural products. The strong polarity of water and other aqueous solvent mixtures precludes the use of normal adsorption chromatography for the examination of polar natural products occurring in aqueous extracts of marine organisms. In reversed phase chromatography, where the adsorbent is less polar than the mobile phase, it is possible to adsorb compounds from aqueous solutions and to elute the bound compounds by decreasing the polarity of the eluting solvent. Reversed phase chromatography has found extensive use in analytical applications and has increasingly been used for preparative work. The reversed phase adsorbent must possess low polarity. Two general methods may be used to obtain such adsorbents. Polymers of suitable polarity can

be used directly, or other adsorbents can be modified so that their polar groups are substituted by non-polar groups.

Amberlite XAD resins are examples of polymeric materials which can be used for reversed phase chromatography. These resins have large specific surface areas and are used as adsorbents for organic materials. Amberlite XAD-2 is a porous polystyrene-divinylbenzene copolymer, while Amberlite XAD-7 is a cross-linked acrylate polyester. The structural differences parallel differences in adsorption properties. Amberlite XAD-2 thus is a nonpolar adsorbent and is used to adsorb nonpolar substances from aqueous solutions, while Amberlite XAD-7 is more polar and is used to adsorb polar compounds from nonpolar solvents. It also finds useful application for aqueous marine extracts, where nonpolar compounds may be adsorbed from polar solvents. Information on the use of Amberlite XAD-2 as a chromatographic phase is available [12–16]; we have reported applications with Amberlite XAD-7 [17]. The following eluotropic series have been established for Amberlite XAD-2 [18] and Amberlite XAD-7 [17], and are detailed in Table 3.

In practice, it is not necessary to proceed past 100% acetone as the eluant, since almost everything that is going to be desorbed by an organic solvent will have been desorbed. Amberlite XAD-2 has a greater affinity for aromatic compounds (benzene, naphthalene, anthracene) than Amberlite XAD-7 [17]. In methanol, the capacity factor [$k' = (Ve - Vo)/Vo$ where Ve is the elution volume and Vo is the void volume] for naphthalene is 3.42 on Amberlite XAD-2 and 0.41 on Amberlite XAD-7 [17]. However, Amberlite XAD-7 has a greater affinity for more polar compounds. For *N,N'*-dimethylthiourea in water, Amberlite XAD-7 had a capacity factor of 2.97, while Amberlite XAD-2 had a capacity factor of 1.72 [17]. Of possibly greater relevance for the isolation of bioactive constituents is the observation that catecholamines are eluted at the void volume with methanol on Amberlite XAD-2, but are measurably retarded by Amberlite XAD-7 [17]. Thus catecholamines can be adsorbed from aqueous solutions by Amberlite XAD-7 and eluted by water/methanol mixtures or methanol depending on the specific compound.

Application to four crude aqueous extracts of marine organisms is shown in Table 4. The samples dissolved in water were applied to columns packed with the

Table 3. Eluotropic series for Amberlite XAD-2 and XAD-7

XAD-2	XAD-7	
	In methanol	In water
Water	Ethyl acetate	Dimethyl sulfoxide
Methanol	Diethyl ether	Methanol
Dimethyl sulfoxide	Acetone	Acetone
Acetone	Dichloromethane	Tetrahydrofuran
Diethyl ether	Tetrahydrofuran	Diethyl ether
Dichloromethane	Benzene	
Ethyl acetate		
Tetrahydrofuran		
Benzene		

Table 4. Behavior of crude aqueous extracts on Amberlite XAD-2 and XAD-7

Organism	Sample in water	Resin	Resin volume	Weight adsorbed and eluted		Approx. % of organics adsorbed and eluted	Approx. % of organics irreversibly bound
				Total (g)	mg/100 ml resin		
Brown alga	5.9 g in 100 ml	XAD-2	400 ml	1.08	270	25	31
Cystophora retorta	5.9 g in 100 ml	XAD-7	400 ml	1.56	390	36	24
Brown alga	5.4 g in 100 ml	XAD-2	400 ml	0.60	150	18	1
Cystophora congesta	5.4 g in 100 ml	XAD-7	400 ml	0.88	220	26	9
Sea anemone	1.7 g in 220 ml	XAD-2	90 ml	0.09	100	7	15
Bunodactis chrysobathis	1.6 g in 210 ml	XAD-7	90 ml	0.099	110	8	20
Sponge	1.3 g in 70 ml	XAD-2	90 ml	0.072	80	10	8
	1.2 g in 70 ml	XAD-7	90 ml	0.09	100	14	15

resin; the column was washed with water to remove salts and eluted with the series water, water/methanol, methanol, acetone, which yielded all elutable material which had been adsorbed. An estimate of the amount of adsorbed organics (approximate % of organics adsorbed) was made from the salt content of the crude extracts as determined by elemental analysis for chlorine (salt content = $1.806 \times \%Cl$ [19]).

If the compound of interest can be adsorbed onto the column and is easily removed, Amberlite XAD-2 and XAD-7 can be useful adsorbents. In experiments with crude extracts, a significant amount of irreversible binding to the resin took place. The estimate of the extent of binding is based on the estimated salt content. This irreversible binding limits the use of these resins. Amberlite XAD-2 was used successfully for initial purification of a bioactive constituent from *Gracilaria edulis* giving a 100-fold purification from the crude extract [4]. Both resins are suitable for the concentration of organic components from aqueous extracts. Large volumes of aqueous extract with low concentrations of organic materials can be passed through the resin and the adsorbed organics can be eluted in a small volume of organic solvent.

The majority of reversed phase adsorbents which have found routine use in analytical applications are chemically modified silicas. The free silanol bonds of silica are functionalized via silylation. In principle any functional group could be introduced. Commercially available adsorbents are available with a wide range of functional groups.

For a long time, reversed phase adsorbents were specifically made for analytical applications (3–10 μm particle size) and the organic chemist interested in the isolation of compounds on a preparative scale only had available HPLC columns up to about 20 mm in diameter. In the last few years commercially available reversed phase adsorbents have become available with larger particle sizes so that they can be used for flash or medium pressure chromatography. Thus it is possible to carry out normal flash or medium pressure liquid chromatography followed by high pressure liquid chromatography as is routinely employed by natural products chemists working with lipophilic constituents. Octadecyl (C18) silica is the most popular reversed phase adsorbent but silicas with a variety of other chain lengths are available, for example octyl (C8) and ethyl (C2). In addition adsorbents with other groups such as phenyl and cyclohexyl are available and a variety of more polar groups such as cyanopropyl, diol, aminopropyl have also been introduced. Indeed, this diversity with its associated difference in binding properties should allow many compounds that previously were very difficult to isolate to be adsorbed from aqueous extracts and fully purified.

I would predict that the use of functionalized silicas will greatly increase the number of water-soluble marine natural products that will be isolated in the future.

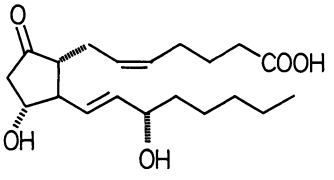
3 Examples of Separations

3.1 Gracilaria edulis [4]

The aqueous extract of the red alga *Gracilaria edulis (lichenoides)* displayed anti-hypertensive properties when given intravenously (i.v.) to anesthetized, deoxycorticosterone acetate-salt hypertensive rats. Preliminary diafiltration and gel permeation chromatography revealed that the extract contained at least two anti-hypertensive agents of apparent molecular weights greater than 5000 (not potent) and less than 500 (potent). Preliminary experiments also revealed that the active constituent was adsorbed by both Amberlite XAD-2 resin and octadecyl silica.

For large scale work-up Amberlite XAD-2 was used for initial purification, followed by gel permeation chromatography and reversed phase HPLC. One hundred-fold purification of the potent anti-hypertensive agent was obtained on Amberlite XAD-2. An aqueous solution (2 L) of the extract (260 g) was passed through Amberlite XAD-2 (53 × 5 cm) at 2 mL min^{-1}. The column was washed with water until the conductivity of the eluate was zero (3 L water) and the active fraction (1.99 g, 0.37% dry weight) was eluted with methanol (4 L). The active fraction was chromatographed on Pharmacia Sephadex G-25 (53 × 5 cm) in water and the active component was eluted at Ve/Vo 2.00–2.73 (780 mg, 0.15%). Chromatography on Waters μ-Bondapak (10 μm, 30 × 2 cm) with a stepwise gradient of water, water/methanol (9:1, 8:2, 7:3, 6:4, 4:6, 2:8) concentrated all activity in the water/methanol (6:4) fraction (220 mg, 0.04%). HPLC on Waters μ-Bondapak (10 μm, 30 cm × 7.8 mm) in water/methanol (6:4) yielded an active fraction shown to be prostaglandin E_2 (PGE_2) (**3**) (27 mg, 0.006%).

This was the first reported occurrence of prostaglandins in a plant. $PGF_{2\alpha}$ was found in the alga in addition to the antihypertensive PGE_2.



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3.2 Tedania digitata [2,3]

The crude aqueous ethanolic extract of the sponge *Tedania digitata* displayed a number of pharmacological activities [2, 3, 20–22]. At a dose of 1 g/kg in mice marked muscle relaxation with hindlimb paralysis and an associated hypothermia was observed. This effect was highly reproducible and provided a suitable bioassay for monitoring fractionation. Preliminary diafiltration revealed that the active constituent passed through an ultrafilter with a nominal molecular weight cut-off of 500 dalton. Chromatography of the diafiltrate on Pharmacia Sephadex G-10 in water showed that the active constituent was retarded. Figure 3 shows the chromatogram, monitored at 254 and 280 nm as well as by conductivity. The

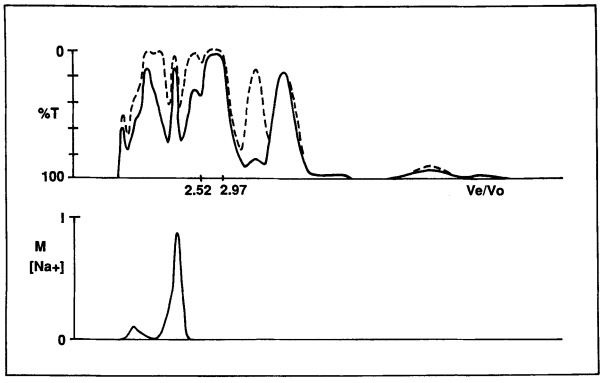


Fig. 3. Chromatography of diafiltrate (4.0 g) of the crude extract of *Tedania digitata* in water (2 ml) on Pharmacia Sephadex G-10 (52 × 5 cm, Vo = 330 ml). Eluant: water, UV monitor: --- 254 nm, — 280 nm, 100–0%T, flow rate: 66 ml h^{-1}

active constituent eluted after the salt peak and lyophilization of the combined active fractions yielded 42 mg of material. This procedure was effective but unsuitable for the processing of large amounts (100–500 g) of crude extract.

Investigation of the active fraction from Sephadex G-10 showed that the active constituent could be absorbed onto cation-exchange resins from dilute acid solutions and eluted with ammonia solutions. A method was developed to chromatograph the crude extract directly on the sulfonic acid cation-exchange resin Bio-Rad AG50W-X8. The selectivity of this chromatography was discussed previously in Sect. II B. Full details follow. Crude extract A (205 g) was suspended in ammonium formate (pH 3.5, 0.1 M in formate), sonicated and heated to effect solution. After the solution was cooled, the pH, which had risen to 4.2, was adjusted to 3.5 with formic acid. Centrifugation removed insoluble material. The solution was applied to a column of Bio Rad AG50W-X8 (ammonium form, 200–400 mesh, 40 × 5 cm) at a rate of 150 mL h^{-1}. After thorough washing of the column with the equilibration buffer (ammonium formate, pH 3.5, 0.1 M formate) the column was eluted with ammonium formate (pH 5.3, 0.1 M formate). The active material was eluted in a large UV-absorbing peak. The active fractions from two separations were combined and rechromatographed under the same conditions. After lyophilization, the active fraction was recrystallized from water, yielding 1-methylisoguanosine (**2**).

1-Methylisoguanosine accounted for all pharmacological activities of the crude extract and its pharmacological properties have been reported [20–27]. It is widely used as a tool for pharmacological investigations and is available commercially [28].

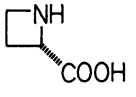
1-Methylisoguanosine was isolated from two other sources. Digestive glands of the nudibranch *Anisodoris nobilis* were homogenized and dialyzed (Spectrapor 1) at 5° C against water. The combined dialyzates were concentrated and lyophilized. The methanol-soluble portion was chromatographed on Bio Rad Bio-Gel P-2 in water. Active fractions were chromatographed on silica gel (Woelm, 30–60 μm) in tertiary butyl alcohol/ethyl acetate/water/acetic acid (40:10:2:1). The active fractions were recrystallized and yielded 1-methylisoguanosine [29].

Isolation of 1-methylisoguanosine from the coral *Madracis mirabilis* [30] was achieved from a crude aqueous extract. The extract was extracted with hot ethanol; the ethanol was evaporated; the residue triturated with chloroform; and the insoluble fraction was recrystallized from methanol. The crude recrystallized material was chromatographed on Pharmacia Sephadex G-10 with water, followed by chromatography on Avicel microcrystalline cellulose in ethanol/water (7:3); recrystallization from water/methanol yielded 1-methylisoguanosine.

3.3 Haliclona sp. and Chalinopsilla sp. [7]

The crude aqueous ethanolic extract of the sponges *Haliclona* sp. and *Chalinopsilla* sp. had specific in vitro activity against *Trichophyton mentagrophytes* having minimum inhibitory concentrations of 0.625 and 2.5 μg ml^{-1} respectively.

Initial diafiltration of an aqueous ethanolic extract of *Haliclona* sp. using membrane ultrafilters having nominal molecular weight cut-offs of 10000, 1000 and 500 dalton established that the active constituent had passed through all three membranes. The crude extract was diafiltered through a 500 dalton cut-off membrane and the diafiltrate subjected to gel permeation chromatography on columns of Pharmacia Sephadex G-15 and G-10 connected in series. The active constituent was eluted in a fraction between Ve/Vo 1.86 and 2.02. Trituration of the dried fraction with dimethylsulfoxide and recrystallization from methanol/water yielded L-azetidine-2-carboxylic acid (**4**).



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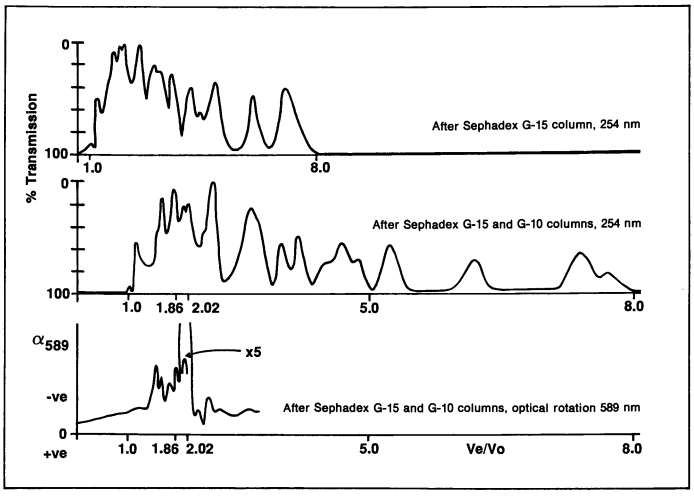


Fig. 4. Chromatography of the diafiltrate (4 g) of the crude aqueous ethanolic extract of *Haliclona* sp. on Pharmacia Sephadex G-15 (41 × 5 cm) and Sephadex G-10 (51 × 5 cm) in water connected in series (V_0 600 ml), flow rate: 2.0 ml min^{-1}

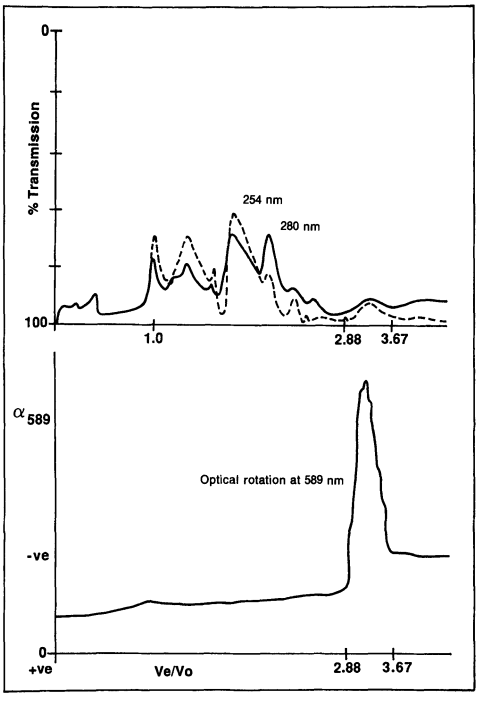


Fig. 5. Chromatography of the gel permeation fraction Ve/Vo 1.86–2.02 (see Figure 4) (37 mg) on Merck microcrystalline cellulose (48 × 1.5 cm, Vo 33 ml) in n-butanol : acetic acid : water (3 : 1 : 1) at 1300 kpa

Figure 4 compares the difference in resolution between a single and a combination of columns in gel permeation chromatography. Higher recovery of the active constitutent was obtained by cellulose partition chromatography under pressure. Chromatography on Merck microcrystalline cellulose with n-butanol/acetic acid/water (3 : 1 : 1) established that there was only one active constituent and that it was L-azetidine-2-carboxylic acid. Figure 5 shows the elution profiles for cellulose chromatography.

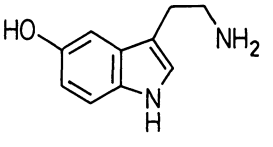
L-Azetidine-2-carboxylic acid was isolated as the only active constituent of *Chalinopsilla* following a similar procedure.

L-Azetidine-2-carboxylic acid is known to act as an L-proline analog, inhibits the growth of *Escherichia coli*, and becomes incorporated into proteins of higher animals. Low concentrations of 4 were found to be active in vitro against recent clinical isolates of the fungal dermatophytes. *T. mentagrophytes, Epidermophyton floccosum* and *Microsporum audouini*. 4 has systemic activity against sub-cutaneous infections of mice by *T. mentagrophytes*, with an ED_{50} of 82 mg kg^{-1} s.c and 400 mg kg^{-1} p.o. However chronic toxicity in mice was observed at 400 mg kg^{-1}/day s.c. after 3 days of treatment and topical application of 4 failed to cure guinea pigs with experimental skin infections of *T. mentagrophytes*. It was concluded that 4 does not have any therapeutic value as a topical or systemic agent against fungal dermatophytes [7].

3.4 Pentacter crassa [5]

A crude aqueous extract of the holothurian *Pentacter crassa* showed antihypertensive activity when administered intravenously to pentobarbitone-sodium anesthetized deoxycorticosterone-salt hypertensive rats. Crude extract (350 g) in water (3 L) was pumped through a column (52 × 45 cm) of Amberlite XAD-2 resin which was then washed with water (5 L) and methanol (9 L). Evaporation of the methanolic fraction yielded 16 g of active material which was partitioned between ethyl acetate and water and resulted in an active aqueous fraction (9 g). This active fraction was chromatographed on octadecyl silica (7 × 10 cm) with a 0→100% methanol in water step gradient, which concentrated activity in the water/methanol (1 : 1) fraction. Repeated chromatography on Pharmacia Sephadex LH-20 in methanol yielded the active constituent, 5-hydroxytryptamine (**5**), (0.003% dry weight) at V_e/V_o 2.20–2.53.

5-Hydroxytryptamine is a neurotransmitter and has been shown to be a constituent of some species of molluscs, sea anemones, annelids, tunicates and gorgonians. 5-Hydroxytryptamine causes a triphasic response on blood pressure after intravenous administration [5].



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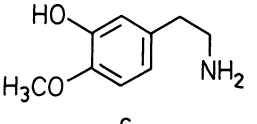
3.5 Nephthea sp. [6]

A crude aqueous extract of the soft coral *Nephthea* sp. increased heart rate and blood pressure when administered intravenously to pentobarbitone-sodium anesthetized deoxycorticosterone-salt hypertensive rats. An aqueous solution of the crude extract (200 g, 2.4 L) was diafiltered through Millipore Pellicon cell containing cut-off filters of 1000 molecular weight. The diafiltrate (130 g) in water (1.5 L) was passed through a column of Amberlite XAD-2 (47 × 5 cm). Elution of the resin with methanol (2.5 L) yielded an active fraction (2.2 g). The active fraction was retained on Pharmacia Sephadex G-25 (fine) in water but active material (93 mg) was eluted with acetic acid (1.7 M).

Chromatography on Pharmacia Sephadex G-15 in acetic acid (1.7 M) yielded an active fraction between Ve/Vo = 1.89–2.66. HPLC on Merck LiChrosorb RP-8 (4.6 × 250 mm) resulted in an active fraction which was eluted with methanol/water (1 : 1). Preparative thin layer (0.25 mm) chromatography on silica gel with n-butanol/water/acetic acid (3 : 1 : 1) yielded 3-hydroxy-4-methoxyphenethylamine (**6**) (2.3 mg, 0.00027% of dry weight) as the active constituent.



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In some cases adsorption occurs when gel permeation resins are used. Such adsorption can be useful in purification schemes provided the adsorbed compounds can subsequently be eluted from the resin. Change of solvent, including pH changes, are the usual approaches for removing adsorbed constituents. In this particular case the bioactive constituent was irreversibly adsorbed on Pharmacia Sephadex G-15 in water, but was desorbed with dilute acetic acid. As a consequence, dilute acetic acid was used as the eluant. Under these conditions the bioactive constituent showed normal chromatographic behavior. A variety of aromatic amines, specifically histamine, tyramine, dopamine and/or octopamine were detected in the extract using high resolution mass spectrometric selective ion monitoring, but none of these amines was responsible for the observed cardiovascular properties of the crude extract. This was the first report of 3-hydroxy-4-methoxyphenethylamine from a marine source, while it has been reported in cacti and human urine. The cardiovascular properties of the compound had not previously been reported [7].

3.6 Hapalochlaena maculosa [8]

An acidic (water/acetic acid, 97:3) extract of the posterior salivary glands of the blue-ringed octopus, *Hapalochlaena maculosa*, was toxic to mice. Crude extract was diafiltered with 3% acetic acid through an Amicon Diaflo UM2 ultrafilter (1000 dalton cut-off). The diafiltrate was chromatographed four times by ion-exchange chromatography on the weakly acidic carboxymethyl resin Pharmacia CM-Sephadex C-25 (ammonium form) using ammonium acetate buffer at pH 6.0 with an ionic strength gradient from 0.1 to 0.4 M. Figure 6 shows the CM-Sephadex C-25 chromatography of the diafiltrate and it can be seen that the toxic fractions overlapped a UV-adsorbing peak. The initial ion-exchange chromatography yielded 179 mg of toxic material from 6.3 g of diafiltrate corresponding to a 23-fold increase in toxicity. It required three additional ion-exchange chromatographies to remove the contaminating material to yield 1.8 mg of tetrodotoxin (**7**)

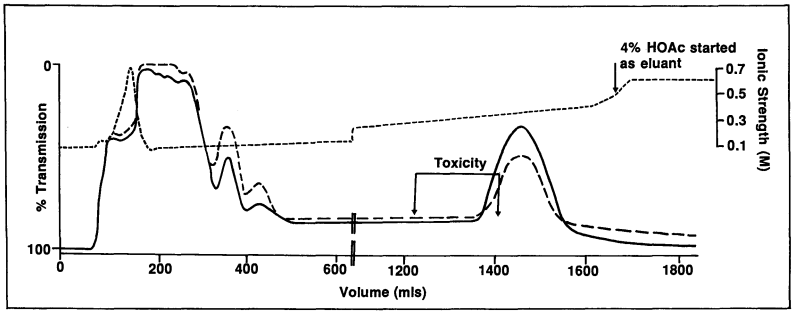
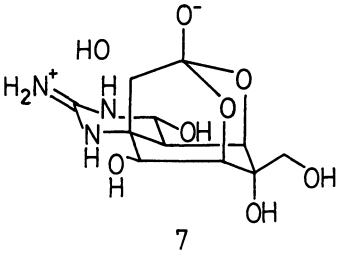


Fig. 6. Chromatography of the diafiltrate (6.3 g) of the crude acetic acid extract of the posterior salivary glands of *Hapalochloena maculosa* on Pharmacia CM-Sephadex C-25 (ammonium form) in pH 6.0 ammonium acetate. Eluant: ammonium acetate pH 6.0, 0.1 to 0.4 M followed by 4% acetic acid. UV monitor: — 254 nm, --- 280 nm, 100–0%T. Gradient monitored by a flow through conductivity cell

with a further 100-fold increase in toxicity. The blue-ringed octopus was the first species in which tetrodotoxin had been found in extracts of the venom gland. In other species it occurs as a poison in the skin, muscle, liver, ovaries or eggs. The role of tetrodotoxin in *H. maculosa* is perhaps more obvious than in other species, since *H. maculosa* uses its venom to immobilize or kill its prey of small crayfish and crabs. A number of human fatalities have been attributed to the bite of this octopus [8].



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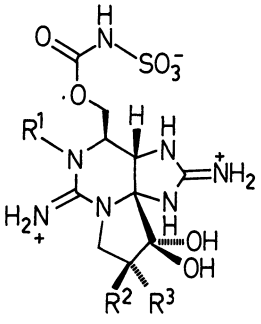
4 Constituents Isolated from Aqueous Marine Extracts

Isolation of water-soluble marine natural products has recently been reviewed by Shimizu [31]. One aim of his article was to assist those traditional natural products chemists who wish to venture into working with water-soluble compounds. Shimizu stated that water-soluble fractions could not be ignored if the objective was the isolation of biologically active constituents. Since 1984 periodic reviews of the literature of marine natural products have been available in Faulkner's reports [32–34]. The reports covering the literature between October 1983 (for algal metabolites) or July 1984 (for invertebrate metabolites) and July 1985 list 514 marine natural products [34]. Shimizu had observed that in a 1977 review [35] listing more than 300 marine natural products only six (2%) were water-soluble compounds. This ratio has improved slightly in the intervening years. Of the 514 marine natural products listed by Faulkner [34] 62 (12%) were obtained from aqueous extracts. These are discussed below. For easy reference the same organization will be used [34].

4.1 Marine Micro-Organisms and Phytoplankton

The isolation of gonyautoxins C3(**8**) and C4(**9**) from the dinoflagellate *Protogonyaulax catenella* was achieved from a mixture of toxins (C1, C2, C3, C4) [36]. Preparative chromatography under neutral or weakly acidic conditions on a number of media failed to provide resolution. Chromatography on Bio Gel P2 in 0.1 M pyridine resulted in the elution of toxins C3 and C4 ahead of, and well separated from, toxins C1 and C2. Toxins C3 and C4 were resolved from each other by chromatography on Biol Gel P2 with either water or 0.1 M acetic acid.



	R^1	R^2	R^3
8	OH	H	OSO_3^-
9	OH	OSO_3^-	H
10	H	H	H
11	OH	H	H
12	H	OSO_3^-	H

Gonyautoxins (GTX)-V(**10**), VI(**11**) and VIII(**12**) were obtained from cell extracts of *Gonyaulax tamarensis* [37]. The cell extracts were adjusted to pH 5.7 and charged onto a Bio-Gel P2 column. The column was eluted with water followed by 0.03 N acetic acid, which yielded GTX-VIII as an early eluting peak. In a similar experiment, toxin extract at pH 5.8 was charged onto a Bio-Gel P2 column and after washing with water, a mixture of toxins was eluted with 0.03 N acetic acid. The lyophilized toxin fraction was dissolved in water, adjusted to pH 5.8 and charged onto a Bio-Rex 70 column (acidic form). Elution with an acetic acid gradient (0 to 0.01 N acetic acid) yielded GTX-V together with GTX-IV and GTX-I as an early eluting toxic fraction. GTX-V was isolated by two separations on Bio-Rex 70 with a gradient of 0 to 0.3 N acetic acid followed by a single chromatography on Bio-Rex 70 with a gradient of 0 to 0.015 N acetic acid. GTX-VI had been obtained from earlier work.

Bio-Rad Bio-Gel P-2 is a porous polyacrylamide gel permeation medium. Reversible adsorption of the toxins to the resin aided purification. Bio-Rad Bio-Rex 70 is a weakly acidic cation exchanger containing carboxylic acid groups on a macroreticular acrylic polymer lattice.

4.2 Blue-green Algae (Cyanobacteria)

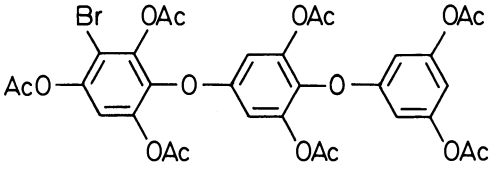
No water soluble constituents were reported.

4.3 Green Algae

No water soluble constituents were reported.

4.4 Brown Algae

Phlorotannins have been isolated as their peracetates from brown algae. While most of the work in this area has involved investigation of the ethyl acetate-soluble phenols followed by acetylation [38,39,40], a series of brominated phlorethols and phlorotannins were isolated from the aqueous ethanolic extract



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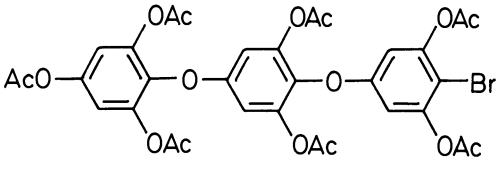

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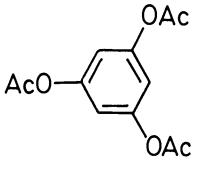

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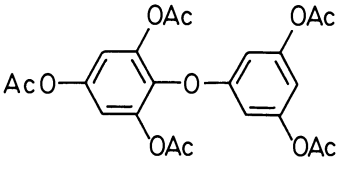

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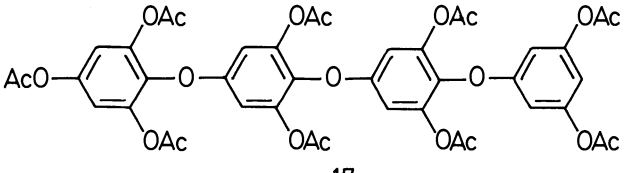

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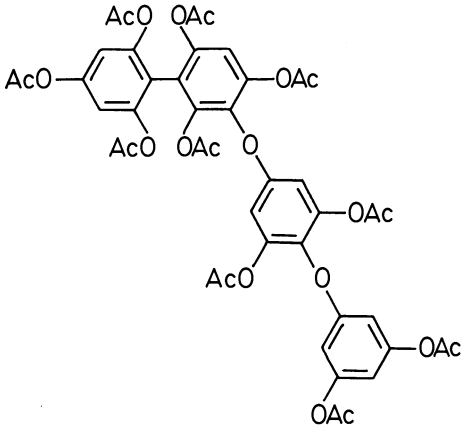
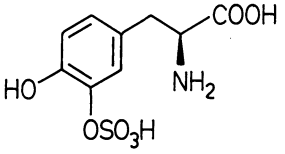


18

of *Cystophora congesta* [41]. An aqueous solution of the extract was passed through an Amberlite XAD-2 column and the phenolics eluted with methanol. After acetylation the first brominated members of the series, bromotriphlorethol-A_1-heptaacetate (**13**) and bromotriphorethol-A_2-heptaacetate (**14**), were isolated. In addition, phoroglucinol triacetate (**15**), diphlorethol pentaacetate (**16**), tetraphlorethol-C-nonaacetate (**17**) and fucodiphlorethol-D-decaacetate (**18**) were isolated [41]. Three phlorotannins containing a dibenzo-p-dioxin skeleton, eckol, 2-phloroeckol and dieckol were isolated from a methanol extract of *Ecklonia kurome*, but no isolation details were published [42].

The 3-sulfate of L-DOPA was isolated from *Ascophyllum nodosum* [43]. *A. nodosum* was soaked for two to three days in 1 mM hydrochloric acid. Charcoal (200 g) was added to the extract, which was collected by filtration. The charcoal was washed with water (1 L) and material desorbed by suspending the charcoal in 50% aqueous ethanol adjusted to pH 5.5 with sodium hydroxide. The aqueous ethanolic filtrate was concentrated and passed through a column of Dowex 50 × 8 (hydrogen form). Elution with water yielded a retarded fraction, which was purified by paper electrophoresis at pH 3.5 (pyridine-acetate buffer, Whatman 3MM paper) and at pH 1.8 (8% acetic acid, 2% formic acid) to yield L-3,4-dihyroxyphenylalanine-3-sulfate (**19**) [43].



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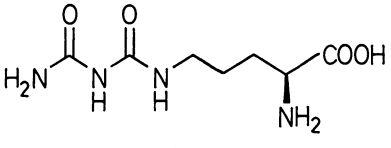
4.5 *Red Algae*

Lividine (**20**) was obtained from an aqueous extract of *Grateloupia livida*. The crude aqueous extract was subjected to ion exchange chromatography on Amberlite IRCG-120 (ammonium form) and **20** eluted in a number of fractions with 1 M ammonium hydroxide. Chromatography of the combined fractions on the weak acid cation resin (COO^-) Amberlite IRCG-50 (ammonium form) with a gradient from water to 1 M ammonium hydroxide, repetition on Amberlite IRCG-50 (ammonium form) and elution with 1 N ammonium hydroxide, preparative thin layer chromatography on Merck silica gel with n-butanol/acetic acid/water (4:1:2), paper electrophoresis at pH 3.5, precipitation from water yielded pure **20** (7 mg from 260 g dried algae) [44].



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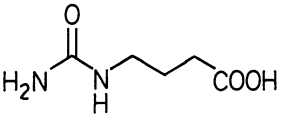
Grateloupine (**21**) was obtained from a 50% aqueous methanolic extract of *G.filicina*. The crude extract was subjected to ion exchange chromatography on Amberlite IRCG-120 strong acidic cation exchanger (SO_3^-) (ammonium form) and **21** eluted slowly with water. The combined fractions were chromatographed on the weakly basic [$-N^+(R)_2$] Amberlite IRCG-4B (acetate form) and eluted with 2 M acetic acid, followed by chromatography on Amberlite IRCG-120 (pyridine form) and elution with 0.1 M pyridine formate. Crude **21** precipitated from the fractions and was recrystallized from water, which yielded pure grateloupine (**21**) [44].



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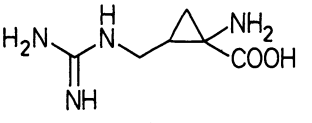
Carnosadine (**22**) was obtained from an aqueous extract of *Grateloupia carnosa* by ion-exchange chromatography on Amberlite IRCG-50 (no details were published) [45].

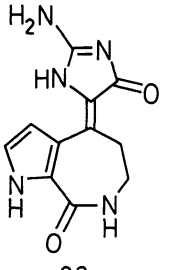


22

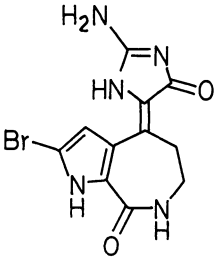
4.6 Sponges

The sponge *Hymeniacidon aldis* (1.5 kg wet weight) was extracted with 10% aqueous methanol. The extract (130 g) was treated with 90% aqueous methanol, which yielded a soluble portion (35 g). Repeated gel permeation chromatography on Toyopearl HW-40 Fine and Toyopearl HW-40 Superfine, elution with 90% aqueous methanol yielded **23** and hymenialdisine (**24**) and a fraction containing **25** which was further purified on a Toyopearl HW-40 Superfine column and eluted with methanol/chloroform/water (10:2:1) yielding pure **25** [46].

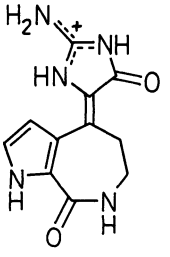
Compound **24** had previously been isolated [47] from *Axinella verrucosa* and *Acanthella aurantiaca*. The sponges were extracted with acetone and after evap-



23



24



25

oration of the solvent, the aqueous residue was extracted with diethyl ether and n-butanol. The butanol extracts were suspended in methanol, the insoluble material collected by filtration and purified by repeated precipitations from hot methanol and finally hot water, which yielded **24**. Compound **25** had previously been isolated from the sponge *Phakellia flabellata* [48] by chromatography of the aqueous extract of the sponge on a Sephadex G-10 column.

4.7 Coelenterates

4.7.1 Soft Corals

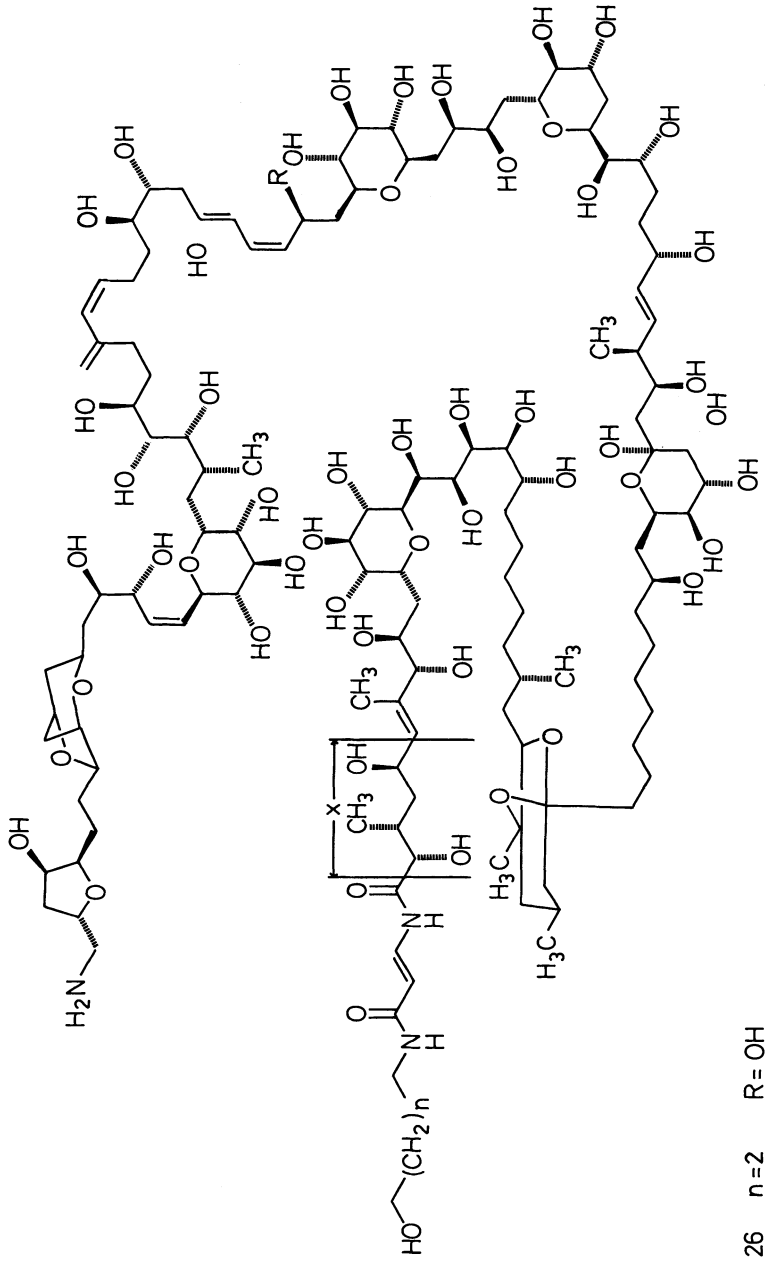
No water soluble constituents were reported.

4.7.2 Gorgonians

No water soluble constituents were reported.

4.7.3 Other Coelenterates

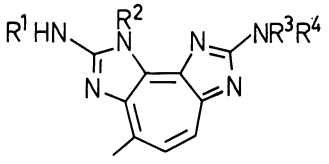
Four minor toxins, homopalytoxin (**26**), bishomopalytoxin (**27**), neopalytoxin (**28**) and deoxypalytoxin (**29**) have been isolated from *Palythoa tuberculosa* and from another *Palythoa* species [49]. *Palythoa tuberculosa* (45 kg) was blended in 75% aqueous ethanol (45 L). The extract was stored in a freezer for 2 days, filtered through Hyflo Super-Cel and the filtrate was concentrated to about one twentieth of its volume under reduced pressure below 40° C. The aqueous solution was charged batchwise (five runs) onto a Toyo Soda TSK G 3000S polystyrene gel [10 × 17 (o.d.)cm]. The column was washed with water to remove inorganic salts and non-bound material; crude toxins eluted with 75% aqueous ethanol (10 L). The combined 75% aqueous eluants were evaporated under reduced pressure to remove ethanol. The aqueous solution was chromatographed on a Pharmacia DEAE-Sephadex A-25 column in phosphate buffer at pH 6.9. Toxins, which are weakly basic, were not adsorbed to the basic anion exchanger and eluted with the solvent front. The toxic fraction was desalted by adsorption onto a TSK G 3000S column, washed with water, 40% aqueous ethanol, and eluted with 75% ethanol. The residue (5.8 g) in pH 4.6 phosphate buffer (10 mL) was chromatographed on a CM Sephadex C-25 column (80 × 2.4 cm) with pH 4.6 phosphate buffer to give two toxic fractions which were desalted as before using TSK G 3000S. One fraction consisted mainly of palytoxin, while the other contained the minor related palytoxins. The mixture in 75% ethanol was chromatographed by HPLC on a Mitsubishi MCI CQP-30 column in pH 6.9 phosphate buffer. Rechromatography on the same system yielded palytoxin contaminated with a small amount of neopalytoxin (4 mg, after desalting), deoxypalytoxin (**29**) (4 mg, after desalting) and homopalytoxin and bishomopalytoxin (4 mg, after desalting, 1 : 1 mixture). Separation of neopalytoxin (**28**) from palytoxin (**30**) was



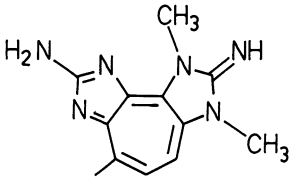
26 n=2 R= OH
27 n=3 R= OH
28 n=1 R= OH
29 n=1 R= H
30 n=1 R= OH

carried out on preparative HPTLC plates of Merck NH_2F_{254} using n-amyl alcohol/pyridine/water (7:7:6) as developing solvent and methanol to elute palytoxin (**30**) (2 mg) and neopalytoxin (**28**) (0.7 mg). Homopalytoxin (**26**) and bishomopalytoxin (**27**) could not be separated chromatographically and the structural elucidation was carried out on the 1:1 mixture.

Seven bases, paragracine I–VII (**31**)–(**37**), have been isolated from the anthozoan *Parazoanthus gracilis* [50, 51]. The wet animals were crushed in a mortar and extracted with methanol at room temperature. The extract was concentrated under reduced pressure and the crude extract partitioned between ethyl acetate and water. The aqueous layer was passed through an Amberlite XAD-2 column and after washing with water the column was eluted with methanol. Chromatography of the methanolic fraction on neutral alumina yielded in the chloroform/methanol (9:1) fraction paragracine I (**31**), which was recrystallised from methanol. The isolation of the minor bases followed a similar procedure except that the methanolic fraction from the Amberlite XAD-2 column was more extensively chromatographed. Paragracine III (**33**) [52] and paragracine IV (**34**) [53] had previously been described.



	R^1	R^2	R^3	R^4
31	CH_3	H	CH_3	CH_3
32	CH_3	CH_3	CH_3	CH_3
33	H	CH_3	CH_3	CH_3
35	H	CH_3	H	H
36	CH_3	H	CH_3	H
37	H	H	CH_3	CH_3



34

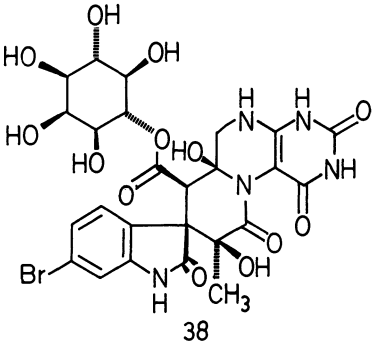
4.8 Bryozoans

No water soluble constituents were reported.

4.9 Marine Molluscs

The total synthesis of surugatoxin (**38**) has been published [54]. This toxin was originally isolated from the Japanese ivory shell, *Babylonia japonica*, which had produced intoxication after ingestion [55]. Mid-gut glands of the gastropod (1 kg) were ground and extracted twice with 5 L of 1% acetic acid. The extract was centrifuged, acetone (ten volumes) added and recentrifuged. The supernatant was

concentrated to 100 mL and defatted with ether. Gel permeation chromatography on Pharmacia Sephadex G-25 in dilute acetic acid (pH 3.9) followed by ion-exchange chromatography on a Pharmacia CM Sephadex C-25 column in the same solvent mixture gave toxic fraction; the isolation was monitored by the onset of mydriasis in mice. The toxic fraction was passed through a Pharmacia Sephadex G-15 column to yield surugatoxin (**38**).



38

4.10 Tunicates

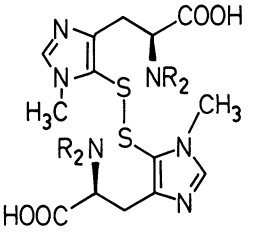
No water soluble constituents were reported.

4.11 Echinoderms

Tetrodotoxin (**7**) has been isolated from the starfish *Astropecten latespinosus* [56]. *A. latespinosus* (1.5 kg) was homogenized with 3 vol. of 1% acetic acid in methanol. The extracts were combined, evaporated in vacuo and defatted with dichloromethane. The aqueous layer was concentrated to 500 mL, adjusted to pH 5.2 with 1 N NaOH and treated batchwise with charcoal. After washing the charcoal with water, the toxic material was eluted with water/ethanol/acetic acid 79:20:1 and evaporated to dryness. The residue was subjected to ion-exchange chromatography on Pharmacia CM-Sephadex C-25 (ammonium form) in 0.1 M ammonium acetate (pH 6) and developed with a linear gradient 0.1 to 0.4 M ammonium acetate (pH 6). Toxic fractions were lyophilized and subjected to ion-exchange chromatography on Bio Rad Bio-Rex 70 (hydrogen form) with a linear gradient of 0 to 0.1 M acetic acid yielding tetrodotoxin (**7**) [56].

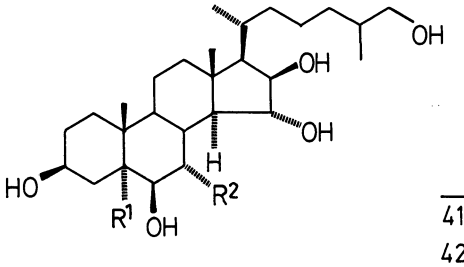
The eggs of several echinoderms were found to contain unusual thiol amino acids which were isolated as the disulphides **39** and **40** [57]. Fresh eggs were homogenized in ethanol/1 M hydrochloric acid (80:20) and left overnight at room temperature. After centrifugation the supernatant was concentrated to a small volume and extracted with ether to remove lipids and pigments. The aqueous phase was evaporated to dryness, taken up in 0.1 M hydrochloric acid and chromatographed on the sulfonic acid cation exchange resin Dowex 50 × 2 (hydrogen form). The column was successively eluted with water, 0.1 M, 0.5 M and 4 M hy-

drochloric acid. The 4 M hydrochloric acid eluant was concentrated to a small volume and oxidized in air at pH 8 to convert 5-histidine thiol(s) into the more stable disulfides. This mixture was acidified to pH 2 and chromatographed on Dowex 50W × 2 using 2 M hydrochloric as eluant. Final purification was achieved by gel permeation chromatography of the fractions on Pharmacia Sephadex LH-20 using 80% aqueous ethanol as eluant. Thus 60 g of fresh eggs of *Paracentrotus lividus* yielded 24 mg of 1-methyl-5-thio-L-histidine disulfide (**39**). From 60 g of fresh eggs of *Sphaerechinus granularis* was obtained 13 mg of N^{α}, N^{α}-1-trimethyl-5-thiol-L-histidine disulfide (**40**). Compound **39** was also found in eggs of the sea urchin *Arbacia liscula*, the holothuroid *Holothuria tubulosa* and the asteroids *Marthasterias glacialis* and *Astropecten aurantiacus* by HPLC of the 2 M HCl fraction from Dowex 50 [57].



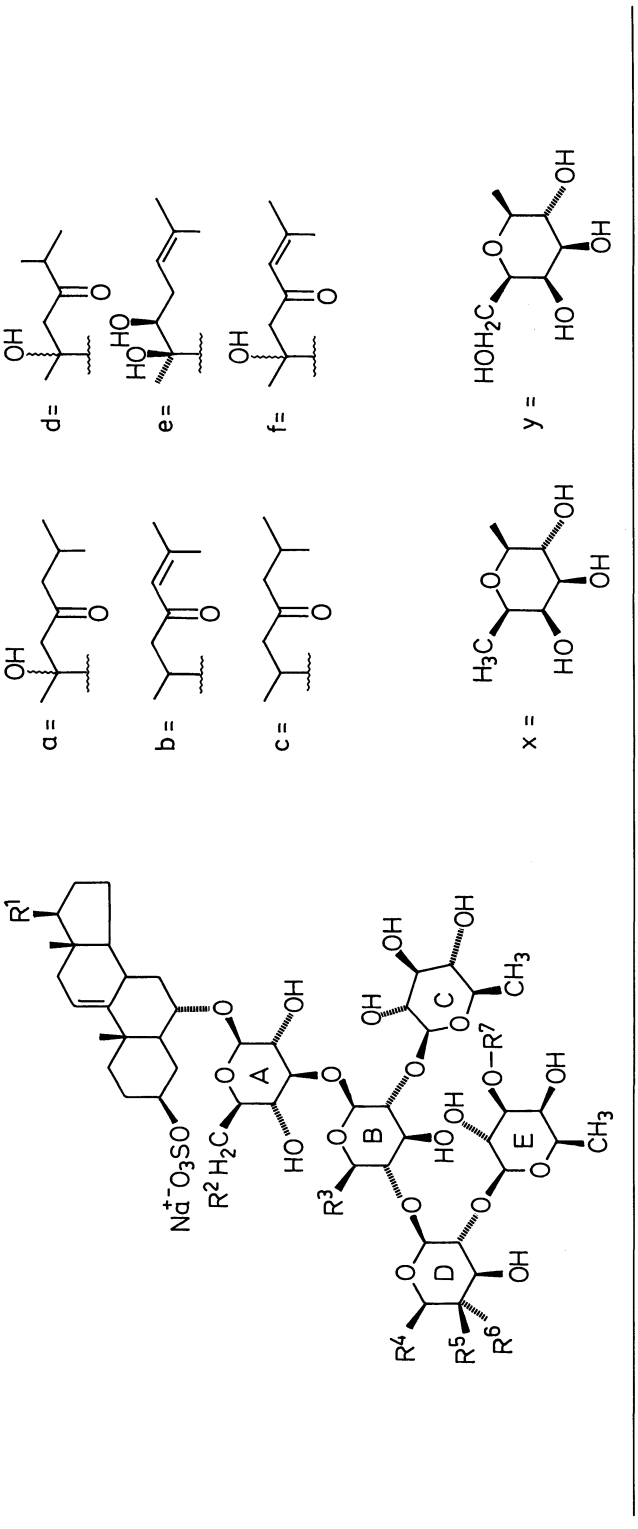
39 R = H
40 R = CH_3

The starfish *Luidia maculata* was found to contain three polyhydroxylated sterols, 5α-cholestane-3β,5α,6β,15α,16β,26-hexaol (**41**), 5α-cholestane-3β,6β,7α,15α,16β,26-hexaol (**42**) and 5α-cholestane-3β,5α,6β,7α,15α,16β,26-heptaol (**43**) [58]. *L. maculata* (5 kg wet weight) was chopped and extracted with water to give after lyophilization 465 g of extract. The extract (220 g) in water (1 L) was passed through a column of Amberlite XAD-2 (1 kg). The column was washed with water (2 bed volumes) and eluted with methanol, which yielded 4.03 g of material. The methanol fraction was subjected to gel permeation chromatography on Sephadex LH-60 in methanol/water (2:1). The steroid fraction was rechromatographed on Sephadex LH-20 in methanol, followed by preparative HPLC on a C-18 μ-Bondapak column with methanol/water (65:35) to yield three polyhydroxylated sterols **41**, **42** and **43** [58].



	R^1	R^2
41	OH	H
42	H	OH
43	OH	OH

	R^1	R^2	R^3	R^4	R^5	R^6	R^7	A	B	C	D	E	F
44	α	H	H	CH_2OH	OH	H	x	quinovose	xylose	quinovose	galactose	fucose	fucose
45	α	H	H	CH_3	H	OH	x	quinovose	xylose	quinovose	quinovose	fucose	fucose
46	b	OH	CH_3	CH_3	OH	H	H	glucose	quinovose	quinovose	fucose	fucose	—
47	c	OH	CH_3	CH_3	OH	H	H	glucose	quinovose	quinovose	fucose	fucose	—
48	α	H	H	CH_2OH	OH	H	H	quinovose	xylose	quinovose	galactose	fucose	—
49	α	H	H	CH_3	H	OH	H	quinovose	xylose	quinovose	quinovose	fucose	—
50	d	H	H	CH_2OH	OH	H	H	quinovose	xylose	quinovose	galactose	fucose	—
51	d	H	H	H	H	OH	H	quinovose	xylose	quinovose	xylose	fucose	—
52	α	H	H	H	H	OH	H	quinovose	xylose	quinovose	xylose	fucose	—
53	α	H	CH_3	H	OH	H	H	quinovose	quinovose	quinovose	arabinose	fucose	—
54	e	H	H	CH_3	H	OH	H	quinovose	xylose	quinovose	quinovose	fucose	—
57	f	H	H	CH_3	H	OH	H	quinovose	xylose	quinovose	quinovose	fucose	—
58	α	H	H	CH_2OH	H	OH	H	quinovose	xylose	quinovose	glucose	fucose	—
59	α	H	H	CH_2OH	OH	H	y	quinovose	xylose	quinovose	galactose	fucose	galactose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e =
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y =
A
B
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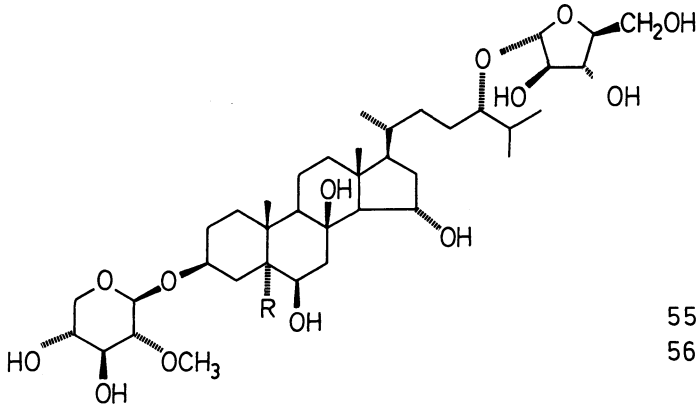
D
E

Saponins from sea stars have been isolated following a general procedure involving extraction with water, chromatography of the aqueous extract on Amberlite XAD-2 and elution of the saponins with methanol. The methanolic fraction was chromatographed on Pharmacia Sephadex LH-60 using methanol/water (2:1) as eluant. The saponin-containing fractions were subjected to droplet counter current chromatography usually with n-butanol/acetone/water (3:1:5) in an ascending mode with the lower phase acting as the stationary phase. Fractions were finally purified by HPLC on a C_{18} μ-Bondapak column with methanol/water.

Marthasterias glacialis yielded marthasterosides A_1 (**44**), A_2 (**45**), B (**46**) and C (**47**) [59]. *Luidia maculata* contains thornasteroside A (**48**), marthasteroside A_2 (**45**), marthasteroside B (**46**), marthasteroside C (**47**) and maculatoside (**49**) [60]. *Ophidiaster ophidianus* and *Hacelia attenuata* contain ophidianosides B (**50**) and C (**51**), while *O. ophidianus* also contains thornasteroside A (**48**) and ophidianoside F (**52**) [61]. *Linckia laevigata* contains thornasteroside A (**48**), marthasteroside A (**44**), ophidianoside F (**52**), maculatoside (**49**) and laevigatoside (**53**) [62]. *Protoreaster nodosus* and *Pentaceraster alveolatus* contain protoreasteroside (**54**) and thornasteroside A (**48**) [63]. *Acanthaster planci* contains isonodososide (**55**) and 5-deoxyisonodososide (**56**) [64].

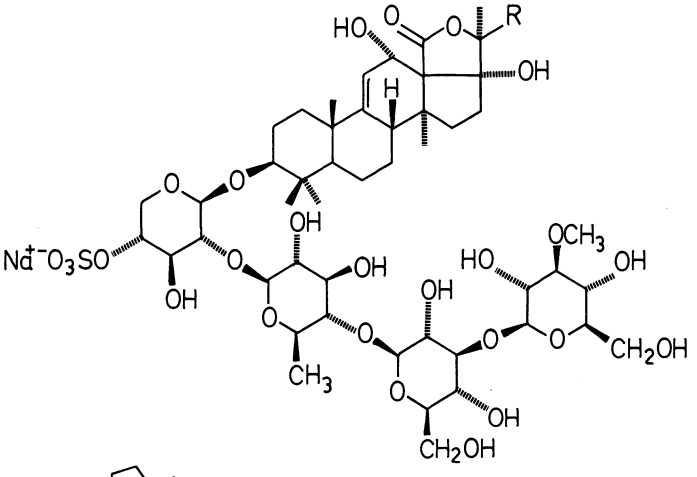
Another series of sea star saponins have been investigated similarly. Extraction with water followed by either filtration through Celite or centrifugation and chromatography of the aqueous extract on Amberlite XAD-2 yielded saponins in the methanol eluant (in one case an additional wash with acetone was used). The crude saponin mixtures from different species were investigated in different manners. Acetone-insoluble material was chromatographed on silica gel with chloroform/methanol/water (6:4:0.5), with n-butanol/n-propanol/water (4:2:1), with chloroform/methanol/water (7:3:0.5), on Pharmacia Sephadex G-15 in water and on Merck Lobar RP-8 with methanol/water (1:1) [65]. In another case, the methanolic eluate from Amberlite XAD-2 was chromatographed sequentially on silica gel with n-butanol/n-propanol/water (3:3:2) and with chloroform/methanol/water (7:3:0.5) on Pharmacia Sephadex G-15 in water, then by droplet counter-current chromatography, ascending method, with chloroform/methanol/water (3:4:2), on silica gel with chloroform/methanol/water/n-

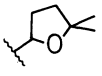


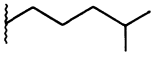
55 R = OH
56 R = H

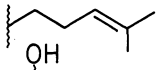
butanol (32:15:4:8), Merck Lobar RP-8 with methanol/water (1:1) to yield a mixture which was solvolyzed to remove sulfate and further chromatographed [66]. In another study, the methanolic eluate from Amberlite XAD-2 was separated sequentially by droplet counter current chromatography with water/butanol, on silica gel with chloroform/methanol/water (7:3:0.5), on Pharmacia Sephadex LH-20 with methanol/water (7:3), on silica gel with n-butanol/n-propanol/water (4:2:1), on Merck Lobar RP-8 with methanol/water (1:1) [67]. Also, the acetone-insoluble part of the methanol eluate from Amberlite XAD-2 was chromatographed on silanized Merck silica gel with 20% methanol and 50% methanol. The saponins were in the 50% methanol fraction and were further chromatographed on silica gel with chloroform/methanol/water (7:3:0.5), on Merck Lobar RP-8 with methanol/water (1:1) [68].

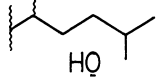
Acanthaster planci contains acanthaglycoside-A (**57**) [65]. *Astropecten latespinosus* contains (**58**) und (**44**) [66]. *Luidia maculata* contains thornasteroside A (**48**), marthasteroside A_2 (**45**), marthasteroside B (**46**), marthasteroside C (**47**) and maculatoside (**49**) [67]. *Asterias amurensis* cf. *versicolor* contains thornasteroside A (**48**) and vericoside A (**59**) [68].

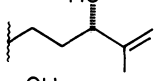


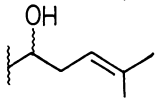
60 R = 

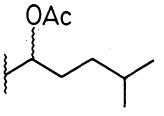
62 R = 

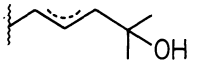
63 R = 

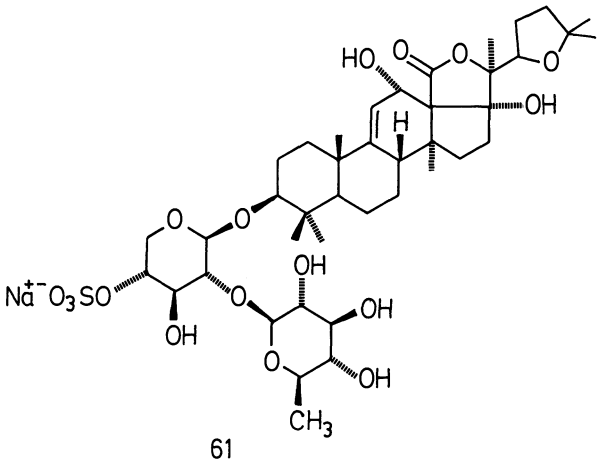
64 R = 

65 R = 

66 R = 

67 R = 

68 R = 



61

The holothurian *Actinopyga flammea* was reported to contain nine triterpenoid saponins. The holothurians were extracted with aqueous ethanol (1:1). The aqueous extract was concentrated, defatted with petroleum ether and the saponins partitioned into butanol from water adjusted to pH 4–5. The concentrated butanol phase was diluted with ether (3 vols) and extracted with water. The aqueous phase was lyophilized, recrystallized from methanol, dialyzed to remove most of the salts, and purified by reversed phase chromatography on Merck LiChroprep RP8 with variable methanol/water ratios (40:60, 45:55, 55:45). Five known saponins holothurin A (**60**), holothurin B (**61**), echinoside A (**62**), 24-dehydroechinoside A (**63**), 22-hydroxy ε-echinoside A (**64**) were isolated together with four new saponins 24*(S)*-hydroxy-25-dehydroechinoside A (**65**), 22ε-hydroxy-24-dehydroechinoside A (**66**), 22ε-acetoxyechinoside A (**67**) and 25-hydroxy-22 or 23-dehydroechinoside A (**68**) [69].

5 The Role of Reversed Phase Chromatography

I have stated in this chapter that chemically modified silicas will allow the isolation of water-soluble constituents of marine organisms at a hitherto unobtainable rate and it is thus appropriate to highlight the already extensive use of reversed phase chromatography in the isolation of marine natural products. Since these separations were achieved in aqueous solvent mixtures, it indicates that slightly more polar related compounds may well have been overlooked in previous studies. This information may help convince natural products chemists that techniques for isolating water-soluble compounds are already in extensive usage and that it is therefore feasible to work with water-soluble extracts. Table 5 lists constituents which, although not isolated from aqueous extracts, were purified by the use of reversed phase chromatography in aqueous solvent systems.

Table 5. The use of reversed phase chromatography to isolate marine natural products

			Refs.
Marine Micro-organisms and Phytoplankton			
Dinophysistoxin 3	Merck LiChroprep RP-8	Methanol/water 20:1	70
	Waters μ-Bondapak C18	Methanol/water 97:3	
Pecetenotoxin 1, -2	Merck LiChrosorb RP-8	Methanol/water 80:20	70
Blue-green Algae			
Debromoaplysiatoxin	Waters Phenylporasil B	Methanol/water 60:40→100:1	71
Anhydrodebromoaplysiatoxin	Whatman Partisil ODS-3	Methanol/water 80:20	71
Aplysiatoxin	Whatman Partisil ODS	Acetonitrile/water 70:30	71
	Waters Bondapak – CN	Ethylacetate/water 8:92	
Oscillatoxin B1, B2	Waters Phenylporasil B	Methanol/water 60:40→100:1	72
30-methyloscillatoxin D	Whatman Partisil ODS-3	Methanol/water 80:20	72
31-noroscillatoxin B	C-18	Acetonitrile/water 50:50	72
Oscillatoxin D			72
Majusculamide D	Waters Phenylporasil B	Methanol/water 60:40→100:1	73
	Whatman ODS-2, ODS-3	Acetonitrile/water 70:30	
Green Algae			
4,9-diacetoxyudoteal	C_{18} silica	Methanol/water 80:20	74
Brown Algae			
Pachydictyol A	Whatman ODS-3	Acetonitrile/water 80:20	75
Dictyoxide		Acetonitrile/water 80:20	
Dictyoxide A		Ethanol/water 80:20	
Red Algae			
Teurilene	JASCO, Finepak-Sil-C18	Methanol/water 85:15	76
Thyrsiferyl 23-acetate			
Thrysiferol			
Sponges			
Polyacetylenes	Waters μ-Bondapak C-18	Methanol/water 80:20	77
18-bromooctadeca-9(E), 17(E)-7,15-diynoic acid	Whatman Partisil-10, ODS-3	Methanol/water 80:20	78
Tedanolide	C-18	Methanol/water 65:35	79
3-bromoverongiaquinol	Merck LiChrosorb RP-18	Acetonitrile/water 10:90	80
3-bromo-5-chloroverongiaquinol		Methanol/water 18:82	

Table 5 (continued)

5-chlorocavernicolin		Tetrahydrofuran/water 2:98	
7β-bromo-5-chlorocavernicolin			
7α-bromo-5-chlorocavernicolin		Methanol/water 20:80→50:50	
5-bromo-7β-chlorocavernicolin			
5-bromo-7α-chlorocavernicolin			
5-bromocavernicolin	Merck LiChrosorb RP-18	Acetonitrile/water 12.5:87.5	81
Kalihinol B, C, E, F	RP-18	Methanol/water 75:25	82
Agelasine A, B, C, D	C18 and C8	No details	83
Agelasine E	C18	No details	84
Ageline A			
Discodermin A, D	Whatman chromedia LPR-1	Methanol/water 64:36	85
Discodermin B, C	LPR-1	Methanol/water 64:36	
	YMC-Shimakyu YMC A-324	Methanol/water 66:34	
Discodermin A	Toyo Sodo LS410 ODS	Methanol/water 65:35	86
Coelenterates			
Soft Corals			
Waixenicin A	Reverse phase	Methanol/water 80:20	87
Waixenicin B	Reverse phase	Methanol/water 75:25	87
		Methanol/water 70:30	
Xeniolone	Cosmosil 5C18	Methanol/water	88
Isoxeniolone			
20-acetoxy-claviredenone-b (20-acetoxy-clavulone II)	Cosmosil 5C18	Methanol/water 86:14	89
20-acetoxy-claviredenone-c (20-acetoxy-clavulone III)			
1α, 3β, 11α-trihydroxysterols	Whatman Partisil ODS-2	Acetonitrile/water 90:10	90
	Altex Utrasphere ODS	Acetonitrile/water 95:5	

Stoloniferone-a, -b, -c, -d	Cosmosil 5C18	Methanol/water	91
4′-0-acetyl-pregnedioside-a, -b	Cosmosil 5C18	Methanol/water	92
3′-0-acetyl-pregnedioside-a			
Gorgonians			
3-chloroguaiazulene	Merck LiChrosorb RP-18	Methanol/water 90:10	93
3-bromoguaiazulene			
Ehuazulene			
Muricins-1, -2, -3, -4	C18	Methanol/water 90:10→95:5	94
Other Coelenterates			
Punaglandin 1, 2, 3, 4	Merck LiChrosorb RP-18	Methanol/water 80:20	95
Bryozoans			
Bryostatin 4	Whatman Partisil-10 ODS-2	Methanol/water 50:50→90:10	96
Bryostatin 8	Whatman Partisil-10 ODS-2	Methanol/water 50:50→90:10	97
Marine Molluscs			
Tunicates			
Echinoderms			
5β-cholestane-3β,4β,6α,8β,15α,16β,26-heptaol	Whatman Partisil ODS	Methanol/water 70:30	98
25ε-methyl-5α-cholestane-3β,6α,8β,15α,26-hexaol			
(22E)-24ε-methyl-5α-cholest-22-en-3β,4β,6α,8β,15α, 26-heptaol			
Nodososide	Waters μ-Bondapak C18	Methanol/water 65:35	99
Attenuatoside S-I, S-II, S-III	Waters μ-Bondapak C18	Methanol/water 58:42	100
Attenuatoside D			
Miscellaneous			
Pavoninin 1-6	Merck LiChroprep RP-8	Methanol/water 80:20	101

6 Conclusion

One remaining problem lies in irreversible adsorption to the chromatographic media. If crude aqueous extracts are applied directly to chromatographic media, invariably significant irreversible adsorption takes place. In the isolation of a bioactive constituent a change of media can overcome this problem. It should be possible to find a chromatographic medium, where the binding is reversible. However, the inescapable problem is that the material bound to the medium will interfere with subsequent chromatography so that it is not possible to re-use the medium. It is recommended that aqueous extracts should be examined by high resolution techniques usually employed by organic chemists, viz. flash or medium pressure liquid chromatography, to be followed by HPLC, with only a change of the chromatographic media. It should be noted that chemically modified silicas are much more expensive than normal silica. A normal progression from open column techniques, to flash or medium pressure chromatography, to high pressure chromatography is feasible as chemically modified silicas of various particle size, are available.

Two major approaches may be used to identify organisms for chemical study, either by chemical or biological screening. The usual chemical screening by thin layer chromatography or nuclear magnetic resonance spectra of crude extracts will rarely reveal the presence of interesting metabolites in crude aqueous extracts. Biological screening, however, is as applicable to aqueous extracts as it is to organic extracts.

The techniques for the isolation of water-soluble constituents of marine extracts are as powerful as the techniques currently used for the isolation of organic soluble constituents. Different adsorbents are necessary for chromatography, but this should not be a deterrent to any chemist who is familiar with chromatography.

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Secondary Metabolites from Echinoderms as Chemotaxonomic Markers

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Contents

Abstract

The structures of some secondary metabolites and their distribution in the Phylum Echinodermata are described. Gangliosides, quinoid pigments and steryl sulfates in echinoderms are widespread, although these compounds are absent or rare in the majority of other marine invertebrates. There are numerous phylogenetic parallelisms dealing with directions of the biosynthesis of low molecular weight natural products between starfishes and sea cucumbers, sea urchins and ophiuroids, sea lilies and starfishes and so on. They indicate a monophyletic origin of all echinoderms. At the same time such metabolites as triterpene glycosides, some gangliosides, $\Delta^{9(11)}$-sterols, steroid oligoglycosides are linked to separate groups of these animals. Of particular interest among them as chemotaxonomic characters are triterpene glycosides from sea cucumbers, which have been utilized in a meaningful classification of Holothurioidea. The application of these and other secondary metabolites for taxonomic purposes is possible only by considering the cases of biochemical convergence and by invoking Vavilov's law of homologous series.

1 Introduction

Marine plants and animals that have been studied in the last two or three decades have yielded a variety of secondary metabolites, which possess novel chemical structures and interesting pharmacological actions. During all this time growing

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attention has been attracted to the problems of their isolation and identification as well as their possible medicinal applications. Although attempts to use chemical data in taxonomy have been made earlier, positive results have not been obtained in most cases. The reason was the lack of chemical information sufficient for taxonomic conclusions. Moreover, such biological phenomena as homology, phylogenetic parallelisms and convergence [1] had sometimes not been taken into account.

The present report is devoted to secondary metabolites from echinoderms. We would like to discuss the distribution of some low molecular weight natural products in different groups of the Phylum, emphasize existing difficulties, and outline perspectives of their application as chemotaxonomic markers. We hope also to touch upon some problems dealing with the chemotaxonomy of all marine organisms using echinoderms as an example.

According to Smith [1] "taxonomy is the theory and practice of classification, and chemotaxonomy incorporates the principles and procedures involved in the use of chemical evidence for classificatory purposes."

Many investigators prefer biopolymers as chemotaxonomic markers [2]. Secondary metabolites were utilized in chemotaxonomic studies mainly of higher plants and microorganisms [1, 3]. It has long been guided by an opinion about the occurrence of fewer low molecular weight natural products in animals in comparison with higher plants. However, a large number of chemically diverse secondary metabolites have been isolated from marine invertebrates [4–7]. Many of these compounds may be useful as biochemical tools in chemotaxonomy. Natural classification that takes into consideration as many characters of an organism as possible creates good conditions for an understanding and prediction of biological phenomena, in the same way as Mendeleev's Periodic Table does for inorganic chemistry. Chemical characters coupled with morphological, anatomical, genetic and others contribute a lot to improving existing classifications and adding to the knowledge of the origin and the evolution of living marine organisms, i.e. of their phylogeny.

Not all kinds of natural products can be used for classification purposes. There are many difficulties in formulating requirements for the application of chemical data. Different chemical characters provide clues that do not provide equal information. It is almost impossible to assign a weight to certain chemical characters ahead of time. Only systematic investigations of the structure, distribution and biological functions of marine natural products help to determine the weight.

In order to use chemical information in taxonomy, it is necessary to take into account several simple considerations. It is obvious that selected natural products, for example, secondary metabolites, must be biosynthesized by the organism under investigation, but not derived from its diet. The presence of natural products in one or several species shows that there is a possibility of selecting these compounds as chemotaxonomic markers. The taxonomic validity of the chemical information is greater, if the compounds present in the organisms came from collections that were not influenced by environmental conditions. Some variations in structures may have greater importance for chemotaxonomy if the compounds are identical within a group of related species, but vary from other distantly re-

lated groups. The biological functions of natural products are very important for the choice of these compounds as chemical characters. The greatest difficulty in their chemotaxonomical application derives from the occurrence of socalled convergent evolution since similar life conditions may lead to the elaboration of similar metabolites. From this standpoint, chemical compounds with important and specific physiological functions carry the greatest weight.

As a principal branch of the animal kingdom the phylum Echinodermata represents an excellent field for chemotaxonomic studies. Echinoderms are deuterostomas related to the Hemichordata. They first appeared in the Low Paleozoic. About fifteen classes, recent and fossil, existed before the Upper Ordovician [8]. At present there exist five classes: the Crinoidea (sea lilies), the Holothurioidea (sea cucumbers or holothurians), the Echinoidea (sea urchins), the Asteroidea (sea stars or starfishes) and the Ophiuroidea (brittlestars). About 6000 species of echinoderms inhabit the bottom of the sea from shallow waters to its maximum depths.

2 Lipids

Earlier works on lipids from echinoderms were reviewed by Fagerlund [9]. Fatty acid composition of these compounds is characterized by the presence of C_{12}–C_{24} compounds, which are characterized by being unsaturated. Eicosaenic (20:1 ω6), arachidonic (20:4 ω6) and eicosapentaenic (20:5 ω3) fatty acids have been identified from many species [10]. Considerable differences were found between fatty acids from the triglyceride and phospholipid fractions and also between the animals feeding on detritus and plankton [11]. The differences arise from branching and from the presence of odd-numbered chains. This would suggest that conversion of dietary fatty acids is a main source of fatty acids of echinoderms. In fact, Dembitsky [12] reported that a starfish, *Henricia* sp., which feeds on sponges, contains long chain acids (C_{24}–C_{30}), which are typical for sponges. It is interesting that the rare cis-14-tricosenic acid was found (1 to 5% of total fractions) in sea cucumbers [13].

On the other hand, some fatty acids are formed through *de novo* biosynthesis in echinoderms. The incorporation of ^{14}C-labelled acetate into saturated and monoenic acids was higher than into polyenic acids [14]. Fatty acids, which are biosynthesized or obtained through specific biotransformation of dietary components, are of greatest interest for chemotaxonomy. They must be absent or rare in other animals. The fatty acid composition of echinoderms generally is similar to that of other marine invertebrates and of fish oils. They include mainly compounds of the ω3 and ω6 series. In most cases the chain length is less than 22 carbon atoms.

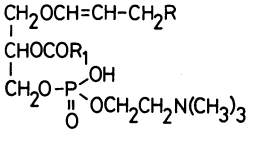
Takagi et al. [15] identified tetracosahexaenic acid (24:6 ω3) in several brittlestars and sea lilies. This compound constituted 5 to 10% of the total fatty acid fractions. Although it had been collected in the same area as ophiuroids studied by Takagi, the sea cucumber *Stichopus japonicus* contained no tetracosahexaenic

acid despite the fact that this animal also feeds on detritus. Therefore, these results do not suggest a dietary source for the acid. Other echinoderms (sea stars, sea urchins and sea cucumbers) seem to be devoid of tetracosahexaenic acid.

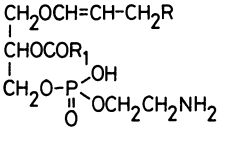
Recently, so-called non-methylene interrupted fatty acids were found in many marine invertebrates [16], including Echinodermata. Takagi et al. [17, 18] demonstrated that unusual cis-5-olefinic compounds, for example 20:2 $\Delta^{5,11}$, 20:3 $\Delta^{5,11,14}$ and 20:4 $\Delta^{5,11,14,17}$ fatty acids, were present in the polar lipids from Japanese and Canadian species of sea urchins; 15 to 20% of the corresponding fractions were represented by these constituents. The order of decrease of cis-5-olefinic acids content for other echinoderm classes was as follows: sea urchins » starfishes [19] » ophiuroids [15] » sea cucumbers and sea lilies [13].

Fatty acids are well known to acylate hydroxyl groups of glycerol and sphingosines during the biosynthesis of both neutral and polar lipids. All living organisms from bacteria to mammals contain lipids with ester or amide bonds. More limited distribution was established for alkyl and alkenyl ethers of glycerol. However such natural products are widespread among marine invertebrates and elasmobranch fishes [9]. The most common in echinoderms, especially starfishes, is batyl alcohol (α-octadecylglycerol ether). So-called plasmalogens or 0-alk-1-enylglyceryl ethers are found mainly in the animal kingdom. Their occurrence in plants is very seldom [20].

Vaskovsky and Dembitsky [21, 22] studied plasmalogen concentrations in 82 species of marine invertebrates belonging to 14 classes from 7 phyla. It was shown that an alk-1-enyl bond is often present in phospholipids isolated from these animals. The most common plasmalogens from marine invertebrates are represented by structures (**1**) and (**2**). The tissues of echinoderms studied were shown to be as rich a source of plasmalogens as mammalian brain [9]. The quantity of phosphatide plasmalogens depends upon the temperature of sea water and increases in summer [21]. A higher level of **1** and especially **2** were found in phospholipids from starfishes and brittlestars [22]. For example, the phosphatidyl ethanolamine fraction from the brittlestar *Ophiura sarsi* [23] contains about 99% of plasmalogen (**2**).



1



2

In contrast to plasmalogens, alkyl ethers in Echinodermata are distributed almost evenly between neutral and polar lipids. Todd and Rizzi [24] found that neutral and phospholipids from the starfish *Asterias forbesi* contained 36 and 27% of glyceryl ethers, respectively.

According to Karnovsky [25] the concentration of ether lipids in starfishes depends on the age of the animals. As follows from the comparative study of Isay et al. [26], which described the determination of ether lipid concentrations in 100 species of marine invertebrates, no striking relationship was evident between

taxonomy and ether lipid content. Concentration of these natural products in echinoderms fluctuated considerably.

Kostetsky and Gerasimenko [27] surveyed phospholipids from 25 species of echinoderms and compared these fractions with those from 175 species of other marine invertebrates. It was shown that evolutionary development is accompanied by the increase of the ratio of choline-containing to ethanolamine-containing compounds, replacement of ether for ester bonds, ceramide aminoethylphosphonate for sphingomyelin, and the decrease of phosphatidyl serine content. Phospholipids of echinoderms, as those of other deuterostomas, contain sphingomyelin and more choline-containing components in comparison with Porifera, Coelenterata and Mollusca. Sphingomyelins often include α-hydroxy fatty acids. The authors had noticed that there were points of similarity between phospholipid composition from Asteroidea and Ophiuroidea within Echinodermata (the high content of phosphatidyl serine, for example).

Thus echinoderms are characterized by the presence of some rare fatty acids and α-hydroxy fatty acids in the neutral and polar lipids as well as the high level of plasmalogens in phospholipids. However qualitative composition of these fractions is more or less unvarying, while the quantitative changes of various components take place due to the nutritional state of the animal. Therefore, these metabolites have more phylogenetic than chemotaxonomic significance.

Uncommon chemical structures are characteristic of some glycolipids, especially of gangliosides, from echinoderms. Gangliosides as one of the groups of sialoglycolipids are known to contain sphingosine acylated by a fatty acid and the oligosaccharide moiety including sialic acid or acids. Warren [28] found that sialic or neuraminic acids are present in vertebrata and in only one invertebrate phylum, Echinodermata. Vaskovsky et al. [29] came to a similar conclusion after a study of sphingolipids from 50 species of marine invertebrates belonging to 15 classes from 8 phyla.

Kochetkov et al. worked on gangliosides from echinoderms for many years [30]. They established structures of several dozens of sialoglycolipids isolated from sea urchins, starfishes and brittlestars (see Table 1). Gangliosides were also detected in sea lilies and sea cucumbers, but were not isolated. It became apparent that, along with *N*-acylated sphingosine or ceramide (Cer), gangliosides from echinoderms also contain *N*-acetylneuraminic acid (NeuAc), *N*-glycolylneuraminic acid (NeuGc), glucose (Glc), galactose (Gal), galactosamine ($GalNH_2$) and arabinose (Ara) as common monosaccharide units.

Gangliosides of sea urchins [31–38] have a general type of structure. The carbohydrate moieties of these secondary metabolites seem to be formed from glucose and sialic acid only. A sialic acid is usually attached to C-6 of glucose. The presence of sulfated gangliosides is probably more typical for sea urchins of the Order Irregularia in comparison with those of the Order Regularia. In general, carbohydrate moieties of sea urchin gangliosides differ markedly from those of starfishes. It may be interesting if one takes the phylogenetic problems of the echinoderms into consideration.

On account of a significant diversity in ganglioside composition of different sea urchin species, sets of gangliosides have the prospect of being utilized for chemotaxonomic purposes. These compounds differ from each other in the struc-

Table 1. Gangliosides from echinoderms

Class, order, species (organs)	Main sphingosin base	Structure of oligosaccharide (for main component of fraction)	Fatty acids	Refs.
Class: Echinoidea				
Order: Regularia				
Strongylocentrotus intermedius (gonads)	C_{16}, C_{18}-phytosphingosins	NeuAc ⟶ Glc ⟶ NeuAc ⟶ Glc ⟶ Cer	$C_{22:1}$; α-hydroxy-$C_{22:1}$	[31, 32]
Strongylocentrotus intermedius (eggs)	C_{16}, C_{18}-phytosphingosins	NeuGc ⟶ Glc ⟶ NeuGc ⟶ Glc ⟶ Cer	$C_{22:1}$; α-hydroxy-$C_{22:1}$	[33]
S. nudus (gonads)	C_{18}-phytosphingosin	NeuAc ⟶ NeuAc ⟶ Glc ⟶ Cer	$C_{16:0}$, $C_{18:0}$; α-hydroxy-$C_{18:0}$, $C_{20:0}$, $C_{22:0}$	[34]
Anthocidaris crassispina (gonads)	C_{18}-sphingosin	NeuAc ⟶ NeuAc ⟶ Glc ⟶ Cer	$C_{22:1}$, $C_{18:1}$, $C_{16:0}$	[35]
Tripneustes ventricosa (gonads)	C_{18}-phytosphingosin	NeuAc ⟶ NeuAc ⟶ Glc ⟶ Cer	$C_{18:0}$	[36]
Order: Irregularia				
Echinocardium cordatum (gonads)	C_{16}–C_{19}-phytosphingosins	8-*O*-sulfate of NeuGc ⟶ Glc ⟶ Cer	$C_{15:0}$, $C_{22:0}$, $C_{22:1}$, $C_{24:6}$	[37]
Echinorachnius parma (gonads)	C_{18}-phytosphingosin	8-*O*-sulfate of NeuAc ⟶ Glc ⟶ Cer	α-hydroxy-$C_{16:0}$, $C_{18:0}$	[38]

Class: Asteroidea				
Order: Forcipulata				
Distolasterias nipon (liver)	C_{18}-phytosphingosin	NeuAc⟶NeuAc⟶NeuAc⟶Gal⟶Glc⟶Cer	α-hydroxy-$C_{18:0}$, $C_{16:0}$, $C_{24:0}$	[39]
Lethasterias fusca (liver)	C_{16}–C_{18}-phytosphingosins	NeuAc⟶NeuAc⟶Gal⟶Glc⟶Cer	$C_{18:0}$	[40]
Evasterias retifera (liver)	n- and isophytosphingosins	NeuAc⟶NeuAc⟶Gal⟶Glc⟶Cer	α-hydroxy-$C_{14:0}$, $C_{16:0}$	[41]
Asterias amurensis (liver)	C_{17}–C_{19}-isophytosphingosins	GalNAc⟶Gal⟶Glc⟶Cer	α-hydroxy-$C_{16:0}$; $C_{22:0}$, $C_{24:0}$	[41]
Ophelasterias japonica (liver)	n- and isophytosphingosins	8-*O*-Me-NeuGc⟶Gal⟶Glc⟶Cer	α-hydroxy-$C_{16:0}$; $C_{22:0}$, $C_{24:0}$	[42]
Order: Spinulosa				
Patiria pectinifera (liver)	n- and isophytosphingosins	Ara⟶Gal⟶8-*O*-Me-NeuAc⟶Gal⟶Gal⟶Glc⟶Cer	α-hydroxy-$C_{22:0}$, $C_{24:0}$	[43]
Order: Phanerozonia				
Luidia quinaria bispina	–	Gal⟶8-*O*-Me-NeuAc⟶Gal⟶Glc⟶Cer	–	[44]
Class: Ophiuroidea				
Ophiura sarsi	C_{13}–C_{22}-sphingosins	8-*O*-sulfate of NeuAc⟶Glc⟶Glc⟶Cer	$C_{18:0}$, $C_{16:0}$	[45]

ture of a carbohydrate moiety, sphingosine base, and in fatty acid composition. It should be noted that it is necessary to take into account variations in ganglioside structure from various organs of the same species.

More complicated structures have been established for gangliosides isolated from several starfish species [39–44]. In contrast to sea urchin metabolites, the full coincidence of the ganglioside structures in different taxonomic groups of Asteroidea is less probable because of a convergence. That is why starfish gangliosides must receive greater emphasis for chemotaxonomy. Kochetkov and collaborators [30] showed that, although these natural products compose one group and all of them are derived in starfishes from a lactosylceramide precusor, they may differ at the subfamily level. In fact, *Distolasterias nipon* and *Lethasterias fusca*, belonging to the subfamily Coscinasteriinae, contain compounds, in which a neuraminic acid is attached to the lactosyl fragment. On the other hand, two other related species (*Asterias amurensis* and *Evasterias retifera*) contain the gangliosides including *N*-acetylgalactosamine attached to the lactosyl unit.

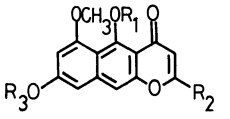
There is a similarity between gangliosides from sea urchins and from *Ophiura sarsi*, the sole species of Ophiuroidea which has been studied [45]. The compounds of *O. sarsi* contain only two sorts of monosaccharides in the oligosaccharide moiety: glucose and neuraminic acid, the latter being sulfated as in some sea urchin gangliosides. However, gangliosides of Echinoidea have glucose between a sphingosine base and a neuraminic unit, whereas the metabolites from *O. sarsi* have a glucobiose unit in its place. Nevertheless, the phylogenetic parallelism regarding the biosynthesis of gangliosides in Echinoidea and Ophiuroidea is a fact.

3 Quinoid Pigments and Related Metabolites

Quinoid pigments occur widely in microorganisms and plants, but in the animal kingdom they are almost exclusively found in echinoderms. These natural products are characteristic of sea urchins and sea lilies (crinoids), although they are also identified in some representatives of other classes in the phylum Echinodermata.

At present four groups of these presumably polyketide-derived constituents of crinoids are known, if differences in degree of *O*-methylation and oxidation are ignored. The linear (**3–6**) and angular (**7–9**) naphthopyrones compose the first group. They have, as a rule, 14 to 16 carbon atoms. These pigments were isolated from specimens of *Comantheria briareus* collected from two areas: a) West Australia; b) the Great Barrier Reef [46–48]; and also from color variants of *Comanthus parvicirrus timorensis* [49]. The animals belong to the most ancient family, Comasteridae. Naphthopyrones differ structurally from other pigments of Crinoidea but have the same polyketide origin.

The second group of pigments is represented by the derivatives of 4-acylanthraquinone (**10–13**) having 16 to 18 carbon atoms. Such natural products were isolated from crinoids of the genus *Comatula* [49–50].

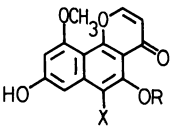


3 $R_1=CH_3$; $R_2=C_3H_7$; $R_3=H$

4 $R_1=CH_3$; $R_2=CH_3$; $R_3=H$

5 $R_1=H$; $R_2=C_3H_7$; $R_3=H$

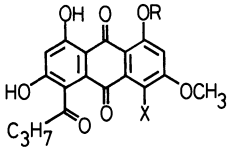
6 $R_1=CH_3$; $R_2=C_3H_7$; $R_3=CH_3$



7 R=X=H

8 R=H; X=OCH_3

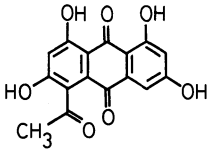
9 $R=CH_3$; X=OCH_3



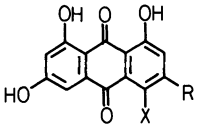
10 R=X=H

11 $R=CH_3$; X=H

12 R=H; X=OH



13



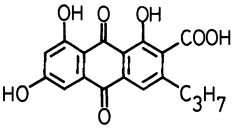
14 $R=CHOHCH_2CH_3$; X=H

15 $R=CH_3$; X=H

16 $R=COCH_2CH_3$; X=H

17 $R=CH_2CHOHCH_3$; X=H

18 $R=CHOHCH_2CH_3$; X=OH

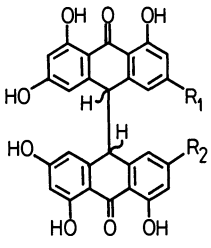


19

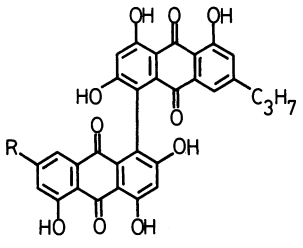
3-Alkylanthraquinones (**15–19**) are most widely distributed in comatulid crinoids (the only order of Crinoidea that has been studied chemically). Such pigments were found in *Comantus benneti* [51], *Ptilometra australis* [52], *Heteronema savignii, Lamprometra klunzingeri* [53], *Cenometra cornuta, Taxometra paupera* [54] and other species.

Finally, related to rhodoptilometrin (**15**) and its derivatives are the dimeric pigments, such as bianthrones and bianthraquinones (**20–24**), which were identified in *Camprometra palmata gigas, Zygometa microdiscus, Hymerometra magnipinna, H. robustipinna, Pontiometra andersoni, Stephanometra oxyacantha* [54]. 3-Alkylanthraquinones in crinoids are often accompanied by minor amounts of dimeric compounds.

Anthraquinoid pigments and naphthopyrones are usually present in the extracts from Crinoidea as monoesters of sulfuric acid. Fishes are deterred from eating food that contains these compounds at the concentration found in corresponding echinoderms. Many richly-colored crinoids inhabit coral reefs and in general are not predated by fish [49]. It may be concluded that sulfates of quinoid pigments may serve to reduce predation.



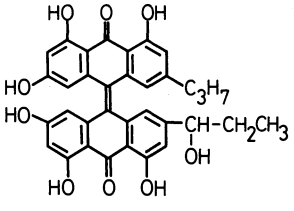
20 $R_1 = R_2 = C_3H_7$



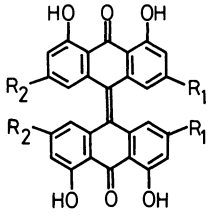
22 $R = C_3H_7$

21 $R_1 = C_3H_7$; $R_2 = CH(OH)-CH_2CH_3$

23 $R = CH(OH)-CH_2CH_3$



24



25 R=Alk

26

Quinoid pigments similar to rhodoptilometrin or its dimers were biosynthesized many million years ago. Support of this conclusion is provided by identification of the fringelites A–H, as hydroxylated phenanthroperylenequinones of structures (**25**), and the related hydrocarbon (**26**) in the fossilized remains of an *Apiocrinus* species from certain Swiss Upper Jurassic beds [55, 56]. Evidently these compounds were formed in the fossils from anthraquinoid pigments.

Some species or at least sub-species, as well as color forms of the same species of crinoid, are characterized by a definite set of pigments. At the same time, concentrations of various components in a set vary from one population or color form to another. For example, both purple and yellow forms of *Zygometra microdisens* [54] contain rhodoptilometrin (**14**), crinemodin (**15**) and dimeric compounds (**20**) and (**23**) but in different ratios. Specimens of *Comantheria briarens* from the Great Barrier Reef and from West Australia have pigment fractions, consisting of linear and angular naphthopyrones **3** and **4**. The capability for specific biosynthesis in particular species has been demonstrated many times [54].

Careful study established that distant families may show considerable overlapping in the composition of their polyketide natural products. Considering the simple structures of these pigments and presumably a defensive function as well as the ancient origin, it may be supposed that the coincidence is a result of the homological development of these chemical characters. It is known that descend-

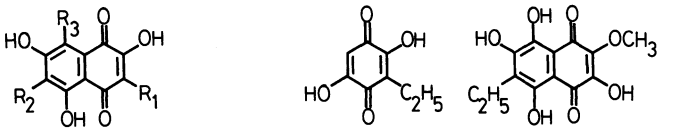
ants from a common ancestor have a tendency to display similar evolutionary trends [57].

Comparison of pigment fractions of three species of *Comanthus* (the Comasteridae family) and two species of *Lamprometra* (the Mariametridae family) led Rideout and Sutherland [54] to the conclusion about "non-chemotaxonomic" distribution of polyketides in comatulids. In fact, *C. parvicirrus* contains linear and angular naphthopyrones, *C. bennetti* yields 3-alkylanthraquinones, and *C. japonica* has a 4-acylanthraquinone, *L. palmata gigas* contains 3-alkylanthraquinones, whereas *L. klunzingeri* provides 4-acylanthraquinones. They postulated that crinoids have had a vast span of time to explore and exploit various polyketide sulfates for defense against predators. Many of these substances remain potentially available in the gene pool of each animal in the form of recessive alleles, and suitable defensive pigments are elaborated by a species as an adaptation to its ecological niche. Thus different populations of the same species may contain various polyketides. Chemical variations were shown but only for specimens of *Lamprometra klunzingeri* [53, 54] from the Persian Gulf and East Africa. At the same time "non-chemotaxonomic" distribution can also be due to defects of crinoid systematics, especially as the *Lamprometra* genus includes several species with characters which are apparently not clear [54]. In any case, polyketide derivatives of crinoids proved to be useless for chemotaxonomy to date.

Recently, Utkina and Maximov found isorhodoptilometrin (**17**) and related pigments, including 2′-*O*-sulfate of **17** in starfishes, *Echinaster echinophora* [58] and *Henricia laviuscula* [59]. It had been considered previously that the distribution of anthraquinoid derivatives is limited to crinoids. Screening of anthraquinoids in Asteroidea showed that the group is present in different species collected both in the Pacific and Atlantic oceans. All these animals belong to the Echinasteridae family (see Table 2). Therefore, in contrast to the Crinoidea there is a taxonomically significant distribution of anthraquinoid pigments in the Asteroidea. Mayr [57] wrote about similar situations: "Every experienced taxonomist knows that a character, which is highly informative in one group of organisms may be altogether useless in another."

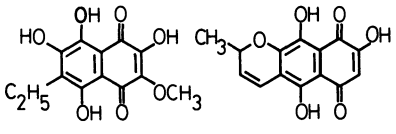
Naphthoquinones from sea urchins first attracted attention in the last century. MacMunn [60] described the red pigment of the perivesceral fluid from *Echinus esculentus* and named this substance echinochrome (**27**). Fifty years later, Kuhn and Wallenfels [61, 62] established the structure of **27** and synthesized it. Extensive works on echinochrome and related spinochromes (**28–32**) from spines and testa of sea urchins were reviewed by Goodwin [63], Thomson [64], Fox and Hopkins [65], Vevers [66], Chang [67] and Grossert [68]. The majority of sea urchin naphthaquinoids such as echinochrome A (**27**), spinochromes A (**28**), C–E (**30–32**) are derivatives of naphthazarin. Spinochrome B (**29**) is derived from juglone [69].

Scheuer et al. [70, 71] showed the wide distribution of **27–32** in the Echinoidea and proposed the modern nomenclature of these compounds [72]. Many components related to **27–32**, for example benzoquinone (**33**), methylated pigments (**34, 35**), pyranonaphthazarin (**36**), dimeric compound (**37**), and others, were identified [73, 74]. Maximov et al. [75, 76] isolated a pigment identical to **37** from *Strongylocentrotus dröebachiensis* and *S. intermedius* and revised its structure to

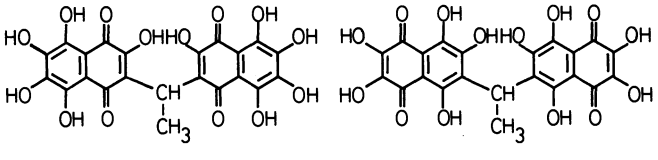


27 R_1=R_3=OH; R_2=C_2H_5
28 R_1=$COCH_3$; R_2=H; R_3=OH
29 R_1=OH; R_2=R_3=H
30 R_1=$COCH_3$; R_2=R_3=H
31 R_1=R_3=OH; R_2=H
32 R_1=R_2=R_3=OH

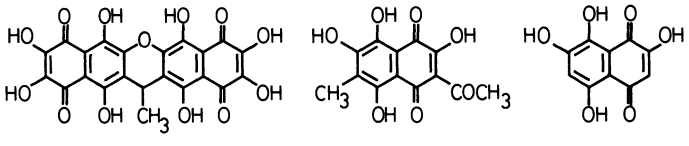
33 34



35 36



37 38



39 40 41

38 by a ^{13}C NMR study. This pigment is usually accompanied by the anhydroderivative (**39**). The uncommon naphthoquinoid (**40**) with two carbon-containing substituents, and naphthazarine derivative (**41**) were found in *S. nudus* [77].

Pigments have important significance for sea urchins [63–66, 78]. They demonstrate interesting physiological activities, especially echinochrome A, which is a bactericidal substance in the celomic fluids of the animals [79]. Recently, echinochrome A proved also to be an active inhibitor of lipid peroxidation [80] in developing sea urchin embryos. In some sea urchin species quinoids are contained in the oocytes, in others they appear at the moment when the gastrula is transformed into the pluteus [81, 82]. Many other hypotheses have been suggested for biological functions of the pigments in adult animals; however the majority of them have not so far been confirmed experimentally [78].

Pigment composition of sea urchins usually includes 2–6 components, although it may be more complicated. Both the extent of quinoids and their concentration within mixtures vary dependent on environmental conditions, the stage of animal development, and from one color variant to another. However, the main components of a mixture seem to be characteristic of a species [83]. Moreover, as may be seen in Table 2, some but not all genera of the Echinoidea contain characteristic groups of quinoids. For example, the genus *Arbacia* elaborates predominantly echinochrome A (**27**) and spinochrome C (**30**), while the genus *Strongylocentrotus* yields spinochromes A, C, D (**28, 30, 31**), binaphtha-

Table 2. Polyketide pigments from echinoderms by Species

Class, order, species	Main pigments			Refs.
	Naphthoquinoids	Anthraquinoids	Others	
Echinoidea				
Aulodonta				
Centrostephanus nitidus	**27**	–	–	[63–65]
C. rodgersii	**27, 28**	–	–	[64]
Diadema antillarum	**27, 32**	–	–	[63, 64]
D. paucispinus	**28**	–	–	[63, 64]
D. savignije	**27, 35**	–	–	[84]
D. setosum	**27, 28, 35**	–	–	[63, 84]
Echinothrix calamaris	**28**	–	–	[63–65]
E. diadema	**28**	–	–	[64]
E. setosum	**27, 28**	–	–	[64]
Cidaroida				
Chondrocidaris gigantea	**28, 30, 31**	–	–	[64]
Cidaris cidaris	**27, 29, 30**	–	–	[64]
Eucidaris thouarsii	**27–30, 32**	–	–	[63, 64]
E. metularia	**28–30**	–	–	[64]
Goniocidaris tubaria	**28**	–	–	[64]
Phyllacanthus irregularis	**27, 28, 32**	–	–	[64]
P. parvispinus	**27, 28, 30, 32**	–	–	[64]
Prionocidaris bispinosa	**28–30**	–	–	[64]
P. hawaiiensis	**28, 30, 31**	–	–	[64]
Clepeasteroida				
Dendraster excentricus	**27, 31, 32**	–	–	[64]
Echinocyamis pusillus	**27**	–	–	[64]
Echinorachnius parma	**27**	–	–	[64]
Encope emarginata	**27**	–	–	[64]
Mellita sexiesperforata	**27, 29, 31**	–	–	[64]
Scaphechinus mirabilis	**27**	–	–	[63]
S. griseus	–	–	–	[85]

Table 2 (continued)

Class, order, species	Main pigments			Refs.
	Naphthoquinoids	Anthraquinoids	Others	
Comarodonta				
Amblyneustus oveum	**28**	–	–	[64]
Anthocidaris crassispina	**28–30**	–	–	[64]
Colobocentratus atratus	**28**	–	–	[64]
Echinometra lucunter	**27–30, 32**	–	–	[64]
E. mathaei	**27–30, 32**	–	–	[64, 85]
Echinometra oblonga	**28, 30, 41**	–	–	[64, 65]
Echinus acutus	**28–30, 32**	–	–	[63]
E. elegans	**27–30, 32**	–	–	[64]
E. esculentus	**27–30, 32**	–	–	[64]
Exinostrephus aciculatus	**28–30**	–	–	[64]
Hemicentrotus pulcherrimus	**28–30**	–	–	[64]
Heterocentrotus mammilatus	**28–30**	–	–	[64]
Loxechinus albus	**27**	–	–	[64]
Paracentrotus lividus	**27–30, 32**	–	–	[63]
Psammechinus miliaris	**28–30, 32**	–	–	[63, 64]
Pseudocentrotus depressus	**28**	–	–	[64]
Sphaerechinus granularis	**27, 28, 30**	–	–	[64]
Sterechinus neumayari	**27, 28, 30**	–	–	[63, 64]
Strongylocentrotus droebachiensis	**28–32, 35, 38, 39**	–	–	[64, 65]
S. franciscanus	**27–30, 32**	–	–	[64, 65]
S. intermedius	**27–30, 35, 38**	–	–	[64, 65]
S. nudus	**27–30, 32, 40, 41**	–	–	[64, 85]
S. polyacanthus	**27, 28, 30–32, 35, 38**	–	–	[84]
S. purpuratus	**27–30, 32**	–	–	[85]
Toxopneustes pileolus	**28**	–	–	[85]
Tripneustes gratilla	**28, 32, 41**	–	–	[64]
T. ventricosus	**28–30, 32**	–	–	

Spatangoida				
Brisaster latifrons	–	–	–	[85]
B. fragilis	–	–	–	[85]
Echinocardium cordatum	**27**	–	–	[64]
Meoma ventricosa	**27, 28**	–	–	[64]
Pourtalesia jeffreysi	–	–	–	[85]
Spatangus purpureus	**27, 31**	–	–	[64]
S. raschi	**38**	–	–	[64]
Striridonta				
Arbacia incisa	**27, 30**	–	–	[63, 64]
A. lixula	**27, 28**	–	–	[63, 64]
A. pustulosa	**27, 30**	–	–	[63, 64]
Stomopneustes variolaris	**27**	–	–	[64]
Tetraphygus niger	**27, 28**	–	–	[64]
Asteroidea				
Phanerozonia				
Luidia quinaria bispina	–	–	–	[59]
Spinulosa				
Acanthaster planci	3,6 and 3,7 di-OCH_3-**32**	–	–	[64, 86]
Echinaster echinophorus	–	**14, 15**	–	[59]
Henricia knipowitschi	–	+	–	[59]
H. leviuscula	–	**14**	–	[59]
H. ochotensis	–	+	–	[59]
H. sanguinolenta	–	+	–	[59]
Patiria pectinifera	–	+	–	[59]
Forcipulata				
Aphelasterias japonica	–	–	–	[59]
Asterias amurensis	–	–	–	[59]
Distolasterias nipon	–	–	–	[59]
Evasterias echinosoma	–	–	–	[59]
Lisostrasoma anthosticta	–	–	–	[59]

Table 2 (continued)

Class, order, species	Main pigments			Refs.
	Naphthoquinoids	Anthraquinoids	Others	
Holothurioidea				
Aspidochirota				
Polycheira rufescens	7-OCH_3-**32**	–	–	[64, 87]
Stichopus japonicus	–	–	–	[85]
Dendrochirota				
Cucumaria fraudatrix	–	–	–	[85]
C. japonica	–	–	–	[85]
Ophiuroidea				
Ophiurae				
Amphipholis kochii	–	–	–	[88]
Ophiocoma echinata	–	–	–	[88]
O. erinaceus	2,7 and 2,6-dideoxy-**27**	–	–	[86, 64]
O. nisularia	2,6-dideoxy-**27**	–	–	[86, 64]
O. riisei	+	–	–	[88]
O. scolopendrius	–	–	–	[88]
Ophioderma brevispinus	–	–	–	[88]
O. cinereum	–	–	–	[88]
Ophioderma rubicundum	–	–	–	[88]
Ophiopholis aculeata	–	–	–	[88]
Ophiura sarsi	–	–	–	[85]
Ophrotrix suensonii	–	–	–	[88]

Crinoidea				
Articulata				
Antedon sp	7-hydroxy naphthazarin	–	–	[86, 64]
Cenemetra cornuta	–	**14, 19**	–	[54]
Comactinia meridianalis meridianalis	–	**13**	–	[54]
Comantheria briareus	–	–	**3–5, 7**	[45, 57]
C. perplexa	+	–	**3, 4**	[45, 86]
Comanthus parvicirrus timorensis	–	–	**7–9**	[48, 54]
C. benneti	–	**14, 17**	–	[51]
C. japonicus	–	**10**	–	[54, 89]
Comantula pectinata	–	**10–12**	–	[49]
C. rotolaria	–	**10, 11**	–	[49]
C. cratera	–	**11**	–	[49, 52]
C. solaris	–	**10, 11**	–	[49]
Hymerometra magnipinna	–	**14, 15, 20**	–	[54]
H. robustipina	–	**14, 16**	–	[54]
Heteronema savignii	–	**14**	–	[53]
Lamprometra kluinzingeri	–	**13, 14**	–	[53]
L. palmata gigas	–	**14, 15, 20, 21, 23, 24**	–	[54]
Pontiometra andersoni	–	**14, 20**	–	[54]
Ptilometra australis	–	**14, 15, 19**	–	[52]
Stephanometra oxyacantha	–	**14, 20**	–	[54]
Tropiometra afra microdiseus	–	**19**	–	[89]
T. afra	–	**19**	–	[52]
Zigometra microdiscus	–	**14, 15, 20, 22**	–	[54]

quinones and a little echinochrome A (**27**) and spinochrome B (**29**). It is of taxonomic interest, when there is a deviation from the norm within a group. Maximov et al. [77, 90] noted the distinction between the quinoids of *Strongylocentrotus nudus* and those from other representatives of the genus *Strongylocentrotus* (see Table 2). It is a difficult problem to determine the significance of similar deviations, since natural systematics can be based only on the presence or absence of several characters. At the same time, the distribution of pigments in *Strongylocentrotus* spp. agrees with some morphological data. It was shown [91] that *S. nudus* is quite distinct from two other *Strongylocentrotus* species by the presence of primary pedicellariae and by the development of a larval skeleton. This raises the question of the necessity to revise this genus.

It is well known that quinoids (naphthoquinoids and anthraquinoids) are typical metabolites of the Crinoidea and the Echinoidea. They were found also among isolated representatives of other echinoderm classes. Methylated spinochromes E were identified in the starfish *Acanthaster planci* [86] and, complexed with proteins, in the sea cucumber *Polycheira rufescens* [87]. Some naphthazarin derivatives have been isolated from the ophiuroid *Ophiocoma erinacens* and *O. insularia* [86]. Nitrogen-containing quinoids of unknown structure were found in *O. erinaceus, O. insularia* [86], *0. riisei* [92], and in the sea urchin *Echinothrix diadema* [64]. It is possible that naphthoquinoids were more widely distributed an ancient echinoderms in comparison to living forms. In that case, the majority of sea cucumbers, starfishes and brittlestars have lacked quinoid pigments altogether, as well as some sand dollars [80]. If this hypothesis is correct, quinoids in the starfish *Acanthaster planci*, the sea cucumber *Polycheira rufescens*, and some ophiuroids are "chemical fossils".

The portion of naphthoquinoids is fixed in the skeletons of adult animals as calcium or magnesium salts. Therefore, there is a theoretical possibility of finding polycondensed compounds like binaphthaquinones in fossilized remains of ancient sea urchins (starfishes? sea cucumbers? ophiuroids?).

4 Triterpenoid and Steroid Glycosides

4.1 Triterpenoid Glycosides

Triterpenoid and steroid oligoglycosides (saponins) have been isolated from a great number of terrestrial plants [93] and utilized as chemical characters for plant chemotaxonomy [1]. In the animal kingdom olygoglycosides have been found exclusively in echinoderms [94, 95]. Starfishes and sea cucumbers biosynthesize these compounds *de novo* [96, 97]. Saponins from both echinoderm classes possess considerable toxicity, including ichthyotoxicity [96, 98–100]. Their biological function is presumably connected with defensive action, discouraging infectious aquatic fungi, protistes, parasites, and predators. Besides, these oligoglycosides, at least in some species of starfishes and sea cucumbers, participate in reproductive processes [101, 102].

In spite of the fact that they belong to one chemical type, saponins of sea cucumbers and starfishes differ considerably from each other. Asterosaponins are sterol derivatives, whereas sea cucumber glycosides have triterpenoid aglycones. None of the aglycones, the monosaccharides, the attachment of carbohydrate moieties, the degree of oxidation in the aglycones are identical for asterosaponins and holothurins. Only the presence of sulfate esters and the sugar quinovose are common features for both groups of these secondary metabolites. Sulfation is typical in the biosynthesis of secondary metabolites in many marine invertebrates, for example sponges [4, 7]. However, in asterosaponins the sulfate is attached to an aglycone, whereas it is attached to the carbohydrate moiety in some sea cucumber glycosides, while others lack sulfate groups. Asterosaponins and holothurins differ in structure almost as much as these groups are distinct from saponins of terrestrial plants. It may be supposed that saponins of both sea cucumbers and starfishes are the result of adaptation during evolution under similar environmental conditions, chiefly due to the action of swimming micro- and macroforms. These metabolites belong to the same chemical type of glycosides due to biochemical convergence rather than because of a phylogenetic relation between Asteroidea and Holothurioidea. Recently, defensive steroid glycosides have also been found in soft corals and fishes [7]. Consequently, the analysis of glycoside distribution within Asteroidea or Holothurioidea at the ordinal, familial, or general level is more useful than the utilization of these data for phylogenetic conclusions at the phylum or class level.

To-date, the study of triterpene glycosides of more than fifty sea cucumber species has been carried out [103–105]. Biogenetic relationship and considerable structural variability are characteristic of these secondary metabolites. Only quinovose (Qui), glucose (Glc), 3-*O*-methyl-glucose (3-*O*-Me-Glc), xylose (Xyl) and 3-*O*-methyl-xylose (3-*O*-Me-Xyl) are included in the carbohydrate moieties of these saponins. All monosaccharides are present in the pyranose form and the glycoside bonds have the β-configuration. The aglycones of sea cucumber glycosides are derived from lanosterol.

There are quite a few series of sea cucumber glycosides. The socalled holothurins (**42, 43**) (this term is sometimes also used for all types of glycosides from Holothurioidea) contain a 12α-hydroxy-9(11)-ene fragment in the aglycone and a disaccharide or tetrasaccharide moieties with the sulfate group attached to C-4 of the xylose residue [106–112]. Acid hydrolysis of **42** or **43** as well as related glycosides provides holothurinogenins (**44a–c**) [113, 114]. Holothurins (**42, 43**) were isolated from Pacific, Indo-Pacific and Atlantic species belonging to the Holothuridae family (Order Aspidochirota), specifically from *Holothuria* and *Actinopyga* spp. (see Table 3).

Five *Bohadschia* spp. from the same family contain glycosides (bohadschiosides) of the general formula (**45**), which yields **44d** as the main artificial genin after acid hydrolysis [115]. These oligoglycosides lack an *O*-sulfate group and have holost-9(11)en-3β-ol and holost-9(11)-en-3β,12α-diol as natural aglycones [116].

In contrast to the holothurins and bohadschiosides, the saponins of another group, socalled stichoposides (**46, 48**) and thelenotosides (**47**) contain true aglycones with a 7(8)-double bond and an acetoxy group at C-23 [117–120]. Glycosides (**46–48**) were isolated from Pacific and Atlantic species of the genera *Sticho-*

Table 3. Triterpene glycosides from sea cucumbers by species

Species	Locality	Structures (main components of mixtures)	Refs.
Aspidochirota			
Holothuriidae			
Holothuria atra	New Guinea	**42, 43** tr.[a]	[103, 107]
H. atra	Nauru Island	**42, 43**	[103, 107]
H. atra	Samoa	**42, 43**	[103, 107]
H. arenicola	Lord Howe Island	**43**	[103]
H. cinerascens	Funafuti Island	**43**	[103]
H. coluber	New Hebrides	**42, 43**	[103]
H. cubana	Cuba	**42, 43**	[104]
H. difficilis	Lord Howe Island	**43**	[103]
H. edulis	Gilbert Islands	**42, 43**	[103, 112]
H. floridana	Cuba	**42, 43**	[104, 109]
H. fuscocinerea	Lord Howe Island	**42, 43**	[103]
H. gracilis	New Caledonia	**42, 43**	[103]
H. grisea	Cuba	**42, 43**	[104, 111]
H. hilla	Samoa	**42, 43**	[103]
H. impatiens	Samoa	**42, 43**	[103]
H. leucospilota	New Hebrides	**42, 43**	[103, 110]
H. mexicana	Cuba	**42, 43**	[104]
H. nobilis	Fiji	**43**	[103]
H. pervicax	New Caledonia	**42, 43**	[103]
H. pulla	Funafuti Island	**42, 43**	[103]
H. scabra	New Hebrides	**42**	[103]
H. surinamensis	Cuba	**42, 43**	[104]
H. species	New Guinea	**42, 43**	[103]
Actinopyga agassizi	Cuba	**42, 43**	[104]
A. mauritana	Nauru Island	**42, 43**	[103]
A. miliaris	Samoa	**42, 43**	[103]
A. echinites	New Caledonia	**42, 43**	[103, 108]
A. lecanora	New Caledonia	**42, 43**	[103]
A. species	Gilbert Islands	**42, 43**	[103]
Pearsonothuria (Bohadschia) graeffei	New Guinea	**42, 43**	[103]
Bohadschia argus	New Hebrides	**45a, b**	[103, 105]
B. bivittata	Okinawa	**45a, b**	[116]
B. marmorata	Gilbert Islands	**45a, b**	[103, 105]
B. vitiensis	Seychelles Islands	**45a, b**	[105]
B. tenuissima	New Hebrides	**45a, b**	[103, 105]
B. species	Seychelles Islands	**45a, b**	[103]
Aspidochirota			
Stichopodidae			
Stichopus chloronotus	Great Barrier Reef	**48a–c, 46a, b** tr.	[117, 119, 120]
S. variegatus	Fiji	**48a–c; 25(26)-**dehydro-**48a, b**	[103, 119]
Astichopus multifidus	Cuba	25(26)-dehydro-**48a–c**	[119]
Thelenota ananas	Seychelles Islands	**47a, b**	[118]
T. anax	Seychelles Islands	**47a, b; 48a–c**	[103]
Stichopus regalis	Cuba	–	[104]
Isostichopus badionotus	Cuba	–	[104]

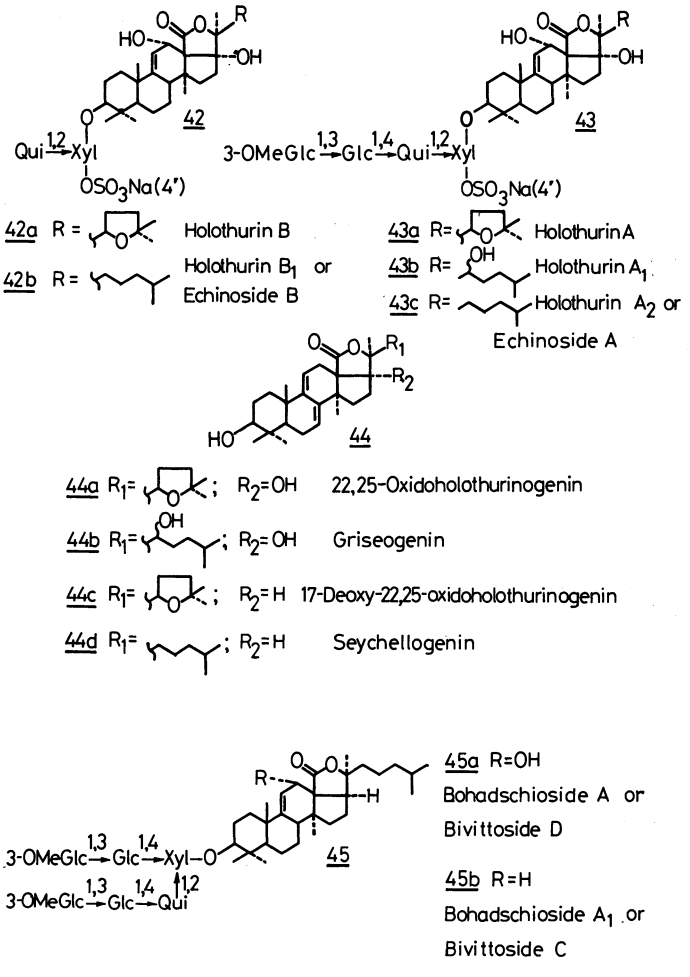
Table 3 (continued)

Species	Locality	Structures (main components of mixtures)	Refs.
Parastichopus californicus	Pacific coast of USA	**49b, d**	[133]
Parastichopus (Stichopus) japonicus	Sea of Japan	**49a–d**	[121, 122]
Dendrochirota			
Cucumariidae			
Cucumaria japonica	Sea of Japan	**52**	[124]
Eupentacta (Cucumaria) fraudatrix	Sea of Japan	**51a–d**	[105, 123]
E. pseudoquinquisemita	Kurile Islands	**51d**	[105]
Dendrochirota			
Psolidae			
Psolus fabricii	Kurile Islands	**53, 54**	[125]
P. species	Kurile Islands	**53**	[105]
Dendrochirota			
Phyllophoridae			
Afrocucumis africana	Madagascar	**57**[b]	[103]
Neothyonidium magnum	New Caledonia	**56**	[127]
Duasmodactyla kurilensis	Kurile Islands	**57**[b]	[129]

[a] tr., traces.
[b] Only structure of the aglycone is known.

pus, Thelenota and *Astichopus* (the Stichopodidae family of the same order). Related holotoxins with the 9(11)-double bond and general formula (**49**) were found in *Stichopus japonicus* and *Parastichopus californicus* from the same family [121, 122].

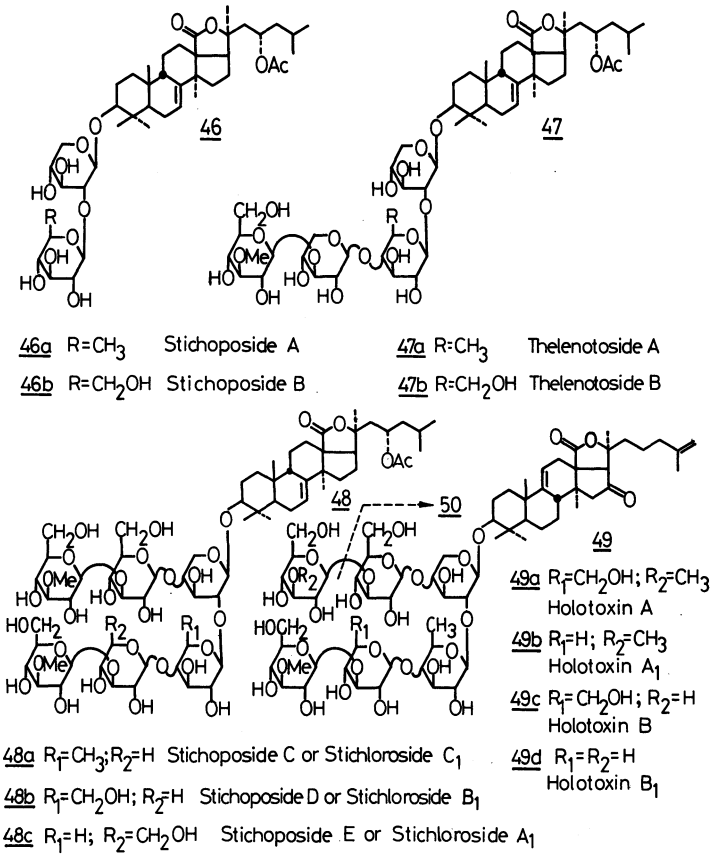
From several species of the Order Dendrochirota several different groups of glycosides were structurally identified. For example, an unidentified species of this order contains the glycoside (**50**) differing from holotoxin A_1 (**49b**) only by the absence of one monosaccharide residue in the carbohydrate moiety. Cucumariosides G_1, C_1, C_2, H (**51a–d**) from *Eupentacta fraudatrix* and *E. pseudoquinquisemita* [105, 123] combine some features of the stichoposides and holothurins. Thus, like holothurins, some cucumariosides have the sulfate group at the xylose residue. Like stichoposides, they include the 7(8)-double bond in the aglycones. The cucumarioside A_2-2 (**52**) from *Cucumaria japonica* [124] closely resembles the holotoxins with respect to the structure of the aglycone, but the structure of carbohydrate moiety in **52** is similar to that of some saponins from *E. fraudatrix*. Polar glycosides psolusoside A (**53**) and B (**54**), were isolated from *Psolus fabricii* and *P.* sp. [125, 126]. The compound (**53**) contains holotoxinogenin as the native aglycone and two sulfate groups in monosaccharide residues attached to C-6 of the sugars. Psolusoside B (**54**) includes both the uncommon aglycone with an 18→16 lactone and an unprecedented tetrasaccharide moiety with three glucose residues. Sulfated oligoglycosides (**55, 56**), having holotoxinogenin as the native aglycone were found by French researchers in the sea cucumber *Neothyonidium magnum* [127] and by us in an unidentified species of the Order Dendrochirota.



Like psolusoside B (**54**), saponins from *Duasmadactyla kurilensis* are derived from a non-holostane aglycone. According to Habermehl and Volkwein [128] holostane is the 18→20 lactone of 18-carboxylanostan-3β,20ξ-diol. The majority of isolated sea cucumber oligosaccharides have aglycones of the holostane type. The natural aglycone of the saponins from *D. kurilensis* seems to be a 27,26,25,24,23,22-hexa-*nor*-lanostane derivative (**57**). Acid hydrolysis of these compounds liberates socalled kurilogenin (**58**) with an α®β-unsaturated ketone chromophore [129].

Thus, saponins are widely distributed in the class Holothurioidea. A correlation exists between the systematic position of the animals and their glycoside structures (see Table 3). As a consequence, these secondary metabolites may serve as chemotaxonomic markers as an aid to the systematics of sea cucumbers.

For example, the sea cucumber *Bohadschia graeffei*, in contrast to all other representatives of this genus, yields holothurins [130] and other members of this genus contain bohadschiosides [103]. There are also essential morphological distinctions between this species when compared with other animals from the genus

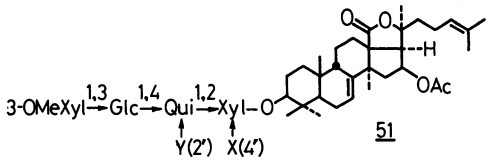


Bohadschia. This chemical and morphological evidence was used for the revision of *Bohadschia*. As a result, there have been suggestions to assign *B. graeffei* to a separate genus named *Pearsonothuria* [131, 132]. It also seems to be advisable to discuss with systematists the possibility of separating the Holothuriidae family into two subfamilies. The first may comprise the genera *Holothuria, Actinopyga* and *Pearsonothuria*, all of which contain holothurins, whereas the second subfamily includes the genus *Bohadschia*, which biosynthesizes bohadschiosides.

Analysis of glycoside distribution in the Stichopodidae family showed that the taxonomic status of *Stichopus japonicus* must be changed: the glycoside structures of the animal are the same as those of *Parastichopus californicus*, but differ from the corresponding structures from *S. chloronotus* and *S. variegatus*. Morphological studies confirmed the conclusion that *S. japonicus* and *P. californicus* are congeneric species [132, 133].

In contrast to the compounds from *Cucumaria japonica*, saponins of *Eupentacta (Cucumaria) fraudatrix* are identical to those isolated from *E. pseudoquinquisemita*. That is why it is more correct to consider *E. fraudatrix* belonging to the genus *Eupentacta* rather than *Cucumaria*.

The validity of triterpene glycosides as chemotaxonomic markers is far greater than that of other metabolites mentioned because of their taxonomic distribution, variability, and complex structures.



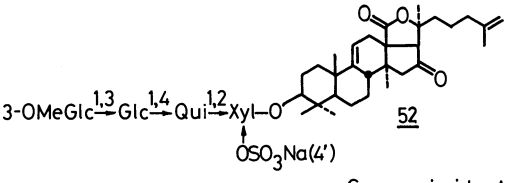
51

51a X=OSO_3Na; Y=H Cucumarioside G_1

51b X=H; Y=Xyl; 22(23)-dehydro(Z) Cucumarioside C_1

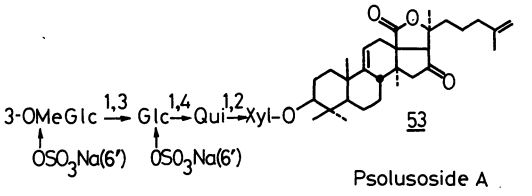
51c X=H; Y=Xyl; 22(23)-dehydro(E) Cucumarioside C_2

51d X=OSO_3Na; Y=Xyl; 22(23)-dehydro(E) Cucumarioside H



52

Cucumarioside A_2-2



53

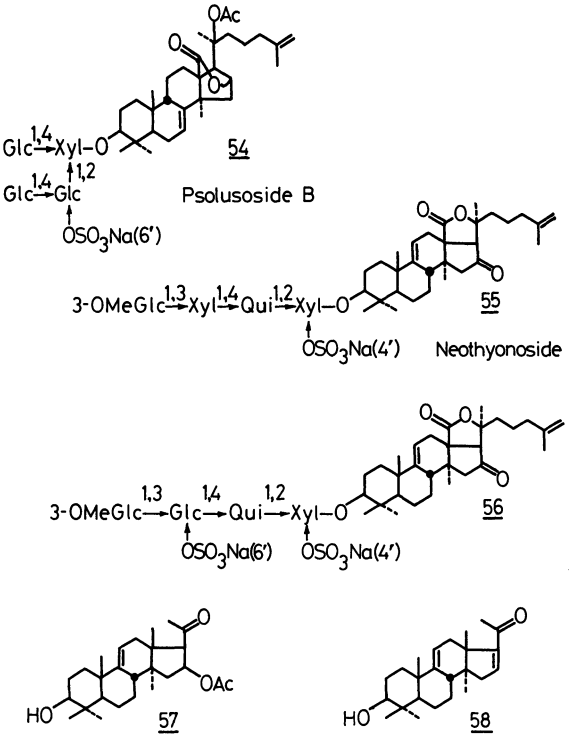
Psolusoside A

The phylogenesis of triterpene glycosides from sea cucumbers was presumably connected with the selection of the most active compounds. As it was shown by Sheikh and Djerassi [134], the biosynthesis of the saponins proceeds via lanosterol (**59**).

An hypothetical approach from **59** to glycoside aglycones in all animals belonging to the Order Aspidochirota involves the oxidation at C-18 and C-20 with the formation of the 18→20 lactone ring (Scheme 1). The 8(9)-double bond of **59** migrates to the position 9(11) in representatives of the Holothuriidae family. Subsequent hydroxylation at C-12 (the genus *Bohadschia*) and at C-12, C-17, C-22 positions (*Holothuria, Actinopyga, Pearsonothuria* genera) provides bohadschiosides and holothurins, respectively.

In the species of the Stichopodidae family which have been studied the initial steps of lanosterol biotransformation are identical to those of the Holothuriidae. But in most cases the 8(9)-double bond of the precursor migrates to the 7(8)-position. Subsequent hydroxylation at C-23, acetylation, and saturation of the 24(25)-double bond yields substances of the general formula (**66**). Only *S. japonicus* and *P. californicus* convert the 8(9)-double bond of **59** to the 9(11)-position, similar to sea cucumbers of the family Holothuriidae. However, the biotransformation in these cases is completed by oxidation at C-16.

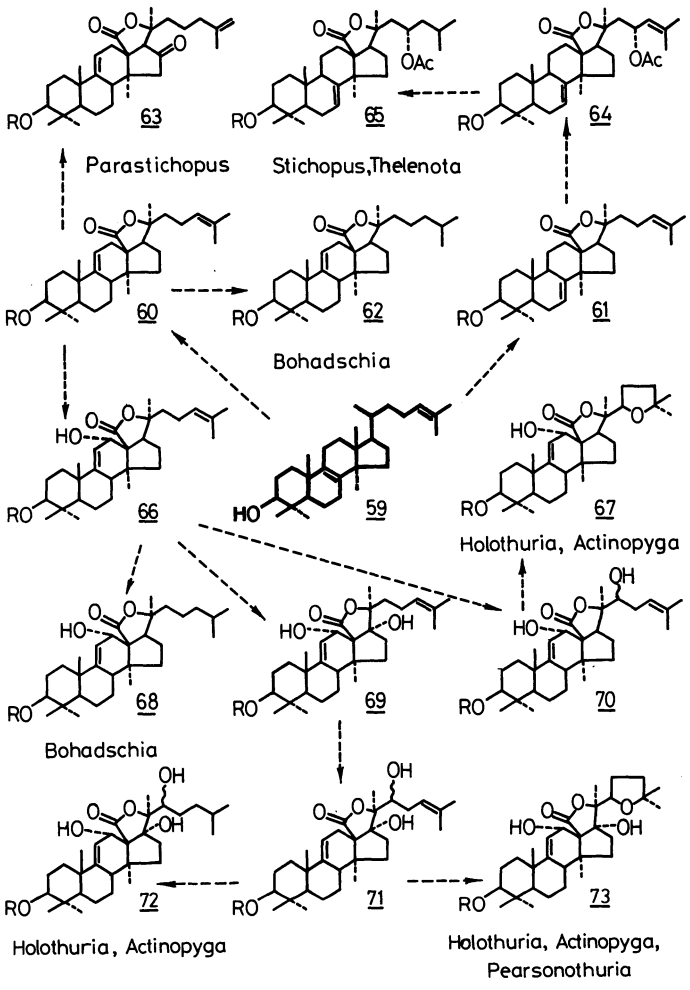
It is interesting that to-date no glycosides with odd numbers of monosaccharides in the carbohydrate moiety have been found in the Order Aspidochirota.



Together with a comparison of glycoside structures this may suggest that the formation of the carbohydrate moieties occurs by successive joining of several bioside blocks to an aglycone [105].

Saponins of *Dendrochirota* spp. have more varied structures. Their biosynthesis utilizes mono- as well as bioside precursors; that is why glycosides with four, five, and six monosaccharide residues are found in animals of this Order. Sea cucumbers of the genera *Cucumaria, Neothyonidium* and *Eupentacta* follow a biosynthetic pathway analogous to that of *Aspidochirota* spp. (Scheme 2). Holotoxinogenin is the natural aglycone for these substances. Glycosides of *C. japonica* contain the $\Delta^{7(8)}$-isomer of holotoxinogenin as the native aglycone, whereas *Eupentacta* spp. provide related saponins of the general formula (**85**). In contrast to the holotoxin triterpenes, glycosides of these species have either an odd number of monosaccharides or a sulfate group in the carbohydrate moiety. Other animals from the Order Dendrochirota (genera *Afrocucumis* and *Duasmadactyla*) biosynthesize aglycones through C-20 and C-16-oxidation, yielding **75**. During biosynthesis of psolusoside B (**54**) the sequence of oxidative reactions is altered when compared with the biosynthesis of the holotoxins. Oxidation at C-18 and C-16 and formation of the 18→16 lactone are followed by including the acetoxy group at C-20, thus resulting in aglycone (**54**).

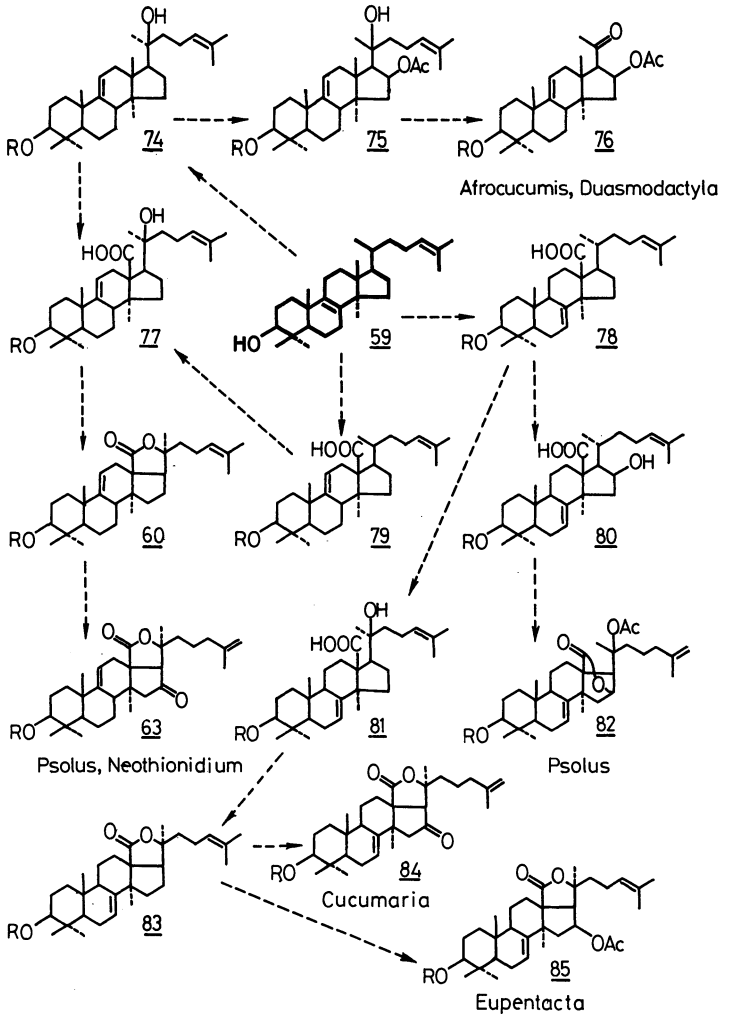
The similarity, and even overlapping, of taxonomic characters in related species are often based on genetic reasons. According to Vavilov's law of homological series [135], "genetically related species and genera are characterized by similar series of hereditary variability – families are characterized by a definite pattern



Scheme 1. Postulated biosynthetic sequence for glycosides of Aspidochirota

of variability, which pervades all genera and species comprising these families". As a result, homological characters can appear in related systematic groups. This is of greater concern to chemical characters than to morphological ones, since chemical structures depend directly on genotype.

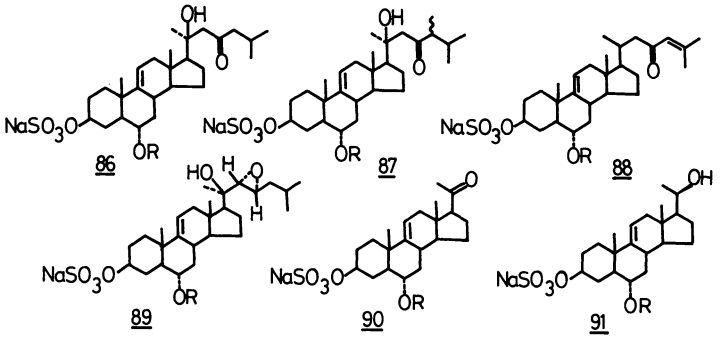
Homology may also be seen in structures of triterpene glycosides from sea cucumbers. For example, oxidation at C-16 occurs during biosynthesis of glycosides from the genus *Parastichopus* (Order Aspidochirota) as well as from the genera *Cucumaria, Eupentacta, Neothyonidium* and *Duasmodactyla* (Order Dendrochirota). Identical carbohydrate moieties were found in glycosides of *Neothyonidium magnum* and *Thelenota ananas*. However, complete overlap of these glycoside structures for distinct taxonomic groups have not been observed to-date. The reason presumably is that phylogenesis of carbohydrate moieties and aglycones of these secondary metabolites occurred independently from each other.



Scheme 2. Postulated biosynthetic sequence for glycosides of Dendrochirota

4.2 Steroid Glycosides

It is known that there are two groups of steroid oligoglycosides and several groups of steroid mono- and biosides in the Asteroidea [95, 136]. Sulfated steroid oligoglycosides derived from sterols usually occur as mixtures that reflect the compexity of the sterols arising through the food chain as well as the presence of varied carbohydrate moieties [136–140]. Sulfated oligoglycosides are characterized by steroid aglycones possessing a $\Delta^{9(11)}$-3β,6α-diol pattern and, as a rule, a 23-oxo function. Carbohydrate moieties are attached to C-6, while the sulfate group is located at C-3. All sugars are in their pyranose forms with β-anomeric configuration. General formulae of these saponins are **86** and **87**. Asterosaponin mixtures also contain components, for example **88, 89**, which may be considered



R=Fuc(1,2)→Fuc(1,4)→Xyl(1,3)→Qui- (a)
Qui(1,2)↑

86a Regularoside B
89a Tenuispinoside B

R=Fuc(1,2)→Gal(1,4)→Xyl(1,3)→Qui- (b)
Qui(1,2)↑

86b Thornasteroside A
24-nor-86b Ophidianoside B
87b Thornasteroside B
89b Tenuispinoside A

R=Fuc(1,2)→Fuc(1,4)→Qui(1,3)→Glc- (c)
Qui(1,2)↑

88c Marthasteroside B
24(25)-dihydro-88c Marthastereoside C

R=Fuc(1,2)→Glc(1,4)→Xyl(1,3)→Qui- (d)
Qui(1,2)↑

86d

R=Fuc(1,2)→Qui(1,4)→Xyl(1,3)→Qui- (e)
Qui(1,2)↑

86e Maculatoside I

R=Fuc(1,2)→Ara(1,4)→Xyl(1,3)→Qui- (f)
Qui(1,2)↑

86f Laevigatoside

R=Fuc(1,3)→Fuc(1,2)→Qui(1,4)→Xyl(1,3)→Qui-(g)
Qui(1,2)↑

88g Marthastereoside A_1
24(25)-dehydro-86g Acanthoglycoside A

R=Fuc(1,3)→Fuc(1,2)→Gal(1,4)→Xyl(1,3)→Qui-(h)
Qui(1,2)↑

86h Versicoside A
88h Marthastereoside A_2
87h Versicoside B

R=Fuc(1,2)→Xyl(1,4)→Xyl(1,3)→Qui- (i)
Qui(1,2)↑

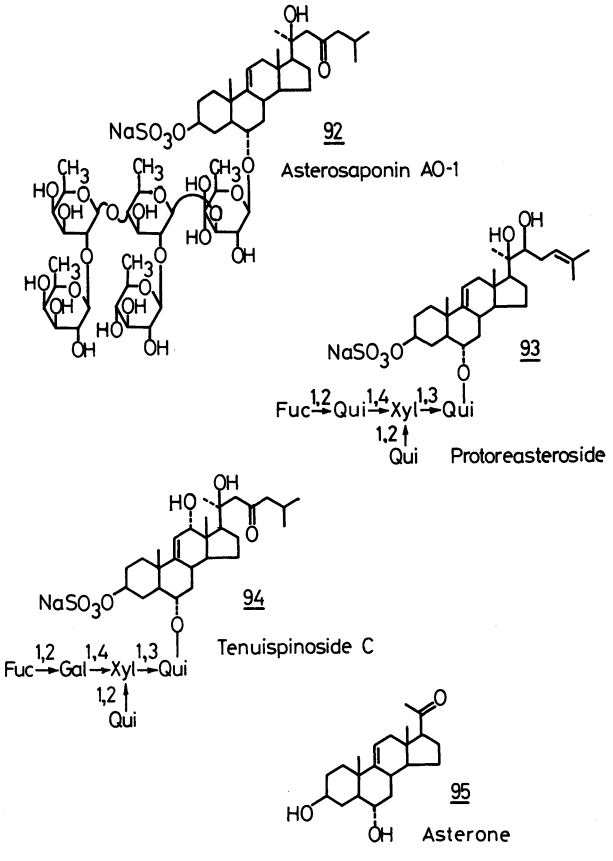
86i Ophidianoside F

R=Fuc(1,2)→Qui(1,4)→Qui(1,3)→Glc- (j)
Qui(1,2)↑

89j Halityloside I
24-Methyl-89j Regularoside A

links of the biosynthetic pathway from sterol precursors to **86** or **87**. Such oligoglycosides predominate sometimes in some species. Other asterosaponins with general formulae **90** and **91** are presumably products of further biotransformation of **86** or **87** in sea stars.

The compounds **86a–c** and **88c, g** are widespread in starfishes. However, some of these animals show a deviation from the norm that is of greatest interest for chemotaxonomy. The Pacific sea star *Asterias amurensis* as well as *A. amurensis versicolor* [141, 142] yielded socalled asterosaponin A0–1 (**92**), whose carbohydrate moiety included the rare monosaccharide 6-deoxyhexosulose. Probably, asterosaponin **92** is derived from **86a** through biooxidation of a quinovose residue. The 6-deoxyhexosulose is a rather delicate molecule, which decomposes under acid hydrolysis conditions. That is why investigators have difficulty in identifying

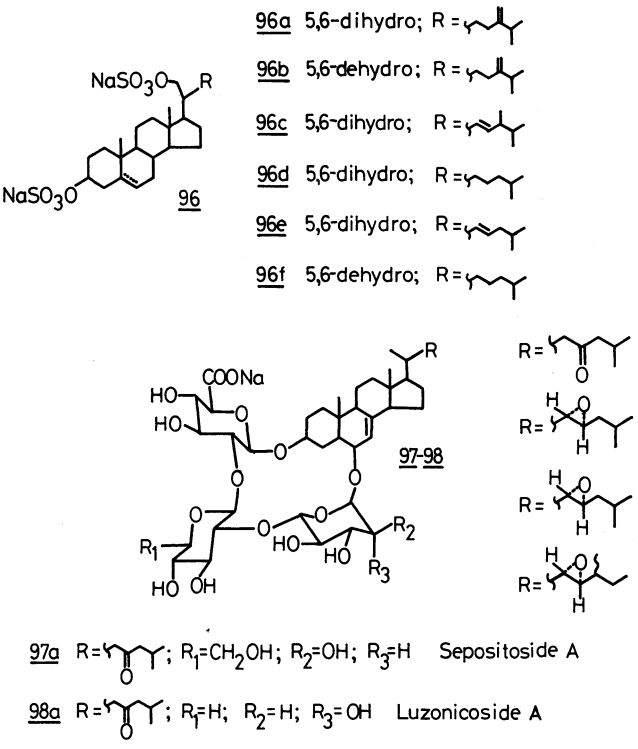


this monosaccharide. At the present time it is unknown whether the distribution of *92* is limited to the genus *Asterias*.

Riccio et al. [143] isolated protoreasteroside (**93**), from *Protoreaster nodosus* and *Pentaceraster alveolatus* which unlike other asterosaponins has C-22 oxygenated. The same researchers identified tenuispinoside C (**94**) as a minor component of the mixture from *Coscinasterias tenuispina* [140]. They reported that **94** contains an unprecedented additional hydroxyl at C-12.

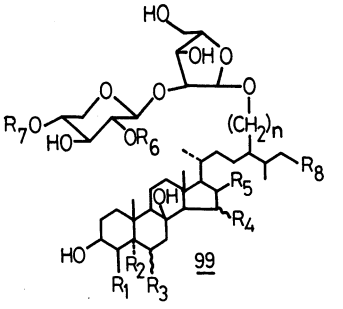
All above-mentioned animals also contain more common asterosaponins. Acid hydrolysis of asterosaponin fractions from the majority of starfishes yields socalled asterone (**95**) as an artificial sapogenin, which is formed by retroaldol cleavage of the side chain in the native aglycones.

On the other hand, some starfishes lack sulfated oligoglycosides. Sometimes derivatives of polyhydroxylated sterols carry out the biological functions of the asterosaponins. Diols (**96**) isolated from the starfish *Euraster insignis* by Italian scientists [144] are examples of this type derivatives. Sulfated oligoglycosides are absent in this echinoderm as well as in animals of the genus *Echinaster*. They provide another group of oligoglycosides with a number of unusual features. These socalled cyclic glycosides (**97, 98**) discovered by Minale et al. [136] are devoid of sulfates. A cyclic trisaccharide in the asterosaponins bridges C-6 and C-3 of a $\Delta^{7(8)}$-steroid aglycone.



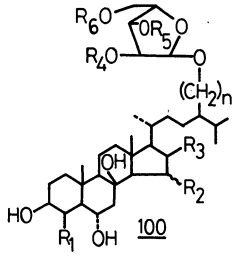
Since members of the Class Asteroidea provide varied structures of oligoglycosides or do not contain these saponins at all, it may be concluded that the secondary metabolites may have some chemotaxonomic significance. In fact, cyclic steroid glycosides have been isolated only from the genus *Echinaster*. On the other hand, such mixtures are less investigated and probably more complicated than corresponding mixtures from sea cucumbers. Six variants of aglycones and three types of oligosaccharide moieties were identified, for example, in ten sulfated saponins of the starfish *Coscinasterias tenuispina* by Riccio et al. [140]. Nevertheless, identical or related predominant compounds were found in animals belonging to different taxa [145–158]. That is why oligoglycosides from starfishes seem to be less informative as chemotaxonomic markers than corresponding saponins of sea cucumbers.

Recent progress of the chemical study of starfishes has revealed a great deal of new information concerning the structure and distribution of steroid monosides and biosides in echinoderms. These series are derived from polyhydroxylated sterols having from five to nine hydroxyl groups. Many species possess such metabolites, which are concentrated predominantly in digestive organs. Usually the animals also contain sulfated asterosaponins. Identification of these polyhydroxylated sterol derivatives provides additional data for steroid biosynthesis and metabolism, which enhance the importance of chemical characters with respect to starfish taxonomy. The new glycoside type shows greater structural variability to-date in comparison with other asterosaponins. The oligoglycoside portions include such sugars as L-arabinose, D-xylose, D-glucose and their methylated or sul-



99

99a n=0; R_1=R_5=R_7=R_8=H; R_2=OH R_3=β-OH; R_4=α-OH; R_6=CH_3 Nodososide
99b n=0; R_1=OH; R_2=R_5=R_8=H; R_3=α-OH; R_4=β-OH; R_6=R_7=CH_3 Culcitoside C_1
99c n=1; R_1=R_5=OH; R_2=R_8=H; R_3=α-OH; R_4=β-OH; R_6=R_7=CH_3 Culcitoside C_2
99d n=1; R_1=R_2=R_8=H; R_3=α-OH; R_4=β-OH; R_5=OH; R_6=R_7=CH_3 Culcitoside C_3
99e n=0; R_1=OH; R_2=R_5=R_7=R_8=H; R_3=β-OH; R_4=α-OAc; R_6=CH_3 Echinosteroside B_1
99f n=0; R_1=OH; R_2=R_5=R_7=R_8=H; R_3=β-OH; R_4=α-OH; R_6=CH_3 Echinosteroside B_2
99g n=0; R_1=R_5=R_7=R_8=H; R_2=OH; R_3=α-OH; R_4=α-OH; R_6=CH_3 6-Epi-nodososide
99h n=0; R_1=R_2=R_5=R_7=H; R_3=α-OH; R_4=β-OH; R_6=CH_3; R_8=H Attenuatoside AI
99i n=0; R_1=OH; R_2=R_5=R_8=H; R_3=β-OH; R_4=β-OH; R_6=R_7=CH_3 Gomophioside A
99j n=2; R_1=OH; R_2=R_5=R_8=H; R_3=α-OH; R_4=β-OH; R_6=R_7=CH_3 Gomophioside B
99k n=0; R_1=OH; R_2=R_5=R_7=R_8=H; R_3=α-OH; R_4=β-OH; R_6=CH_3 Attenuatoside BI

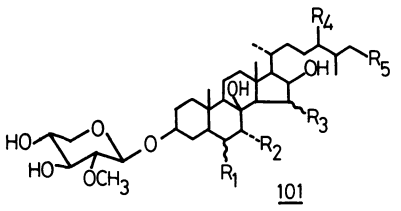


100

100a n=0; R_1=R_3=R_4=H; R_2=α-OH; R_5=CH_3; R_6=SO_3Na Asterosaponin P_1
100b n=2; R_1=R_3=OH; R_2=α-OH; R_4=R_6=H; R_5=CH_3 Asterosaponin P_2
100c n=0; R_1=R_3=R_4=R_6=H; R_2=α-OH; R_5=SO_3Na
100d n=0; R_1=OH; R_2=β-OH; R_3=R_4=R_5=R_6=H Attenuatoside BII
100e n=0; R_1=OH; R_2=α-OH; R_3=R_4=R_5=R_6=H Attenuatoside C

fated derivatives. Aglycones differ from one another by the number, configuration, and placement of hydroxyls, as well as by the presence or absence of a sulfate or acetate group. The carbohydrate moiety may be attached at C-3, C-24 or C-26 of the steroid. These compounds demonstrate, as a rule, only moderate cytotoxic activity and, in contrast to oligoglycosides, they possess other biological functions. Probably some these metabolites participated as emulsifiers in the digestion of food [105]. Riccio et al. [159] discovered this series by the isolation of socalled nodososide (**99a**) from the Pacific starfish *Protoreaster nodosus*. Later we found the asterosaponin P_1 (**100a**) in *Patiria pectinifera* [160].

Glycosides related to nodososide are characterized by disaccharide portions attached to C-24 (or C-28 for ergostane and C-29 for stigmastane derivatives). Such compounds were obtained from extracts of different starfish species. Cul-



101

101a R_1=β-OH; R_2=H; R_3=α-SO_3Na; R_4=CH_3; R_5=OH; Δ^4 Echinasteroside A

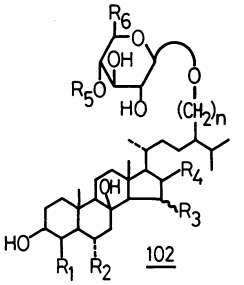
101b R_1=β-OH; R_2=H; R_3=α-SO_3Na; R_4=CH_2CH_2OH; R_5=OH; Δ^4 Echinasteroside B

101c R_1=β-OH; R_2=H; R_3=α-OH; R_4=H; R_5=OH

101d R_1=β-OH; R_2=α-OH; R_3=α-OH; R_4=OH; R_5=OH

101e R_1=β-OH; R_2=H; R_3=α-OH; R_4=CH_2; R_5=OH

101f R_1=α-OH; R_2=OH; R_3=β-OH; R_4=CH_3; R_5=OSO_3Na



102

102a n=1; R_1=R_4=OH; R_2=α-OH; R_3=β-OH; R_5=SO_3Na; R_6=CH_2OH; Δ^{22}

102b n=0; R_1=R_4=R_5=R_6=H; R_2=β-OH; R_3=α-OSO_3Na

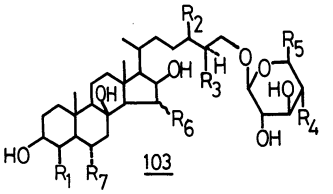
102c n=0; R_1=OH; R_2=α-OH; R_3=β-OH; R_4=OH; R_5=R_6=H

102d n=0; R_1=OH; R_2=α-OH; R_3=β-OH; R_4=R_5=R_6=H;

citosides C_1–C_3 (**99b–d**) were isolated from *Culcita novaeguinea* [161, 162], while echinasterosides B_1 and B_2 (**99e, f**) were found in *Echinaster sepositus* [163]. 6-Epi-nodososide (**99g**) from *Pentaceraster alveolatus* differs from **99a** by the configuration at C-6 only [164]. Attenuatoside AI (**99h**) and gomophiosides A, B (**99i, j**) from *Hacelia attenuata* [165] and *Gomophia watsoni* [166] are other compounds of this type. Nodososide seems to be more abundant than the others. This saponin was also isolated from *Acanthaster planci* and *Linckia laevigata* [167] and related compounds from *Halityle regularis* [168]. The identification of **99a** from representatives of distant genera is an additional example of homological development of chemical characters in echinoderms.

Sulfated monosaccharides have a more limited distribution. Accompanied by **100c**, asterosaponin P_1 (**100a**) was also identified from *Oreaster reticulatus* [169] in addition to *Patiria pectinifera*. Similar saponins, which are devoid of the sulfate group, were found in *Hacelia attenuata* [136, 170].

Several other steroid monosides are known. Compounds of the general formula **101** have 2-*O*-methyl-D-xylose attached at C-3 of the steroid. Compounds **101c–e** were isolated from *Poraster superbus* [171], while corresponding sulfated

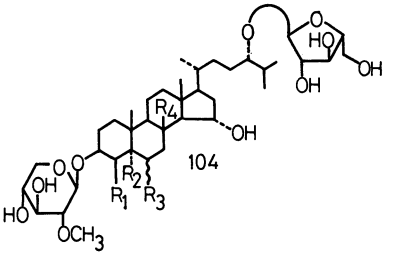


103

103a $R_1=R_3=H$; $R_2=CH_3$; $R_4=OSO_3Na$; $R_5=CH_2OH$; $R_6=\beta$-OH; $R_7=\alpha$-OH

103b $R_1=R_4=OH$; $R_2=R_5=H$; $R_3=CH_3$; $R_6=\beta$-OH; $R_7=\alpha$-OH

103c $R_1=R_5=H$; $R_2=C_2H_5$; $R_3=CH_3$; $R_4=OH$; $R_6=\beta$-OSO_3Na; $R_7=\beta$-OH Attenuatoside SIII



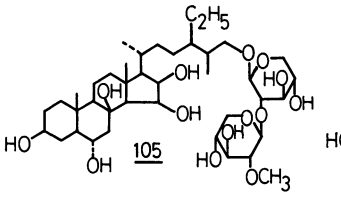
104

104a $R_1=H$; $R_2=R_4=OH$; $R_3=\beta$-OH Isonodososide

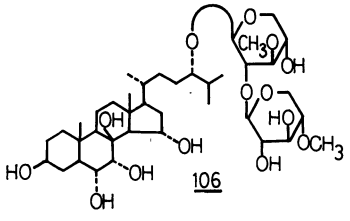
104b $R_1=R_2=H$; $R_3=\beta$-OH; $R_4=OH$ 5-Deoxyisonodososide

104c $R_1=R_4=OH$; $R_2=H$; $R_3=\beta$-OH Granulatoside A

104d $R_1=R_2=R_4=H$; $R_3=\beta$-OH Granulatoside B



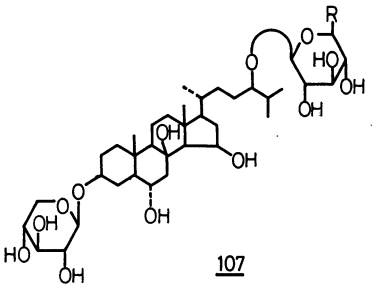
105



106

glycosides **101 a, b** und **f** were provided by *Echinaster saposites* [172] and *Coscinasterias tenuispina* [140], respectively. The later echinoderm is characterized by the most complicated mixture of asterosaponins of all starfishes that have been studied. Monosides **102 a–d, 103 a, b** were isolated from the starfish *C. tenuispina* together with **101 f** and sulfated oligoglycosides [140]. The identification of saponins with aglycones of the stigmastane type, such as **103 c** from *Hacelia attenuata* [173], shows the exogenic origin of the steroid moiety in these compounds. It is well known that starfishes are capable of biosynthesizing *de novo* only C_{27}-sterols [4]. Therefore steroid polyols of asterosaponins are derived from dietary sterols.

Starfishes have also been reported to contain biosides with two monosaccharide residues attached to different positions of their aglycones. It is of interest to note that the starfish *Acanthaster planci* [174] yields not only nodososide (**99 a**), but also isonodososide (**104 a**) and 5-deoxyisonodososide (**104 b**). This means that *A. planci* possesses enzymes for the glycosilation of steroid polyols at both C-3 and C-24. Moreover, glycosidation of hydroxyl groups of the monosaccharide residue also takes place. Consequently, some monosides from extracts of star-

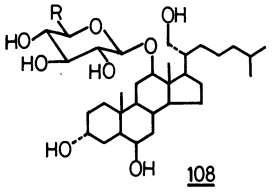


107

107a R = H Distolasterioside D_1

107b R = H; Δ^{22} Distolasterioside D_2

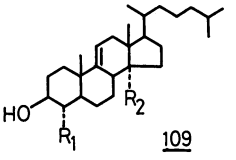
107c R = CH_2OH Distolasterioside D_3



108

108a R = H

108b R = CH_2OH



109

109a R_1 = H; R_2 = CH_3

109b R_1 = R_2 = CH_3

109c R_1 = R_2 = H

fishes may be considered as biosynthetic precursors of corresponding biosides. Methylation and sulfation of sugar hydroxyls probably has significance as a terminating reaction. It stops further biotransformation of steroid precursors.

Granulatosides A and B (**104c, d**) from the starfish *Choriaster granulatus* [175] are structural analogs of isonodososide.

Two uncommon biosides, **105** from *Halityle regularis* [176] and **106** from *Crossaster papposus* [177], include carbohydrate moieties composed of methylated xylose derivatives. In contrast to other related compounds, distolasterosides (**107 a–c**) from the starfish *Distolasterias nipon* [178] contain only non-methylated sugars in their carbohydrate moieties.

From an analysis of the asterosaponin distribution in different starfish species (see Table 4) it may be concluded that no correlation between systematic position of these animals and their glycoside structures has been established to-date. The insufficiency of the chemical data is the main reason for this. Moreover, identical structural types have been encountered in various predatory and detritus eating animals collected from distant areas and at different depths. That is why the similarity and even coincidence of some features of the saponin structure in quite distinct families presumably is not a result of adaptation to similar environmental conditions. It may be explained by the homology of chemical characters in genetically related groups. Homologies complicate the usefulness of chemical compounds for taxonomic purposes, especially in the higher taxa of Order, Class, and Phylum. At the same time, secondary metabolites have useful potential for the

Table 4. Asterosaponins from starfishes by species

Species	Asterosaponins	Refs.
Acanthaster planci	**86b, 87b,** 24(25)-dehydro-**88g, 99a, 104a, b**	[139, 145, 167, 174]
Aphelasterias japonica	**86**[a], **88**[a]	[137]
Archaster angulatus	**86**[a]	[137]
Asterias amurensis	**92**	[141]
A. amurensis versicolor	**86h, 87h, 92**	[142]
A. forbesi	**86**[a]	[95]
A. rubens	**86**[a]	[95]
A. vulgaris	**86**[a]	[95]
Asterina (Patiria) pectinifera	**100a, b**	[169, 179]
Asteropsis corinifera	**86**[a]	[137]
Astropecten latespinus	**86g, d**	[48]
Choriaster granulatus	**104c, d**	[175]
Coscinasterias calamaria	**86**[a], **88**[a]	[157]
C. tenuispina	**89a, b, 94, 101f, 102a–d, 103a, b**	[140]
Crossaster papposus	**106**	[177]
Culcita schideliana	**86**[a]	[95]
C. novaeguinea	**99b–d**	[161, 163]
Diplasterias brucei	**88**[a]	[138]
Distolasterias nipon	**107a–c**	[178]
Echinasterias luzonicus	**98**	[136]
E. sepositus	**97, 98, 99e, f, 101a, b**	[136, 163, 172]
Evasterias retifera	**88**[a]	[137]
E. troschelii	**88**[a]	[95]
Hacelia attenuata	24-nor-**86b, 99h, k, 100c–e, 101d, e, 103c**	[136, 151, 165, 170, 173]
Halityle regularis	**89j,** 24-methyl-**89j, 86a, b, 105**	[151, 168, 176]
Henricia sanguinolenta	**86**[a]	[95]
Hippasteria phrygiana	**86**[a], **88**[a]	[95]
Gomophia watsonii	**99i, j**	[166]
Leptasterias polaris	**86**[a]	[95]
Lethasterias fusca	**86, 88**[a]	[137]
Linckia guildingi	**99b**	[161]
L. laevigata	**86f, 99a**	[155]
L. maculata	**86e**	[154]
Linckia multiflora	–	[95]
Luidia ciliaris	**88**[a]	[138]
Luidia sp.	**86**[a], **88**[a]	[137]
Lysastrosoma anthosticta	**86**[a], **88**[a]	[137]
Marthasterias glacialis	**88c, g, h,** 24(25)-dihydro-**88c**	[155]
Ophidiaster hemprichi	**86**[a], **88**[a]	[137]
O. ophidianus	**86i,** 24-nor-**86b**	[151]
Oreaster reticulatus	**100a, c**	[169]
Pentaceraster alveolatus	**93, 99g**	[143, 164]
P. regularis	–	[137]
Porania pulvillus	**88**[a]	[138]
Poraster superbus	**101c–e**	[171]
Protoreaster lincki	**86**[a]	[95]
P. nodosus	**93, 99a**	[143, 159]
Solaster endeca	**86**[a]	[95]

[a] Glygoside type was determined only from hydrolysis products.

sub-family, genus, or species levels since related species, for example *Asterias amurensis* and *A. amurensis versicolor*, seem to contain characteristic sets of asterosaponins.

In considering the results of the research in steroid glycosides, the apparent phylogenetic parallelism between steroid metabolisms in Asteroidea and Ophiuroidea must be discussed. Longicaudosides A and B (**108 a, b**) were recently isolated by Riccio et al. [180] from the brittlestar *Ophioderma longicaudum*. These saponins are composed of a steroidal polyol and a monosaccharide residue, similar to some asterosaponins. There are points of similarity between **108** and steroid diols of the general formula (**96**) that were isolated from the starfish *Euretaster insignis*. Both secondary metabolite series, from starfishes and from ophiuroids, are oxygenated at C-3 and C-21 of the aglycones and contain sulfate groups.

5 Sterols and Their Derivatives

Glycosides from echinoderms, especially those of starfishes and brittlestars, are biosynthetically connected through their free sterols. Several important works and a general review of sterols from the phylum Echinodermata have been published [181–183]. The sterols of sea cucumbers and starfishes are Δ^7-compounds and stanols, whereas sea lilies, sea urchins and ophiuroids provide predominantly Δ^5-unsaturated sterols. Thus, the first two classes differ from the others in this respect. Sea cucumbers are also remarkable because their sterol content is lower than that of other echinoderms. The presence of Δ^7-sterols in both sea cucumbers and starfishes was regarded as one of a few arguments for establishing a phylogenetic relationship between these animals.

Some recent reults raise doubt about this relationship. Starfishes and sea cucumbers contain appreciable quantities of cytotoxic oligoglycosides. The physiological activity of these metabolites is dependent on their ability to form complexes with membrane sterols. The presence of the cytotoxic agents in echinoderms and also in some plants is connected to biochemical alteration of sterol composition [105]. Both glycoside-containing echinoderms and some saponins in higher plants contain Δ^7-sterols instead of cholesterol or phytosterols of the Δ^5-series. Δ^7-Sterols form complexes with cytotoxic saponins of echinoderms with greater difficulty than with cholesterol. Mackie et al. [184] demonstrated that 5α-cholest-7-en-3β-ol inhibits the hemolytic action of asterosaponins to a greater extent than cholesterol. Analogous observations have been made by Anisimov et al. [185] on the cytotoxic action of holothurins. It may be concluded that the absence of sterols is sensitive to the presence of glycoside as Δ^5-derivatives as well as to the low concentration of free sterols in biomembranes of sea cucumbers and results from the adaptation of the echinoderms to their own cytotoxins.

Italian scientists [136] confirmed the correlation between oligoglycoside content and replacement of cholesterol by other sterols. They found that the starfish *Euretaster insignis*, which lacks the sulfated oligoglycosides, has only a very small content of Δ^7-sterols in comparison with other starfishes.

Some sea cucumbers replace membrane cholesterol for 14α-methylsterols of the $\Delta^{9(11)}$-series. Analogous steroids have been also found in some higher plants. The main component from the sterol mixture of the sea cucumber *Cucumaria japonica* [186] was identified as 14α-methyl-5α-cholest-9(11)-en-3β-ol (**109a**). It was accompanied by **109b** in the sea cucumber *Eupentacta fraudatrix*. The latter substance was also isolated by a Canadian group [187] from the sea cucumber *Cucumaria frondosa*. Sterols **109a, b** were identified as the main components of corresponding fractions from *Psolus fabricii* and *P. phantapus* [188, 189].

According to Goad, ApSimon and collaborators [188, 189], these natural products are biosynthesized *de novo*, whereas minor stanols and Δ^7-sterols are formed through biotransformation of dietary constituents. It was postulated that during biosynthesis squalane yields lanost-9(11)-en-3β-ol (**109c**), which is transformed into both triterpene glycosides and sterols (**109a, b**). Actually the glycosides from *P. fabricii* have the 9(11)-double bond similar to **109a, b**. However glycosides belonging to *Cucumaria* and *Eupentacta* spp. are 7(8)-unsaturated. Thus, glycosides and main sterols in these groups of echinoderms seem to be derived from different precursors.

Among other sea cucumbers, only representatives of the Dendrochirota order elaborate $\Delta^{9(11)}$-sterols. This means that these steroids may be used as markers for the chemotaxonomic study on the Order.

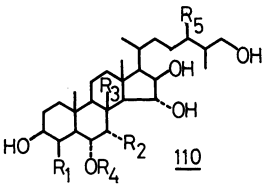
Free sterols are accompanied by steryl sulfates in all five classes of the phylum Echinodermata [95, 183, 190–192]. C_{27}-Components predominate among these sulfates, even when C_{28}- and C_{29}-compounds are present as the major free sterols of the animals. High concentration of steryl sulfates is a characteristic feature of echinoderm chemical composition.

The distribution of free and conjugated sterols in echinoderms was recently reviewed [183]. With the exception of $\Delta^{9(11)}$-derivatives, these secondary metabolites seem to be unimportant for chemotaxonomy.

Varied polyhydroxylated sterols were recently discovered in starfishes and brittlestars. Such derivatives are biosynthetically connected with glycosides of steroid polyols isolated from the echinoderms. At first, compounds **110a–c** were identified by Riccio et al. [193] in the starfish *Protoreaster nodosus* and by us [194] in the starfish *Patiria pectinifera*. The related pentol **110d** was found in *Hacelia attenuata* [195]. It also proved to be the aglycone of nodososide (**99a**) from several starfish species. Octol **110e** from *Patiria pectinifera* [196] and *Oreaster reticulatus* [169] has a sulfate group like some other polyols. Both *P. pectinifera* and *O. reticulatus* show a resemblance of their steroid metabolites that is of interest from a chemotaxonomic point of view. Sulfated polyhydroxylated steroid **110f** form *Poraster superbus* [171] and minor polyols **100g–i** from *Protoreaster nodosus* [197] belong to the same structural series.

Steroids with the general formula **111** differ from **110** in the configuration at C-6 or C-15. This series is composed of **111a–c** from *Luidia maculata* [198], **111d** from *Halityle regularis* [168] and **111f** from *Gomophia watsoni* [166].

New sterol derivatives with the unusual feature of 8β,14α,15α-hydroxylation were isolated by D'Auria et al. [199] from the starfish *Archaster typicus*. Nanaols **112** constitute the most hydroxylated steroids isolated from natural sources.



110

110a $R_1=R_2=R_4=R_5=H$; $R_3=OH$

110b $R_1=R_4=R_5=H$; $R_2=R_3=OH$

110c $R_1=R_2=R_3=OH$; $R_4=R_5=H$

110d $R_1=R_2=R_3=R_4=R_5=H$

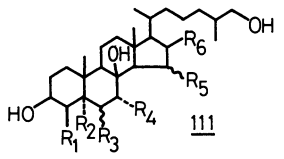
110e $R_1=R_2=R_3=OH$; $R_4=SO_3Na$; $R_5=H$

110f $R_1=R_2=R_4=H$; $R_3=OH$; $R_5=CH_2OSO_3Na$

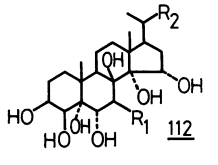
110g $R_1=R_3=OH$; $R_2=R_4=H$; $R_5=CH_3$

110h $R_1=R_2=R_4=H$; $R_3=OH$; $R_5=CH_3$

110i $R_1=OH$; $R_2=R_4=H$; $R_3=OH$; $R_5=CH_3$; Δ^{22}



111



112

111a $R_1=R_4=H$; $R_2=R_6=OH$; $R_3=\beta$-OH; $R_5=\alpha$-OH

111b $R_1=R_2=H$; $R_3=\beta$-OH; $R_4=R_6=OH$; $R_5=\alpha$-OH

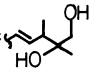
111c $R_1=H$; $R_2=R_4=R_6=OH$; $R_3=\beta$-OH; $R_5=\alpha$-OH

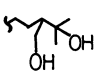
111d $R_1=R_2=R_4=R_6=OH$; $R_3=\alpha$-OH; $R_5=\beta$-OH

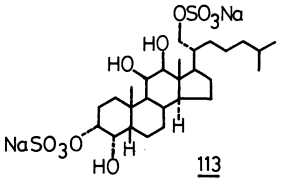
111e $R_1=R_6=OH$; $R_2=R_4=H$; $R_3=\alpha$-OH; $R_5=\beta$-OH

111f $R_1=OH$; $R_2=R_4=R_6=H$; $R_3=\alpha$-OH; $R_5=\beta$-OH

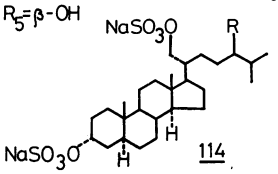
112a $R_1=OH$; $R_2=$ 

112b $R_1=H$; $R_2=$ 

112c $R_1=H$ $R_2=$ 



113



114

114a R=H

114b R=H, Δ^5

114c R=H, $\Delta^{5,22}$

114d R=CH_3, $\Delta^{5,24(28)}$

First results of the study of steroid polyols demonstrate a wide variety of these secondary metabolites exist in starfishes. There are very good prospects to discover new steroids of this type and establish whether a correlation exists between their structures and the systematical positions of the corresponding echinoderms.

The sterol derivatives **113, 114** from the ophiuroid *Ophioderma longicaudum* [200] are also steroid polyols. These polar metabolites contain two sulfate groups and are characterized by oxidation at C-21. In addition, we have isolated the 3-*O*-sulfate of cholest-5-en-3α,4β,21-triol from the ophiuroid *Ophiura sarsi*. Hydroxylation at C-21 in steroids from starfishes and ophiuroids may be of phylogenetic significance.

6 Some Conclusions

During the past decade a great deal of new information on structures of secondary metabolites from echinoderms has become available. A comparative analysis of the distribution of sterols and their derivatives, of glycosides, of polyketides and of some lipids reveals that Echinodermata represents a rich source of rare natural compounds. Gangliosides, polyketides and a high concentration of steryl sulfates have been found in all classes of the Phylum. Echinoderms also possess characteristic phospholipid composition. This confirms the monophyletic origin of these animals.

At present we do not have enough chemical and other data to establish unequivocally the phylogenetic relation among the classes of the phylum Echinodermata. To-date, phylogeny of echinoderms has more difficulties and problems than achievements with chemical evidence. In fact, the presence of long chain fatty acids and anthraquinoids relates Crinoidea and Asteroidea. According to Balker [201], only Ophiuroidea and Echinoidea lack batyl alcohol, and both groups contain gangliosides with oligosaccharide moieties composed of glucose and neuraminic acid. Steroid polyols and their glycosides have been isolated only from Ophiuroidea and Asteroidea. In contrast to other classes, Holothuriidae and Asteroidea provide oligoglycosides. The complex and contradictory picture of the distribution of secondary metabolites in echinoderm classes is connected with the previously mentioned phylogenetic parallelisms, which also indicates a common ancestry of all echinoderms.

The opinion that chemical evidence tends to favor a phylogenetic relationship of sea cucumbers and starfishes [95] seems to be convincing. We sometimes exaggerate a superficial resemblance and do not take essential distinctions into account. The relationship of Holothurioidea and Asteroidea is usually based upon the presence of oligoglycosides and Δ^7-sterols as well as some similarity of their larval types. However oligoglycoside groups from these classes differ considerably from each other, both with respect to structure and biosynthesis. The presence of Δ^7-sterols is a result of biochemical convergence, i.e. an adaptation to the action of inherent cytotoxins. We are of the opinion that the biochemical resemblance of starfishes and ophiuroids (and probably ophiuroids and sea urchins) is more evident than that of sea cucumbers and starfishes.

The problems of chemotaxonomy of echinoderms essentially fall into two categories, selection of chemical characters and limitations of their utilization. From all the above-mentioned secondary metabolites, only triterpene glycosides proved to have significance as chemotaxonomic markers. These compounds have been used to confirm the systematics of sea cucumbers of the Order Aspidochirota. Gangliosides and steroid glycosides are potential chemical characters of starfishes, as are $\Delta^{9(11)}$-sterols of sea cucumbers. A chemotaxonomic meaningful distribution of quinoid pigments seems to take place in some sea urchin genera.

It is a hard task to determine the field of application of a chemotaxonomic character. Identical or similar chemotaxonomic markers may be very usefull for the improvement of systematics within one taxon but useless for other related taxa. Moreover, a difference exists in taxonomic significance of diverse invertebrate groups having formally the same systematic rank.

Homologies of the structure of secondary metabolites from echinoderms make the chemotaxonomic utilization of these compounds difficult, especially on Phylum and Class levels. Phylogenetic parallelisms for classes and the action of Vavilov's law of homological series for related families and genera yield overlapping and even coincidence of structures of naphthoquinoid pigments (distant sea urchin taxa), steroid glycosides (starfishes) and gangliosides (sea urchins and starfishes). However, these secondary metabolites may be utilized, while triterpene glycosides have already been applied as chemotaxonomic markers of species, genera and, in some cases, subfamilies.

The intensive growth of the chemical information dealing with echinoderms opens up new possibilities for the joint work of taxonomists and chemists to gain new insight into the systematics of echinoderms.

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The Chemical Ecology of Alcyonarian Corals

Coelenterata: Octocorallia

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Contents

Abstract

Evolutionary success in Indo-Pacific octocorals is partially attributable to the occurrence of toxic secondary metabolites, particularly terpenes, in their tissues which function as anti-predator and anti-competitor adaptations, and in reproduction. ~50% of Great Barrier Reef alcyonaceans are ichthyotoxic, toxicity varying widely between species. Feeding deterrence characteristics (olfaction and palatability) also vary widely and are not necessarily correlated with ichthyotoxicity. Toxicity is negatively correlated with anti-predatory morphological adaptations, but only at a high level of taxonomic resolution and in traits clearly related to predator defense. Some nudi-

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branchs and other molluscs are adapted to feed on toxic soft corals, chemically altering toxins within their digestive tract; other nudibranchs are immune to toxins and store them in cerata which can be autotomized to discourage predators.

Some toxins function as species-specific allelopathic agents, allowing colonies to effectively compete for space, causing tissue necrosis either by direct contact or transmission through the water column. Effects also include stunting of growth in scleractinians. Some soft corals secrete a polysaccharide layer, protecting them from scleractinian tentacles and permitting adherence to and movement across living scleractinian tissue, while others avoid and grow away from each other, maintaining an individual distance. Still others exhibit increased toxin production prior to egg release, both in the parent tissue and the eggs.

Future research directions include a) mechanisms by which toxins disrupt physiological processes in fish; b) identification of feeding deterrents and determination of synergistic interactions with other compounds; c) physiology/biochemistry of detoxification processes in specialized predators; d) histological responses of scleractinian tissue to known cytotoxins, including sites and mechanisms of action; e) mediation of allelopathy by the environment; and f) effects of *Acanthaster* predation on octocoral population dynamics after removal of scleractinian competitors.

1 Introduction

The field of chemical ecology has its origins in terrestrial systems. Much early research was undertaken to increase agricultural yield and therefore focused on plant-herbivore interactions. The field expanded to cover chemically mediated interactions between plants and pathogens, epiphytes, pollinators, as well as between other plants competing for space [1, 2]. Other areas of interest to chemical ecologists include chemically mediated interactions between animals, such as aggregation, communication, predator defense, competition, feeding, etc. [3].

With increasing interest in marine organisms, chemical ecologists looked to the sea to ascertain whether similar processes of chemical mediation occurred there. Marine chemical ecology is now expanding rapidly, building on the field of marine natural products chemistry. Recent significant advances in this field have generally involved interdisciplinary collaborative research by scientists in the fields of marine ecology and marine natural products chemistry.

The most recent review in marine chemical ecology is that published by Bakus [4] which presents a general overview of the chemical ecology of marine organisms. It is encyclopedic and, of necessity, brief in its treatment of any single topic. Earlier general reviews include those of Kittredge [5], Barbier [6], Scheuer [7] and Naylor [8]. This review will cover the chemical ecology of the Alcyonaria – the soft corals – concentrating on the families Alcyonacea and Gorgonacea but also drawing on examples from other families (Stolonifera, Telestacea, Coenothecalia, and Pennatulacea) where information is available. We will review the chemical aspects of defense against predation, competition for space, communication, reproduction, and chemotaxonomy. We will also highlight areas of study which appear to be most promising for further experimental investigation. Extensive coverage of the chemical literature may be found in Scheuer's [9, 10], Baker and Murphy's [11], and Faulkner's [12–14] reviews. The reader is referred to these for details on molecular structure.

2 Alcyonarians as Important Benthic Invertebrates

Surveys of marine communities on hard-bottom have revealed that alcyonarians account for a substantial proportion of living biomass and cover, particularly on coral reefs. In many parts of the Indo-Pacific [15], including the Red Sea [16, 17], a wide variety of alcyonacean soft corals can dominate the benthic community, sometimes surpassing the scleractinians (hard corals) in percent cover. In the Caribbean, gorgonaceans and pennatulaceans are more prominent while alcyonaceans are depauperate in both diversity and abundance [18]. Each group contains numerous representatives which are abundant but appear to possess no obvious physical defense against predators. Furthermore, despite their value as a potential food source, field surveys reveal that the frequency of predation on Great Barrier Reef soft corals is negligible.

The Alcyonaria or Octocorallia are an order within the Anthozoa along with such groups as the Scleractinia. A summary of their taxonomic position within the phylum Coelenterata (Cnidaria) is shown in Table 1. Further details on the taxonomy and suggested phylogeny of the group may be found in Moore [19], Bayer [18], and Verseveldt [20].

Alcyonarians are benthic, sessile, colonial organisms often composed of thousands of individual polyps, each genetically identical to the other, with some polyp specialization in certain species. The colonies often possess zooxanthellae. These endosymbionts, via their photosynthetic activity, are believed to provide oxygen and nutrients to the host coelenterate colonies [21] such as these. Soft corals are, however, heterotrophic, being suspension feeders or active planktivores [22]. They are also capable of utilizing dissolved organic nutrients from seawater [23].

Form can vary widely within the Alcyonaria and also within families (e.g. the Alcyonacea). In fact, a single species can possess ectomorphs ranging from en-

Table 1. A summary of major taxonomic relationships within the Coelenterata, with emphasis on orders within the classes Alcyonaria (Octocorallia) and Zoantharia [19]

Phylum	Subphylum	Class	Order
Coelenterata (Cnidaria)	Medusozoa	Hydrozoa	
		Scyphozoa	
		Cubozoa	
	Anthozoa	Ceriantipatharia	
		Alcyonaria (Octocorallia)	Alcyonacea
			Gorgonacea
			Stolonifera
			Telestacea
			Coenothecalea
			Pennatulacea
		Zoantharia (Hexacorallia)	Scleractinia
			Actiniaria
			Corallimorpharia
			Zoanthiniaria (Zoanthidae)

crusting to erect, depending on local environmental conditions [24]. An account of both general colony and polyp morphology may be found in cited Ref. [22]. More detailed descriptions may be found in Bayer [25].

Alcyonarian corals are an extraordinarily rich and diverse source of novel secondary metabolites. An excellent review of the literature covering the Coelenterata until approximately 1978 may be found in Tursch [26]. This review has been updated by Faulkner [13, 14]. The major classes of compounds identified from the Alcyonaria include steroids, alkaloids, and lipids, some of which have been found to have promising pharmacological value [27]. The majority of compounds, however have fallen into the category of terpenoids [9], known for their toxicity [e.g. 28]. These have mostly been sesqui- or diterpenes, many of which are bioactive. These classes of compounds afford the characteristics of odor, taste, toxicity, allelopathy and chemical communication in such terrestrial plants as pine trees, eucalyptus, sagebrush, and white oak trees [29, 30]. Although macromolecules also play important ecological roles in marine organisms [31], the importance of these compounds as a selectively advantageous class of heritable characters [32] should not be underestimated. In fact, as will become evident in the Alcyonaceae, certain secondary compounds may play multiple ecological roles, as has been observed in the terrestrial environment [e.g. 33].

This review will illustrate that secondary metabolites enhance survival capabilities within the Alcyonaria. Specifically, we will offer evidence of their roles in defense against predation, competition for space, and strategies to help ensure success of reproduction.

3 Defense Against Predation

Predator-prey defenses come in many forms. In relation to the Alcyonaria, we will be discussing a) the distribution of toxicity within the order, b) the relationship of toxicity to feeding deterrence via both olfaction and palatability, and c) the relationship of toxicity to morphological defense against predators. Behavioral responses to predators will also be discussed briefly.

Chemical defense in soft corals is a particularly important aspect of this review. Detailed parallel studies have also been carried out, however, on a variety of other taxa, and articles reviewing a substantial portion of the literature have appeared covering chemical defense attributes of sponges [34, 35], holothurians [36, 37], ascidians [38, 39], algae [40–48], and nudibranches [49–52].

3.1 Distribution of Toxicity Within the Alcyonaria

Predation on coral reefs is intense, particularly in the Indo-Pacific [53], where marine organisms generally possess a higher frequency and higher levels of antipredator adaptations than in the Caribbean [54–60]. Predators of soft corals are usually specialized and include fish [61], molluscs [62], echinoderms [63], and

crustaceans [64]. Most alcyonarians are soft in texture and potentially susceptible to predation. Chemical analysis indicates that in comparison to the Scleractinia, the group is a potentially rich nutritional source of protein, fat, and carbohydrate for marine predators [65]. Also, they are sessile, lacking the ability to flee predators. We have noted that the incidence of predation on this group is low – on the order of 1%–2% – a level consistent with that reported by Tursch [66]. By contrast, scleractinian (or hard) corals constitute an important food source for some groups of common reef fish (e.g. scarids and chaetodonts [61, 67, 68]), asteroid echinoderms (e.g. *Acanthaster* spp. – crown-of-thorns starfish [63, 69]), molluscs [70], crustaceans (e.g. pagurids [71]), and annelids (e.g. polychaetes – fire-worm [72]). Soft corals must therefore possess defensive attributes lacking in scleractinian corals. Chemical analyses of scleractinian corals have not revealed any significant levels of secondary compounds [26], while analyses of soft corals show them to be a rich source of such bioactive molecules [12–14, 26, 73].

While the standard bioassay for toxicity of pure compounds is the LD-50 test [74], high correlates have been obtained between the results of these tests and those of ichthyotoxicity tests on aqueous extracts of marine organisms using common freshwater fish, e.g., *Carassius auratus* (common goldfish) [75] or *Gambusia affinis* Baird and Girard (the mosquito fish) [76], as test organisms. These ichthyotoxicity tests, initiated by Yamanouchi [77] have been further developed by Bakus [36, 75], and Coll [78]. It is particularly effective for rapid field-based assessment of toxicity in marine organisms. They have already been used to assess toxic characteristics in such organisms as sponges [75], holothurians [36], and alcyonarians [78–80]. It should be noted that such aqueous extracts of most marine organisms contain substantial quantities of lipophilic toxins [78]. For a general survey, the facile *Gambusia* bioassay thus permits reliable screening of numerous specimens to confirm the presence of either water-soluble or lipophilic toxins. Subsequent purification of the principal components of a toxic extract and LD-50 measurements can then be used to identify more specifically the chemical class, structure, and toxicity of the active compounds [81].

A positive result from such tests does not necessarily relay information on the ecological role of the compound. The key to understanding the function of a compound is observation of the organism and its interactions with other organisms (including with conspecifics) in its natural habitat. The dilemma here is that if an organism is highly toxic to potential predators, then the probability is high that active predation may not be observed. Many predators will have either learned to avoid the prey [82] or will possess adaptions by which they inherently recognize the prey as being toxic [83]. Secondly, any high occurrence of predation on a known toxic species, particularly if by members of a single species, is usually indicative of a predator in which an immunity to the toxin has coevolved [3]. We will now examine the distribution of toxicity in the Alcyonacea, where a large quantitative database is available, both between and within genera.

With respect to the Alcyonacea, laboratory tests have been performed on *Gambusia affinis* using aqueous extracts of numerous soft corals collected over the full extent of the Great Barrier Reef. The results of tests derived from specimens collected from different geographic regions (northern, central, and southern Great Barrier Reef) have shown that a consistent level of approximately 50% of

Table 2. A summary of the distribution of ichthyotoxicity (using the *Gambusia affinis* bioassay) across species of Alcyonacean soft corals. (Numbers in table represent toxic and non-toxic specimens (≧ number of species; see original Refs. [78–80, 84] for details)

Soft coral species	Specimens	
	Toxic[a]	Non-toxic[b]
Alcyonacea		
Alcyoniidae		
Lobophytum spp	9	1
Sarcophyton spp	13	2
Cladiella spp	8	1
Sinularia spp	28	15
Parerythropodium spp	2	1
Alcyonium spp	0	1
Nephtheiidae		
Lemnalia spp	9	1
Paralemnalia spp	4	2
Nephthea spp	12	7
Dendronephthya spp	4	11
Capnella spp	0	9
Xeniidae		
Xenia spp	6	1
Cespitularia spp	3	4
Efflatounaria spp	4	6
Anthelia spp	1	1
Controls	4	27

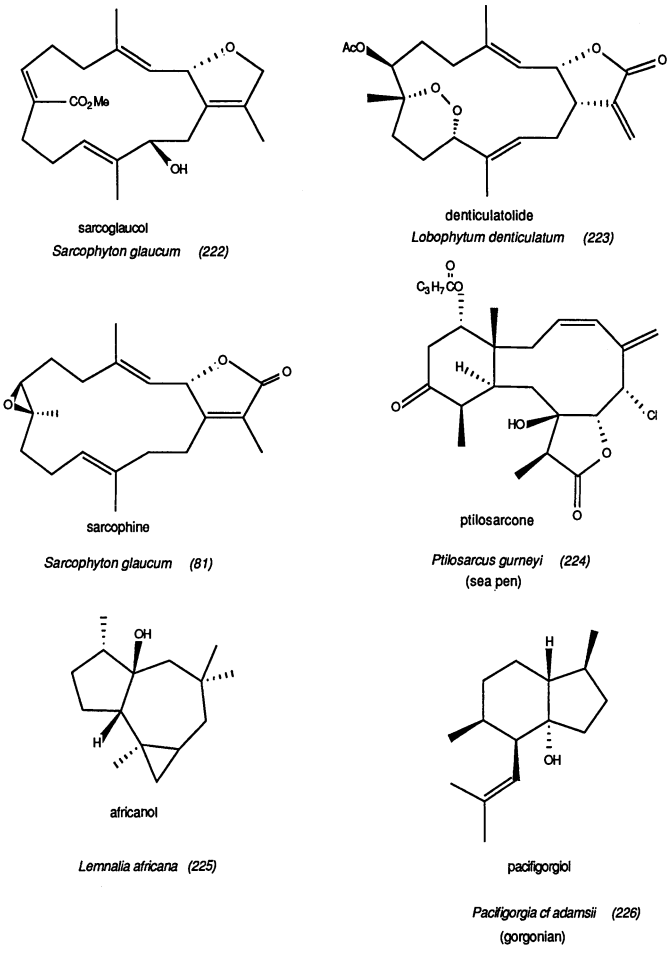
[a] *Toxic:* $1 \leqq n \leqq 6$ fish died in 12 hours of treatment with aqueous soft coral extracts.

[b] *Non-toxic:* No fish died in 12 hours of treatment with aqueous soft coral extracts.

the soft coral extracts exhibit ichthyotoxic characteristics [78–80, 84], a level somewhat lower than that found by Bakus [34]. In addition, the level of toxicity between families varies widely, with specimens ranging from lethal to harmless. This range of variability also applies at the intrageneric level within each family (Table 2). Sufficient data have now been accumulated to support the hypothesis that terpenoid compounds are most likely responsible for the observed toxicity.

3.2 Behavioral and Physiological Responses to Toxins

In the ichthyotoxicity tests, test fish exhibited different symptoms of abnormal behavior and physiological stress. The behavioral responses to immersion in dilute aqueous soft coral extracts include the following: 1) change in the depth of location within the test aquarium; 2) lack of orientation, including lateral and/or vertical rolling of fish; 3) level of general movement and fin activity, which may be depressed or stimulated; and 4) response to visual stimuli such as sudden shad-



Selected alcyonarian derived ichthyotoxins

ing. Any of these responses can be indicative of the site of action of a toxic compound. For example, loss of lateral stability as evidenced by disorientation may indicate an effect on balance receptors, neural transmitters, the central nervous system, or a certain section of the brain [85]. Hyper- or hypoactivity and responses to visual stimuli may indicate central nervous system effects. Direct effects included hemorrhaging from the gill rakers in dead test fish, an indication of the action of a potent vasodilator causing rupture of capillaries, or a surfactant, changing the permeability of respiratory membranes. This symptom has also been documented to occur in *Carassius auratus* [4]. Soft coral toxins have been tested as a shark repellant for this reason, but no conclusive results on their effectiveness have been reported to date [86]. The potential for detailed studies on the physiological effects of these compounds warrants further investigation.

No similar comprehensive ichthyotoxicity surveys of the other orders have been carried out to date, and thus a direct comparison of relative toxicity levels between them is not possible at this stage. Within the Alcyonaria, the Gorgonacea and Pennatulacea appear to have received the greatest attention [12–14].

Some laboratory studies have demonstrated direct toxic effects of cembranolides derived from *Eunicia mammosa, E. succinea,* and *Pseudoplexaura porosa* on zooplankton. Eunicin causes loss of velar cilia and mortality in the nudibranch *Phestilla sibogae* Bergh [87, 88]. The compounds also caused mortality in the predatory amphipod *Parhyale hawaiensis* Dana.

Variability in the frequency and levels of toxicity in alcyonaceans and perhaps other groups of alcyonarians raises the question of why predation is so infrequently observed in this group in the field. Hypotheses of alternate anti-predator defenses must be invoked to explain this. We believe that a type of diffuse Batesian mimicry may be operating here [29]. It is well known that predators learn to discriminate between toxic and non-toxic prey on the basis of visual cues [82]. It has also been demonstrated that mimicry, whether Batesian or Mullerian, can act as an effective refuge from a predator for non-toxic prey. In the terrestrial environment, it has been demonstrated that protection derived from Batesian mimicry of a toxic species can be extended to several other morphologically similar species lacking toxicity [29, 82]. It is possible that the wide variability of ichthyotoxicity among alcyonaceans and the overall similarity in form in many instances in this group, both at the whole organism and individual polyp level, may distribute the advantage of limited toxicity over a wide range of species. Thus, we use the term Diffuse Batesian Mimicry [80].

3.3 Feeding Deterrence

3.3.1 General Characteristics

Feeding deterrence is manifested in several ways by potential prey and can act at different stages of predator-prey contact [89]. Adaptations involving secondary compounds may act at 1) the initial encounter stage through olfaction; 2) the initial contact stage through palatability; 3) the ingestion stage through an emetic response, physiological stress, or mortality; or 4) any combination of these.

Gambusia affinis has also proven to be an appropriate test organism in feeding deterrence studies. It is easily trained to feed under experimental conditions in laboratory aquaria, is discriminating in its feeding behavior, and is sensitive to feeding deterrents. It is because of these qualities that both chemists [90] and vertebrate physiologists [91–94] have used it for examining the molecular basis of chemoreception in teleost fish. Freshwater fish such as this will most likely have had no exposure to or contact with soft corals in recent evolutionary time and would not possess coevolved adaptations of resistance to soft coral toxins or tolerance to associated feeding deterrents.

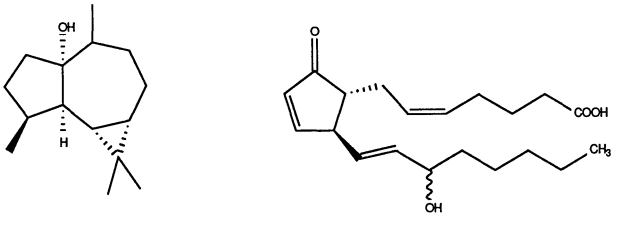
3.3.2 Olfaction and Palatability

In marine systems, chemical cues can play an important role in noncontact communication between organisms. Whittaker and Feeny [3] categorize these allelochemical communicative substances as 1) kairomones – those allelochemicals of

adaptive advantage to the receiver, and 2) allomones – chemicals of adaptive advantage to the producer. An example of kairomones is the substance released by phytoplankton detectable by copepods, allowing the latter to respond to a chemical concentration gradient by altering its directional movements and capture its food [95, 96]. An example of an allomone may be found in the nudibranch *Cadlina luteomarginata* which, upon disturbance, releases chemicals into the water acquired from sponges upon which it has fed [52]. These substances serve to deter potential predators. Here, we will primarily discuss allomones.

Feeding deterrence characteristics in the Alcyonaria have been studied to a fair degree. Characteristics of feeding deterrence have in particular received attention more recently in the Alcyonacea. The earliest work in soft corals was carried out by Tursch et al. [c.f., 26], in which feeding deterrent properties were associated with the sesquiterpene palustrol. Some terpenoid compounds are known to have feeding deterrent properties in various taxa [83, 97–100]. In a wider ranging, preliminary survey of alcyonacean soft corals for feeding deterrent characteristics, *Gambusia affinis* was again utilized as a test organism [80]. Standard tropical fish food was impregnated with the same soft coral extracts used in the ichthyotoxicity studies (see above) over a range of concentrations. Samples of food containing the highest concentration elicited feeding deterrence in almost 88% of the tests. At one fourth of this concentration, 48% of the samples still elicited detectable levels of deterrence. In 22% of the cases, avoidance of test particles after initial recognition indicated the presence of an effective olfactory component. Recent analysis of these data have revealed that there was no significant relationship between the presence of negative olfactory cues and negative palatability cues ($p \gg 0.05$, Kendall's Rank Correlation Test). The study was also unable to identify any positive relationship between ichthyotoxicity and feeding deterrence, through either olfaction or taste. Ongoing studies are examining the nature of the chemical cues implicit in these results [101].

Among the most profound sub-lethal effects which a secondary compound may exert on a predator are the emetic ones and those inducing temporary or permanent disability. These compounds can be debilitating, which is why they have received so much attention from the public health sector. The best known marine example of this type of poisoning in man is ciguatera [reviewed in 102]. The toxin (ciguatoxin) cannot be detected by man, illustrating how olfaction, taste, and toxicity are not necessarily associated [89]. It is also a good example of negative reinforcement in a learned avoidance response [103].



(+) palustrol
Cespitularia aff subviridis (26)

PGA 2
Plexaura homomalla (104)

Alcyonarian derived feeding deterrents

Among the Gorgonacea, Gerhart's studies [104, 105] of *Plexaura homomalla* are probably the most exciting. *P. homomalla* has been reported to contain a prostaglandin PGA_2 at levels as high as 8% of the dry weight of the organism. In feeding experiments, Gerhart observed avoidance by *Fundulus heteroclitus* (common American killifish) of food pellets impregnated with crude lipid extract and pure PGA_2, known to be ichthyotoxic. Food impregnated with the lipid extract appeared to be distasteful, and the level of acceptance decreased significantly with time. Food impregnated with pure PGA_2 was avoided, increasingly so with additional trials. Oral doses (0.1–0.2 mg) induced an emetic response in test fish. Similar results were obtained using a natural predator, *Halichoeres garnoti* (yellowhead wrasse) in the field, which also exhibited a negative learning response in feeding on the material.

3.4 Functional Morphology in Relation to Toxicity: Physical Defense

One of the most basic defenses against predation is structural or morphological defense. Examples are abundant and range from the spines of a sea urchin or thorns of a cactus through the hard exoskeleton of a turtle or a marine alga such as *Halimeda* [29, 106]. Soft corals are highly variable in form and may possess physical properties which protect either the individual polyps or the colony as a whole from predation [25]. Some soft corals are defended by small, sharp calcium carbonate sclerites (spicules). These can be long and needle-like and protect the polyp head in a canopy-like fashion (e.g. *Dendronephthya* spp.), or in other species (e.g. *Sinularia* spp.), the sclerites may be large and tightly packed throughout the colony. In some species, polyps can be retracted completely within such a protected colony, while in others the polyps are constantly exposed (cannot be withdrawn into the colony) and possess no scleritic defense (e.g., *Xenia* spp.).

Intuitively, one might expect that a formidable structural or morphological defense would obviate the need for chemical defense against predators. This problem has been investigated in sponges [35, 61, 75, 107, 108], holothurians [36], starfish [34], nudibranchs [49, 109], ascidians [31, 38, 39], scleractinians [54], gorgonians [61], and the marine algae [43, 110] with respect to fish predation. With the possible exception of the Scleractinia, toxic secondary metabolites have been found to co-occur with structural defenses [e.g., 111]. In other cases, the reverse has been found [34]. Thus, the question of association between toxicity and structural defense has been an elusive and unresolved one.

Until recently, this problem had not been considered for the Alcyonaria. The Alcyonaria are highly variable in form as well as in their levels of ichthyotoxicity. They thus afford a particularly suitable system for analysis of this relationship. A large number of specimens (sixty-eight) drawn from three of the most common families of soft corals (Alcyoniidae, Nephtheidae, and Xeniidae) were assessed for diverse morphological characters [112] related to colony defense. The attributes considered fell into the general categories of gross colony form, consistency or texture of the colony, presence of mucus, color, ability to withdraw polyps, and the nature and distribution of sclerites throughout the colony. This large multi-

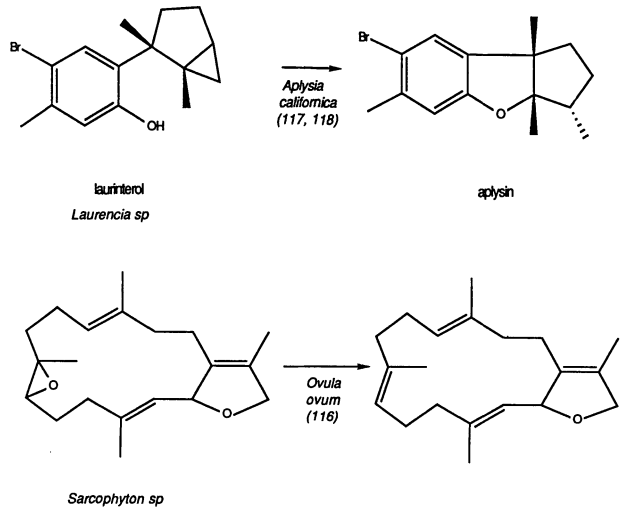
variate data set was compared against ichthyotoxicity data for each specimen and analyzed for association. Initially, no meaningful associations emerged between physical defense and ichthyotoxicity. Most characters showed no association whatsoever with toxicity, and two were positively associated. This suggests that consideration of too wide a variety of both taxonomic representatives and morphological features is likely to yield non-significant or counterintuitive results [e.g. 43]. The reason for this is that any given morphological or chemical character represents the evolutionary end-product of natural selection by innumerable factors, both biological and physical, only one of which may be predation. Such a character, when considered with many other characters, each of which is also responding to numerous selective factors which may or may not overlap with the one in question, would most likely not yield significant results due to the counterbalancing effects of selection in the different characters. In addition, misleading significant relationships may appear.

More reliable results were derived from further analyses of data restricted to the familial or generic level and considering a smaller number or morphological traits clearly related to predator defense. Only the family Nephtheidae and the genus *Sinularia* (Alcyoniidae) were considered along with five specific morphological traits, including superficial armament of the polypary (upper polyp bearing surface of the colony), coenenchymal mass (fleshy matrix), and the anthocodial armament or protection of the individual polyp by calcitic sclerites. These physical attributes were inversely associated with ichthyotoxic characteristics. In a more detailed analysis of the first and last of these physical characters, members of the family Nephtheidae were found to be completely responsible for the significant negative association there, while *Sinularia* spp. were found to be completely responsible for such an association in the second trait. Thus, an inverse relationship has been identified between structural defense characteristics and toxinological defense characteristics; however, these only become clear at a high level of taxonomic resolution (genus or family) and when considering species which show a reasonable amount of variability in both physical characters and toxinological properties [112].

3.5 Coevolved Predators, Selective Predation, and Toxicity

Even with the evolution of highly effective defenses, a small subset of specialized predators capable of overcoming these defenses can often be found. This coevolution of counter-adaptation has been well documented in terrestrial, freshwater, and marine systems [29, 113, 114]. The coevolution of predators specialized to feed on highly toxic organisms is also well documented. Whittaker and Feeny [3] have reviewed this topic for the terrestrial environment. Examples are also known from the marine environment [49, 115].

One mechanism facilitating selective predation on toxic prey is modification of the active secondary metabolite to a less toxic form. For example, the egg cowrie *Ovula ovum* (Mollusca) readily consumes the tissues of *Sarcophyton glaucum*, which are known to contain copious amounts of the toxin sarcophytoxide [116]. This metabolite is converted in the digestive gland of the mollusc to 7,8-deoxysar-



Chemical modifications of ingested metabolites by molluscs

cophytoxide, a markedly less toxic compound, and subsequently deposited in the feces. A similar mechanism occurs in the sea hare *Aplysia californica* (Mollusca, Ophisthobranchia) which ingests certain marine algae (e.g. *Laurencia pacifica*) known to contain the toxin laurinterol, modifying this active compound into aplysin [117, 118]. These chemical modification strategies appear to make available abundant, yet otherwise inaccessible, food sources.

Anthelia edmondsoni, a xeniid alcyonacean, is known to possess two diterpenoids and yet is readily fed upon two molluscs, *Tritonia hawaiiensis* and *Pteraeolidia ianthina* [119]. The toxicity levels of these two compounds are not yet known nor has an anti-feedant role been identified for them. Another general predator of soft corals and one apparently immune to their toxins is the Crown-of-Thorns starfish, *Acanthaster planci* (Echinodermata, Asteroidea).

Predator-prey links have been implicated by isolating and identifying known secondary compounds (e.g. the sterol gorgosterol) from alcyonarians and their supposed predators. This has been shown for *Briareum* spp. and *Xenia elongata* as well as the predators commonly associated with them – *Cyphoma gibbosum* and the crab *Caphyra laevis*, respectively [120]. *Isadascus longispinatus* is a specialized parasite of the gorgonian *Chrysogorgia* cf. *elegans* [64]. *Cyphoma gibbosum* (Mollusca, Ovulidae) is specially adapted to strip the hard and toxic tissue of *Plexaura homomalla* and utilize it as a food source [105, 121, 122]. Most molluscan predators which have coevolved as selective predators of the Alcyonaria utilize the entire colony as a food source [also see 123].

Some predators, however, are specifically adapted to feed only on individual polyps. These include *Chaetodon mellanotis* (Pisces, Chaetodontidae) and *C. capistratus* which have highly specialized mouthparts including a small mouth and jaws on a long, thin extended snout [61, 124]. This raises the question of whether differential levels of toxicity exist between the polyps and the coenenchyme – a question which presently remains open.

There is a higher level of coadaptation which occurs as well. Some predators are not only adapted to utilize toxic prey as a food source but also to exploit the toxic properties of the prey for defense against their own predators [125]. Numerous examples of this phenomenon may be found in the literature relating to predation on sponges by nudibranchs [32, 126]. Among such examples is the aeolid nudibranch, *Phyllodesmium longicirra*, which consumes and selectively stores the terpenoid toxins of *Sarcophyton trocheliophorum* by concentrating them in their cerata [73]. A similar situation has been found to occur between the nudibranch *Armina maculata* and the pennatulacean *Veretillum cynomorium* [127]. In the case of *Phyllodesmium*, the cerata can be autotomized upon disturbance or threat by a would-be predator [128], evoking a distasteful or toxic response. *Abudefduf leucogaster* (Pisces, Pomacentridae) is known to associate itself with the alcyonacean *Litophyton viridis*, swimming amongst its tentacles and stimulating the release of mucus laden with secondary compounds noxious to most fish. The fish itself is tolerant to the toxins [129].

The homilid crab, *Poromola japonica* (Decapoda, Brachyura) is known to actively choose pieces of gorgonians as well as antipatharians and sponges to protect and camouflage itself from predators [130]. This interaction may involve a borrowed chemical defense system and merits further investigation.

3.6 Summary

Evolutionary success of any organism is at least partially dependent upon its ability to defend itself against predators. This section has focussed on the range of predatory defenses exploited by soft corals. These defenses have relied heavily on the secondary chemistry of the organism. More importantly, the adaptations and co-adaptations involving those compounds are both diverse and complex. They involve direct toxicity, feeding deterrence by olfaction or taste, acquired toxicity, sometimes through mere association with toxic organisms, and detoxification strategies. Although the ichthyotoxicity of soft corals has been associated with the presence of specific terpenoid toxins in many instances, the chemical nature of the active anti-feedant compounds has not yet been conclusively identified. The problem of toxic or anti-feedant characteristics being defined in freshwater test fish as opposed to natural predators needs to be addressed. As most natural coral reef predators have evolved in the presence of soft corals, and since many are highly specialized, this problem becomes even more difficult. Perhaps in the future, tests using more generalized predators such as *Halichoeres* spp. [104], *Thallasoma* spp., or *Lutjanus* spp. will provide further insight into the roles of the compounds. Although physical or structural anti-predator defenses are utilized by some soft corals, chemical defense has also clearly played an important role in contributing to their evolutionary success.

4 Competition for Space

Many colonial organisms on coral reefs possess specialized mechanisms which assist them in maintaining and expanding their living space. In a sessile community, space is often a limiting factor for growth and, in colonial organisms, for reproductive capacity as well [131]. In scleractinian and some hydrozoan corals, these adaptations include extracoelenteric digestion by mesenterial filaments [132, 133] stinging nematocysts on tentacles [22], sweeper tentacles [134–136], directed growth [137], and overtopping [138]. Alcyonarian corals, however, possess none of these advantages [e.g., 139] and draw upon a battery of other competitive defense strategies. Chemical strategies play an important role in competition for space, along with other mechanisms not available to scleractinians.

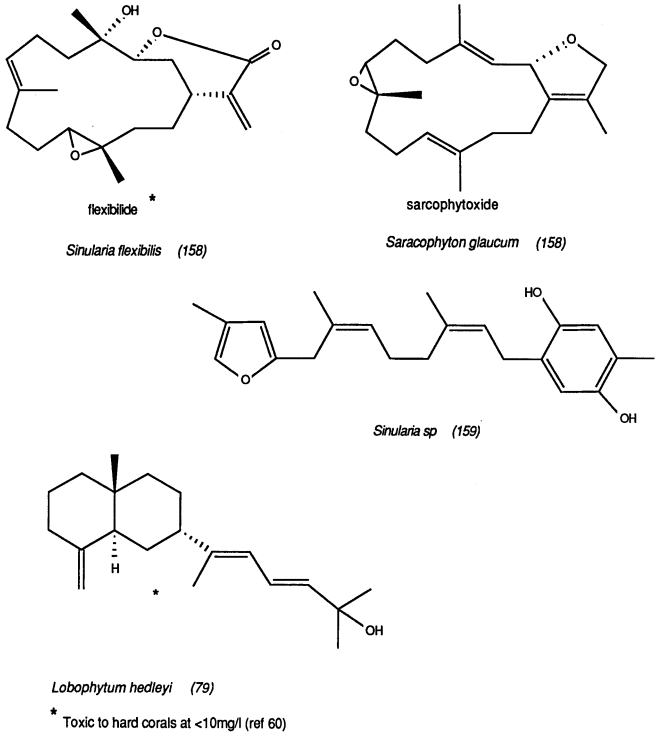
4.1 Allelopathy

One of the striking contrasts between scleractinian and alcyonarian corals is that the latter are rich in secondary compounds, both qualitatively in terms of diversity and quantitatively in terms of abundance or concentration [65]. The implications of this set of characteristics with respect to predation has already been discussed. Its use in competition for space has recently been demonstrated and is now well documented.

The use of chemicals to maintain and expand living space has been known to occur in the terrestrial environment for some time [2, 140, 141]. The term allelopathy is here used to describe "the direct inhibition of one species by another using noxious or toxic chemicals" [2, 29, 142, 143]. Whittaker and Feeny [3] have claimed that such chemical defense plays an important role in niche differentiation and the control of community structure in numerous ecosystems. The possibility that such allelopathic effects could occur in the marine environment has been discussed for many years. Lucas [144–146] and Saunders [147] have stated their belief that exudates, which they term ectocrines, space planktonic community structure and affect the outcomes of competitive encounters and succession in much the same way as in the terrestrial environment. Kittredge et al. [5] expanded this concept to include the marine epibenthos. Experimental evidence offered by such investigators as Kerfoot [113], Folt and Goldman [148], and Seitz [149] have lent strong support to these concepts. Jackson and Buss [150] implicated this relationship to occur between sponges and bryozoans in the Caribbean. Other marine examples where allelopathic interactions have been implicated in competition for space include the sponge *Aplysina fistularis* which releases biologically active metabolites [151, 152], the sponge *Siphonodictyon* sp. which inhibits scleractinian coral growth [153], exudates from the anemone *Condylactis gigantea* as a growth inhibitor for marine algae [154; also see 155, 156] and exudates from the predatory plankter *Epischura nevadensis* which reduces filtering rate in its copepod competitor and prey *Diaptomus tyrrelli* [148].

Soft corals are one of the very few groups in which allelopathy has been experimentally demonstrated to occur in the marine environment [157]. Toxic soft corals may commonly be observed in association with scleractinian corals exhibit-

ing growth retardation and tissue necrosis in the central region of the Great Barrier Reef. Experiments involving certain species of soft corals (*Lobophytum pauciflorum, Sinularia pavida, Xenia* sp. aff. *danae*) with known levels of toxicity which were relocated into stands of scleractinian corals (*Porites andrewsi* and *Pavona cactus*) with appropriate controls confirmed that these effects were significant and reproducible in the field. Local mortality, tissue necrosis, and growth retardation in scleractinian corals were effected with the aid of secondary metabolites through the water column without contact. The presence of the active molecules in the water column around the soft coral was confirmed via a submersible water sampling device which utilized reversed-phase Seppaks^{R} which selectively adsorbed dissolved organic molecules [158]. The diterpenes flexibilide and sarcophytoxide were found to occur in the water column at levels estimated to be $\leqq 5$ ppm. Subsequent laboratory experiments utilizing pure compounds demonstrated that 5–10 ppm of flexibilide were sufficient to cause total mortality in *Acropora formosa* and *Porites andrewsi* [79]. Further laboratory experiments have revealed one possible mechanism by which their competitive superiority is effective. The active compounds have been found to cause an increase in respiration together with decreased photosynthetic output which may be due to an uncoupling of respiration for oxidative phosphorylation (ATP production) [159]. In all cases, the test corals failed to survive 24 hours of exposure to soft-coral derived terpenes at 10 ppm.



Known allelopathic agents released from soft corals

The efficacy of this chemical strategy against scleractinians is not universal. Not all soft corals release toxins into the environment, and not all scleractinian corals are susceptible to damage caused by exposure to a specific octocorallian exudate. That is, a high degree of species-specificity exists in these interactions. This is highlighted by another set of experiments which demonstrated that some soft corals known to contain high concentrations of particularly toxic diterpenes had no allelopathic effects on the same species of scleractinian corals used in earlier experiments [160]. Similarly, the susceptibility of alcyonacean soft corals to the aggressive defense mechanisms of scleractinian corals is equally species-specific, for a single species of scleractinian can cause total colony mortality in one soft coral and have no effect on another.

According to these studies, the chemical defenses possessed by many Indo-Pacific soft corals are effective enough to produce soft coral dominated reefs [15]. A mathematical model of interactions involving effective soft coral allelopathy has supported this contention and revealed that one mechanism which ensures the survival of competitively inferior corals is refuge in space [161]. In addition, both physical and other biological factors may reverse competitive advantage and reestablish competitive balance between the two species. Further field and laboratory experimentation is indicated here to assess the relative importance of these factors.

Interspecific allelopathic interactions have also been experimentally confirmed to occur between soft corals, resulting in severe necrotic effects after only a few days [162]. The effect is ameliorated, however, by avoidance and growth responses (see below).

One case has also been documented whereby the proximity of the gorgonian *Plexaura homomalla* is detected by the hydrozoan coral *Millepora alcicornis* and *M. complanata*, presumably by a water-borne allelochemical, the nature of which remains to be elucidated [137].The hydrozoan responds by subsequently redirecting growth towards the gorgonian and overgrowing it, utilizing the gorgonian's skeleton as a new, pre-formed branching substratum. It is known that this same gorgonian possesses prostaglandin A_2 [104, 105] and that this compound functions as an emetic anti-feedant [104]. Experimentation thus far has not been able to identify any allelopathic role for the compound [163].

4.2 Growth and Avoidance Responses

Allelopathy is not the only form of aggression or defense which soft corals use in competition for space, and this is one of the reasons why these animals vary in their responses and susceptibility to damage from scleractinian corals. One of the responses of soft corals to neighboring organisms is secretion of a protective polysaccharide layer (or cuticle) which protects the soft coral against damage from the nematocysts of neighboring scleractinian corals [160]. It also appears to minimize immunological responses upon contact with other organisms as evidenced by the colonization of fouling organisms on this layer. This polysaccharide cuticle also allows the organism to overgrow live scleractinian coral surfaces by providing a base for colony attachment and expansion. Once the attach-

ment is complete, this same layer allows directional movement of some soft corals across the living surface of some scleractinians, thus utilizing otherwise unavailable living space [164]. Such movements induce undifferentiated calcified neoplasms [165] in the scleractinians, resulting in heavily calcified "trails" following the colony growth patterns. This response has been observed in *Litophyton viridis* in contact with *Porites lutea* [66] and was attributed to an allelopathic interaction. Simple overgrowth through movement may provide a more parsimonious explanation of the observed phenomenon, as indicated by the results of current studies [17, 164].

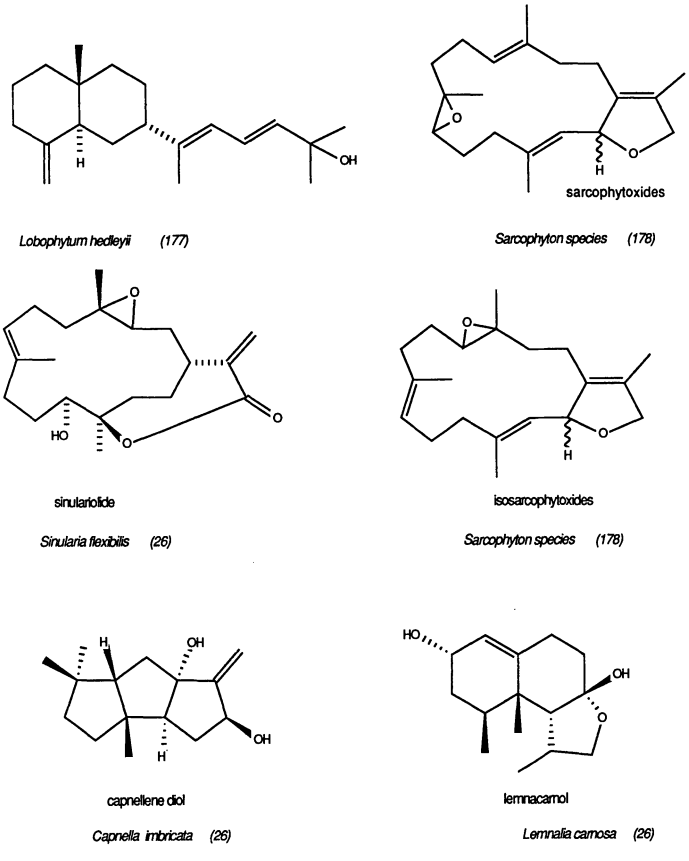
Some soft corals exhibit an avoidance response when placed in contact with scleractinians [162]. Here, the polypary of the soft coral is reoriented so as to minimize interpolypal contact. This response is even more pronounced when soft corals are placed into interspecific contact. Localized tissue necrosis occurs very rapidly. However, within several days, an avoidance response occurs, whereby colonies bend away from each other, thus minimizing the amount of colony damage. In the longer term (in the order of months), the colonies actually move apart through directional growth [17, 166]. They utilize this spacing capability to maintain an individual distance – a trait usually reserved for vagile organisms [167, 168]. Although allelopathic interactions between soft corals and scleractinian corals are observed commonly in the field, observations of such natural interactions between soft corals are much rarer. This is most likely a result of this spacing capability which would greatly lower the observed frequency of the necrotic responses and the probability of detecting this dynamic process at any particular time [169].

4.3 Immunological Responses

Most of the immunologically oriented studies performed on the Coelenterata have involved the Scleractinia [170, 171]. With respect to the Alcyonaria, research has been minimal and restricted mainly to the Gorgonacea. Theodor [172, 173] has demonstrated that *Funicella stricta* (Bertolani) and *Lophogorgia sarmentosa* (Esper) are capable of self-recognition in conspecific encounters. These animals can also differentiate between species, mostly resulting in tissue necrosis of one species or the other. Intraspecific grafts demonstrated self-recognition both at the intra-clonal and inter-clonal levels. The former invariably led to fusion, whereas the latter could lead to tissue necrosis in one, the other, or both of the colonies involved. Unless one examines this problem at the molecular level, discerning between immunological and toxinological effects will be difficult.

4.4 Anti-Fouling Properties

The majority of natural product research groups utilize either an antimicrobial or anti-fungal assay to identify extracts which contain compounds of possible pharmaceutical interest. The individual compounds which have resulted from this screening have been published and collated in several review series. The reader is



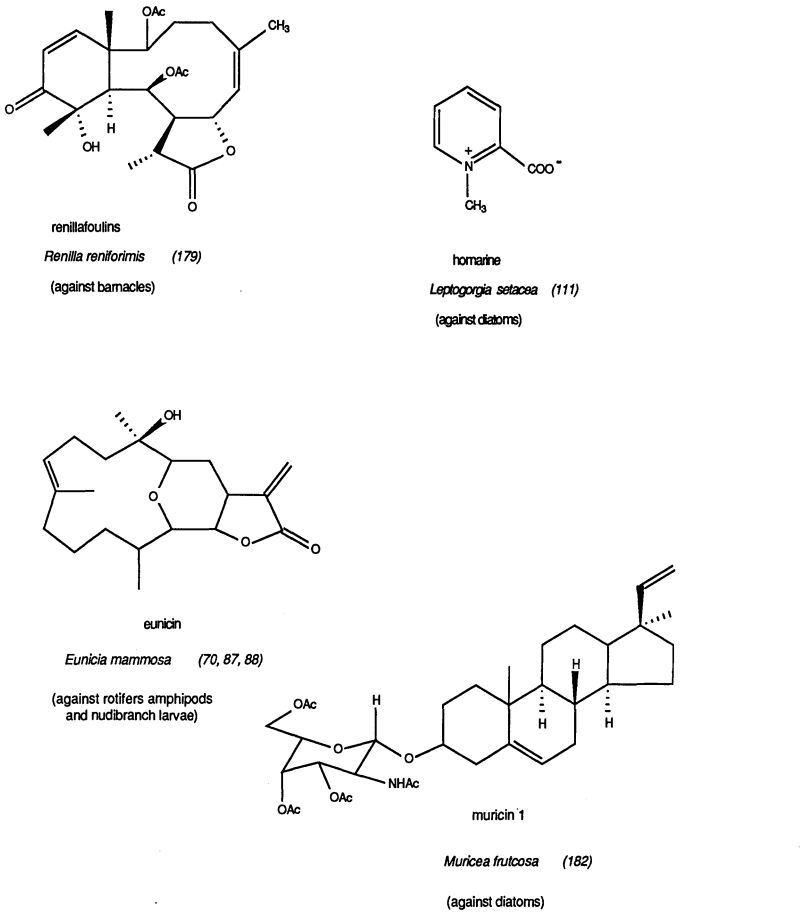
Antialgal compounds from alcyonarians

referred to those of Baker and Murphy [11], Faulkner [12–14], and Scheuer [9, 10].

Quite often, investigators interested in marine natural products chemistry or marine chemical ecology use the absence of bio-fouling on sessile epibenthic organisms as an indicator of potentially useful bioactivity [174, 175]. Few of these studies, however, have been carried out specifically to identify secondary compounds responsible for antifouling or to demonstrate the mechanisms by which they function [38, 176].

It is recognized that the polyparies of alcyonaceans are generally free of fouling organisms. One of the few documented cases of colonization of the polypary of an alcyonacean coral by a marine alga involved the overgrowth of *Lobophytum* sp., a highly toxic soft coral, by *Ceramium* sp. and *Enteromorpha* sp. on the Great Barrier Reef. Significant growth inhibition in the algae was demonstrated experimentally using the major diterpenoid secondary metabolite from this soft coral [177]. A more extensive field study has supported these findings [178].

The pennatulacean *Renilla reniformis* contains three diterpenes (renillafoulins) which inhibit larval settlement in barnacles [179]. Unspecified low-molecular weight compounds which function similarly [180] have also been found in *Lepto-*



General antifouling agents from alcyonarians

gorgia virgulata. The cembranolides eunicin, crassin acetate, and eupalmerin acetate, derived from the gorgonians *Eunicea mammosa, E. succinea*, and *Pseudoplexaura porosa*, are also known to be toxic to rotifers and amphipods at very low concentrations ($\sim$1 ppm) [176]. Both of these studies are consistent with the results obtained by Standing et al. [181] concerning general anti-fouling characteristics in *Renilla* and *Leptogorgia*. It is unfortunate that more specific information is not available from some of these studies [180, 181] concerning the compounds specifically responsible for the observed phenomena. Targett et al. [111] have found that homarine, which may be found in both *L. virgulata* and *L. setacea*, inhibited diatom growth, complementing morphological defenses against predators also present in these organisms.

Some compounds have been identified as having a specific anti-fouling role. Bandurraga and Fenical [182] found that some muricins from *Muricea fructosa* (Gorgonacea) possess anti-fouling characteristics against diatoms but are not ichthyotoxic, cytotoxic, or anti-microbial. Gerhart [163] has demonstrated that prostaglandin A_2 does not serve an anti-fouling role in *Plexaura homomalla*, nor an

allelopathic one, but has an anti-predatory role. Rützler et al. [183] have found that the gorgonians *Plexaura homomalla, Gorgonia flabellum*, and *G. ventalina*, and an unidentified plexaurid, although capable of being artificially infected with black-band disease (*Phormidium corallyticum*; Cyanophyceae), appear to exhibit low natural infestation rates in the field, implying that these species may possess as yet unidentified anti-fouling agents.

5 Chemical Aspects of Reproduction

Secondary metabolites have played an important role in the evolution of reproductive strategies of many organisms, especially in terrestrial plants [184–186]. These compounds may exert their effect at different stages of the reproductive process. In some cases, seeds are surrounded by highly toxic plant tissue. This toxicity may be transient, being restricted to the period of seed development, disappearing entirely upon maturation of the seed. Dispersal is then facilitated via ingestion and subsequent deposition [187, 188]. In other cases, the seeds themselves are toxic while the surrounding tissue is not, yet the toxicity is contained within the seed by a coating highly resistant to digestive breakdown.

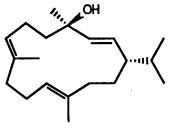
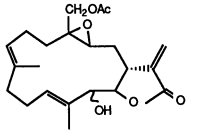
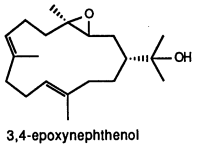
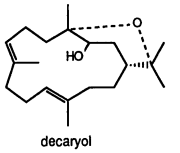
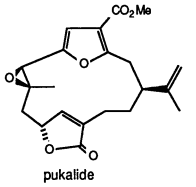
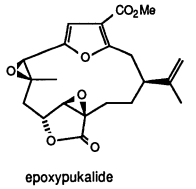
In the marine environment, the ovaries of some animals such as pufferfish are more toxic than muscle tissues [189]. In others, both the eggs and planktonic larvae may be toxic, e.g. bipinnaria larva of *Acanthaster planci* [190, also see 32].

Until recently, little information was available concerning sexual reproduction in the Alcyonaria; much effort had been concentrated on asexual aspects. Through asexual reproduction, the toxic traits of the parent may be transferred directly to the new clone. This can occur via fragmentation, production of stolons, or simple division and colony growth [66, 191–194]. By contrast, some secondary metabolites have been found to be specifically associated with the process of sexual reproduction. For example, in *Palythoa tuberculosa* (Zoantharia) and other species of *Palythoa*, the maximum concentration of palytoxin coincides with maturation of the gametes [195].

Evidence is accumulating that a similar process occurs in the Alcyonaria. Sexual reproduction has now been studied in various Indo-Pacific alcyonaceans, beginning with Gohar's [196] research on *Xenia* sp., continuing with, for example, Hartnoll's [197] work on *Alcyonium digitata* and Yamazoto's [198] work on *Lobophytum crassum*, and Benayahu and Loya's [199] on *Parerythropodium fulvum fulvum*. In none of these cases, however, was the chemistry of the organism examined in relation to its reproductive state. Kashman et al. [200], however, did note that there was a seasonal component to the chemical composition of some xeniid soft corals.

Recent studies of mass spawning of Great Barrier Reef corals in the Scleractinia and Alcyonaria [201] have provided an opportunity to make collections of mature gametes for a number of alcyonacean corals. The earliest studies [202] revealed chemical differences between the parent colony and the eggs of *Lobophytum compactum*. The diterpene thunbergol was present in the eggs and undetectable in the parent colony. Subsequent experimentation has shown that the con-

Table 3. A summary of chemical differences between freshly released alcyonacean eggs and the coral tissues of the releasing colony. (Figures refer to percent dry weight of eggs and coral tissue, respectively, represented by the terpenes)

Species	Terpene	Eggs	Coral
Lobophytum compactum (202)	 thunbergol	(1.3%)	(0%)
Lobophytum crassum (202)	 13-hydroxylobolide	(0%)	(0.5%)
Lobophytum microlobulatum (177)	 3,4-epoxynephthenol	(2.5%)	(0%)
Sinularia spp. (4 species) (204)	 decaryol	(>0%)	(0.1%)
	 pukalide	(0.25%)	(0%)
	 epoxypukalide	(0.25%)	(0%)

centration of thunbergol in the planktonic larvae rapidly disappears over a period of less than one week, while the other diterpenes remain constant [203]. This variation implies that the action of thunbergol is defined with time. Its specific function, however, still needs to be elucidated (Table 3).

Another example was found in *Lobophytum microlobulatum*, where the eggs contained 3,4-epoxynephthenol, which was absent from the parent tissue [177]. Several other diterpenes were present at comparable levels in the eggs in both cases. In *Sinularia* spp., the eggs of as many as four different species with different chemical profiles were found to contain pukalide and often epoxypukalide, which

were absent in the coral tissue [204]. In addition, these compounds increased in concentration during the period immediately preceding ovulation (i.e. one month) and disappeared after egg release. The latter two compounds did not appear to be ichthyotoxic nor do they possess appreciable levels of antibiotic activity against a range of marine microorganisms [205, 163]. Field observations during spawning confirm that soft coral eggs are readily consumed by reef fish, including some pomacentrids and chaetodonts. It is interesting to note, however, that precisely the same compound found in five *Sinularia* species – 11β,12β-epoxypukalide – has been found to occur in a number of gorgonian species [204, 206]. The question of the role of these compounds in nature remains unresolved. (Table 3)

Two possible roles may be suggested: 1) a chemical trigger for release of the eggs, or 2) a sperm attractant. Preliminary results indicate that chemotaxis is involved in the attraction of the sperm of *Lobophytum compactum* to its eggs and that the attractant is a lipophilic molecule [203]. Further work is clearly indicated for the alcyonaceans. To date, no similar studies have been reported for the Gorgonacea.

6 Secondary Metabolites and Chemotaxonomy

Marine chemotaxonomy is an area of research that is receiving an increasing amount of attention. The results of these studies have been more promising in some groups of marine invertebrates than in others. For example, identification has been a continuing problem in the sponges, and chemotaxonomy has served well to complement classical taxonomy there [207].

The first use of chemical analysis for taxonomic purposes in the Alcyonacea was Kashman et al.'s [208] use of sesquiterpenoid "fingerprints" within the genus *Sinularia* by gas liquid chromatography. Although some species-specific compounds exist in the Alcyonacea, it is common to find a compound or a set of closely related compounds to occur in relatively distantly related groups – i.e. across families. For example, some of the same compounds, which may be found in the Alcyoniidae, can also be found in the Nephtheidae [209].

A more promising approach to this problem may be found in the technique of numerical taxonomy [210] using quantifiable chemical characters as the basis for association. This can then be compared with the results of more classical techniques utilizing morphological characters, such as sclerites and colony form. This has been attempted with some success in the Gorgonacea where the co-occurrence of secondary metabolites (terpenes) produced associative relationships between species paralleling those determined with the aid of morphometric data [211]. The application of this technique to the Alcyonacea is planned [212].

One of the limitations of this technique is that the complete chemical composition in terms of secondary metabolites of any one organism is usually not known. This is particularly true in the Alcyonacea, where only novel compounds have been reported; known compounds are usually omitted from published reports. Thus, collation of data on presence or absence of compounds across many species derived from varied reports is likely to be incomplete. In addition, many

secondary compounds may not have been present in sufficient quantity for characterization and identification in earlier studies. Over the past 20 years, the amount of a pure compound required for complete characterization has decreased from ~50 mg to ~500 μg; therefore, earlier reports most likely underestimate the range of terpenoid metabolites present in the alcyonarians.

Another complicating factor which must be considered in the use of secondary metabolites for attempting to establish phylogenetic relationships among organisms is the problem of convergent evolution. Morphological traits in general will tend to be more conservative, exhibiting a comparatively slower rate of evolution [213]. The dynamics of chemical biosynthesis are adapted in such a way that only minor chemical modifications in cyclization can result in apparently major changes in skeletal type. For example, cyclization of geranyl geraniol can easily afford five, six, or more different diterpenes [214]. The co-occurrence of similar structural types in widely disparate taxa does not necessarily imply phylogenetic proximity. An example may be found in the occurrence of iso-neocembrene-A, which occurs in both termites and soft corals [215]. A single step alteration in a chemical pathway could easily result in the appearance of a novel compound. The probability that this might occur in unrelated organisms possessing the same pathways integrated over evolutionary time would be much higher than that for the same morphological trait evolving, requiring numerous mutations and gradual directional changes in structure over long periods of time [see 213, 216].

Another possible confounding factor which must be considered in the use of secondary metabolites as an indicator of phylogenetic proximity is the presence of endosymbionts. Many marine invertebrates carry endosymbiotic zooxanthellae which may be capable of contributing to the production of terpenoid-based compounds. While aposymbiotic gorgonaceans have clearly been shown to produce complex diterpenes [182, 217], and while detailed stable isotope analyses strongly implicate the octocoral as the producer of terpenoids in the presence of zooxanthellae [218], evidence continues to emerge which suggests that in certain cases the zooxanthellae are capable of carrying out the elementary steps of terpene biosynthesis [219] and, in some cases of the terpene itself [220]. The question of the use of chemotaxonomy in organisms possessing endosymbionts, at this time, remains open.

7 Discussion and Summary

7.1 Overview

Alcyonarians are among the most abundant and diverse group of organisms on coral reefs in the Indo-Pacific. Among the characters which have contributed to their evolutionary success is the occurrence of toxic secondary metabolites. Recent studies suggest that these secondary compounds, primarily terpenes, play important ecological roles which enhance survival from predation and competition for space and promote reproductive success. The frequency of ichthyotoxicity in Great Barrier Reef alcyonaceans alone is estimated to be ~50%.

Toxicity in this group as determined by laboratory tests varies widely among species in both frequency and degree. Feeding deterrence characteristics also vary widely in the group, both from the perspectives of olfaction and palatability. This feeding deterrence, however, does not appear to be correlated with ichthyotoxicity, thereby suggesting that a variety of compounds may induce the response in different species. Toxicity, however, is related in some ways to the amount of physical protection that soft corals possess; i.e. the more heavily armored a species is, the less likely it is to be toxic to fish. This relationship only becomes clear when examined at a high level of taxonomic resolution and when morphological traits clearly adapted as predator defenses are considered. Some animals, particularly nudibranch molluscs, have coevolved in such a way as to specialize in feeding on certain toxic soft corals by chemically altering the toxins within their digestive tracts. Other nudibranch molluscs are immune to the toxins and are specialized to feed on particular species of soft corals, storing the toxins in their cerata. These structures may then be autotomized and left behind to discourage predators.

With respect to competition, toxins assist some soft corals in maintaining living space. Many common reef organisms, particularly scleractinian corals, appear to lack toxic secondary metabolites but possess effective morphological features such as nematocyst-bearing tentacles which help them to survive. Although soft coral tentacles do not possess stinging nematocysts, some colonies expand horizontally through allelopathic interactions. These toxic allelopathic agents can cause destruction of living tissue of their competitors, whether scleractinian or soft corals. From the limited data presently available, it appears that soft coral-derived ichthyotoxins are the allelopathic agents in these cases. Local mortality in neighbors can be caused by direct contact, or, more importantly, in the absence of contact via toxins released into, and carried through, the water column. Effects include local tissue necrosis and stunting of growth in scleractinian corals, where the interactions are highly species-specific in nature. Some soft corals can also secrete a polysaccharide layer to protect themselves from the nematocysts of scleractinians. This layer also permits them to adhere to the surface of a live scleractinian coral, and eventually move across the coral through overgrowth. Some alcyonaceans also have the ability to bend away and avoid each other, later spacing themselves and growing away from each other entirely. In this way, these sessile colonial organisms are actually capable of maintaining an individual distance, like many vagile organisms.

With respect to reproduction, some soft corals increase production of their toxins prior to release of their eggs. In these cases, high concentrations of the toxins may also be found in the eggs themselves.

7.2 Further Studies

A link between the presence of terpenoid secondary metabolites and ichthyotoxicity has in many cases been established. The actual mechanisms by which these toxins destroy respiratory membranes, disrupt physiological processes related to respiration, affect orientation and navigation, slow muscular response, block sensory reception of visual stimuli, etc. is unknown at present and warrants further investigation.

The feeding deterrent properties and olfactory warning signals of soft coral-derived secondary metabolites have only been attributed to a pure compound in one instance [26]. Further research is clearly justified in this area. The Alcyonaria are characterized by the presence of numerous classes of secondary compounds, including terpenoids, sterols, and a range of fatty-acid derived metabolites. Feeding deterrence may be attributed to any one of these or may be the result of synergism between two or more. Only additional laboratory and field experimentation will reveal which compounds are responsible for the symptoms observed. Where a feeding response is evoked, the problem of whether the cue is olfactory, visual, or taste-related remains to be solved. When one of these cues evokes a positive feeding response and a toxic prey item is consumed, how does the predator physiologically deal with the toxins? Detoxification or vacuolization/isolation of the toxins is known to occur in molluscs specialized to feed on certain toxic soft corals, but the problem remains to be addressed in specialized predatory fish (e.g. chaetodonts).

Soft coral derived compounds shown to be ichthyotoxic are now known to be responsible for allelopathy in interactions with scleractinian corals. The detailed mechanism by which this cytotoxicity is effected on scleractinians represents an attractive area for investigation. In addition, the manner in which these chemically mediated competitive interactions can be further controlled by both physical and biological factors of the environment needs to be elucidated, for competitive dominance is not absolute and can be altered by shifting competitive advantage through physiological stress or predation pressure.

Among the types of biological disturbances which can occur on Indo-Pacific coral reefs is population explosions of *Acanthaster planci* (Crown-of-Thorns starfish) [63]. This organism can remove almost all of the living scleractinian tissue from a reef, while the level of predation on alcyonarian soft corals is much lower. This produces a gross imbalance in abundance of the two groups and affords a distinct competitive advantage to the soft corals. With their moderate growth rates [66, 194], which are still faster than those of scleractinian corals they could easily dominate a reef [16]. This phenomenon could shift the community to a new stable point [221], with the dominant fauna changing from scleractinian to alcyonacean corals. Opportunities for subsequent recolonization of scleractinian coral larvae might then be reduced. This aspect of coral reef ecology also deserves further investigation.

The structure of the chemotactic agents in the attraction of sperm to eggs in the Alcyonaria requires elucidation. The roles of the accumulation and shifts in composition and concentration of secondary metabolites in the eggs and parent colony also require attention.

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Chemical Defense in Fishes

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Contents

Abstract

Chemical defense is a protective measure employed by living organisms to evade predation or infection, by the use of chemical substances which affect the physiology of potential predators. These substances may be classified into two categories. Toxins associated with mechanical devices that are injected directly into the body of enemies constitute one category and are usually called venoms. The other category comprises those that reach sensory organs of enemies through environmental media. This chapter covers only substances of the latter category which play roles in the defense of fishes, since little is known about chemistry of fish venoms except that they are all proteins as far as is known to-date. While chemical defense occurs only sporadically in vertebrates, little phylogenetic relation is found among the fishes using chemical defense and their defense substances are therefore fairly diverse in terms of their chemical structures.

1 Introduction

Colorful fishes which may be seen cruising on coral reefs in rather leisurely fashion are valued as pets, but they are not so common on our dinner tables. In contrast, our common food fishes are fast swimming pelagic fishes, such as jacks, mackerel, and their companions. This correlation may be attributed to differences in feeding habits: colorful reef dwellers are largely herbivores, whereas the pelagic

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species tend to be carnivores. (Somehow the opposite is true for terrestrial mammals.) If fishes were conscious of these phenomena, they would have to swim swiftly because they are tasty, provided their palate is similar to ours. Tasty yet leisurely swimming fishes would have perished a long time ago.

Contemporary organisms have survived for specific reasons. Among these are defensive tactics against their predators, secure nutrition, and efficient reproduction; those organisms which have established their habitat in an environment where others cannot survive are an exception. In the evolutionary period when predatory animals were still embryonic, chemical substances were perhaps almost entirely responsible for the protection of individuals and species from predation. This chemical defense continues to be important for many plants and sessile animals. As predators evolved motion, vision and other senses, or masticatory and digestive systems, those preyed upon have also developed physical defenses, such as escape, camouflage, and nesting, as well as mechanical or structural defenses, such as cuticulation of the epidermis as principal defensive measures. While this process was going on, chemical defenses have also evolved and may be observed in some higher animals. Terrestrial representatives among these are insects, frogs, and other tiny animals, while skunks and weasels are well-known among mammals.

Among aquatic organisms, unsavoriness is a passive form of chemical defense, which is presumed to be important for sessile organisms with no apparent mechanical defense, such as algae and soft corals. When a nasty taste arises from a certain substance, this is qualified as a defensive substance. Such substances, however, are usually termed distinctly as antifeedants. As a more positive form of defense, there exist offensive chemicals, which arouse discomfort in potential predators. Many of the fishes that possess this positive form of chemical defense were initially recognized through incidents when smaller fishes died when they had been placed in the same aquaria.

The body surface of fishes is usually slippery because their epidermis contains mucus cells secreting mucopolysaccharides, which are considered to play a role in the reduction of swimming resistance [1]. Certain fishes have different types of epidermal mucus cells in addition, as distinguished by coloration on staining, where they store material toxic to other fishes, the so-called ichthyotoxins. These mucus cells have various names, such as club cells, clavate cells, and proteinaceous cells, and the toxins therein are called mucus toxins, skin toxins, or ichthyocrinotoxins. Cameron and Endean reasoned that these toxins function as defensive substances because poor physical and mechanical defense, such as inefficient swimming and undeveloped (or atrophied) squamae, appears to be common among fishes possessing such cells, in spite of having habitats which require a harsh struggle for existence, typically on coral reefs [2]. Although the premises are not always valid, as is seen in some examples below, the authors' conclusion is further supported by the fact that the toxins are released when the fish is irritated. Besides, the offensive role of the toxins to capture prey, which is suspected from their ability to kill or stun other fishes in aquaria, is probably minute or non-existant in the open sea, whose constant movement rapidly dilutes and carries them away [3].

Although indirect evidence strongly indicates that the toxins are repellent defensive substances, the cases are rare, where either the toxins themselves, secre-

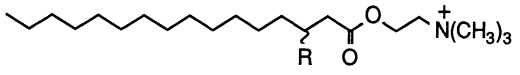
tions containing them, or the fish itself, have actually been demonstrated to repel predatory fishes. Scientific proof that these toxins are defensive substances therefore remains for future research. Because of the experimental difficulty of this proof, many of these substances were originally reported as ichthyotoxins while their significance in defense has frequently been hypothesized. Ichthyotoxicity of these substances, however, can constitute indirect evidence of their defensive function. Wild animals in general avoid toxins by instinct as long as the latter exist in their natural habitat. For instance, terrestrial animals keep away from the bitterness of toxic plants or rotten smells. In the marine world on the other hand, many ichthyotoxins have been reported from algae and sessile or sluggish animals which appear to need chemical defenses. In other words, predators have survived through natural selection by sensing these toxins, while the would-be prey have survived as well by possessing perceptible toxins. It is also reasonable that a repellent substance, which must act on a sensory organ of a predator, can be fatal to them when it acts on another certain organ. Such toxins as suspected repellent substances have been reported from the following fishes.

2 Trunkfishes and Boxfishes

Trunkfishes and boxfishes compose the family Ostraciidae in the order Tetraodontiformes, and are characterized by rectangular carapaces covering their bodies as the names indicate. Members of this family have been known to kill other fishes in aquaria for a fairly long time. It was described as far back as in 1918 that a trunkfish, *Lactophrys bicaudilis*, which lives along the coast of the Virgin Islands in the West Indies, possessed toxic jellylike material in its "labial glands" [3]. A Pacific boxfish, *Ostracion lentiginosus*, was also observed to kill other fishes while confined in an aquarium. Thompson prepared a crude toxin from the mucous secretion of the boxfish through centrifugation and dialysis; he conjectured that the toxic principle might be similar to the echinoderm saponins, because of its surfactant and hemolytic properties, and named it ostracitoxin [4]. The toxic mucus was inferred to emerge from "labial glands" found in the buccal region of this fish as well as from club cells seen in its epidermis [5].

Boylan and Scheuer meanwhile observed that the same boxfish released the toxic secretion when placed in distilled water; they isolated the toxin through extraction with *n*-butanol and subsequent silicic acid chromatography, and named it pahutoxin after the Hawaiian word for the boxfish, *pahu* [6]. The crude aqueous solution lost its toxicity gradually on standing, but butanol extraction not only enriched it twentyfold, but also stabilized it. The instability of toxicity in the aqueous solution was attributed to a coexistent enzyme in the secretion, as is the case in other instances, but it is uncertain whether the instability exists so that the fish eludes self-intoxication, as was speculated.

The chemical structure of pahutoxin was elucidated by spectroscopic studies and hydrolytic degradations to be *O*-(3-acetoxypalmitoyl)choline (**1**), and was further established by a chemical synthesis. Natural pahutoxin showed an optical



1 (R = CH_3CO_2)
2 (R = H)
3 (R = $CH_3CH_2CO_2$)

rotation arising from chirality at the acetoxylated carbon, though its absolute configuration remains unknown. Although synthetic pahutoxin was racemic, it was as ichthyotoxic and hemolytic as optically active natural pahutoxin. The authors proceeded to synthesize analogs of pahutoxin with shorter alkyl chains, in order to correlate chain length with bioactivity. As a result, the C_{14} analogue, shorter than pahutoxin by two methylenes, was shown to be several times less ichthyotoxic and hemolytic. The diminution of activity was still greater from the C_{14} to C_{12} analogs. The results paralleled the hemolytic activity of sodium alkanoates, or classic soap, where C_{14} to C_{18} are considered to be the most active [6]. Moreover, Kikuchi and Wakabayashi recently observed in their environmentally oriented research that sodium alkyl sulfates became more ichthyotoxic as the alkyl chain was elongated from C_{12}, C_{14}, then to C_{16} [7]. Incidentally or not, surfactant properties of these sulfates increase proportionally [8]. A correlation of the surfactant property with repellency or ichthyotoxicity of many fish repellents exists also in other cases described below.

The chemical structure of pahutoxin was the first to be determined among the repellents of marine animals, when it was reported more than two decades ago; it remained the sole example from fish for a long time, until Goldberg et al. reported deacetylpahutoxin, or *O*-palmitoylcholine (**2**), as the major ichthyotoxin in the secretion of smooth trunkfish *L. triqueter* collected off the Virgin Islands [9]. Its C_{17} homologue was also identified as a minor component in a mass spectrometric analysis of the crude toxin. Fusetani and Hashimoto recently isolated, in addition to pahutoxin, its homologue as the secondary toxin in the secretion of a Japanese boxfish, *O. immaculatus* [10]. The additional methylene in the homologue, named homopahutoxin (**3**), resided in the side chain in this case, that is, propionate replaced an acetate in pahutoxin.

3 Soapfishes

Soapfishes are so called because they release a surfactant secretion which makes sea water foam in a small container where they are kept. They used to be classified as members of the bass family, Serranidae, but were separated into an independent family, Grammistidae, based on histological characteristics of their skin with its peculiar secretory cells [11]. A representative of this family is the golden striped bass *Grammistes sexlineatus*, which has showy markings; a number of longitudinal bright stripes against a blackish background. When a juvenile *G. sexlineatus* was fed to a lionfish, *Pterois volitans*, the lionfish at once took the prey into its

mouth but immediately spat it out and never went for it again [11]. This observation indicated learning had taken place in the lionfish, where the showy markings of the bass were presumed to assist in the learning. Because of the attractive appearance of this fish, it has been highly valued as a pet, and consequently has become notorious for killing other fishes during transportation. Members of this family, in general, have such conspicuous markings, so-called aposematic (or warning) coloration, that they are not attacked by mistake. Soapfishes *Aulacocephalus temmincki*, with a yellow dorsal band on a lapis blue background, and *Diploprion bifasciatum*, with a black traverse band separating the pale yellow fore and the darker yellow hinder halves of its body, are also known as pets. The bright coloration also draws the attention of Japanese coastal fishermen so that they do not place them with other fishes in the same tank.

A soapfish, *Pogonoperca punctata*, is occasionally caught accidently during game fishing around the Ryukyus of southern Japan and it resembles sea basses with its protruding lower jaw. It appears a relatively conservative member of the family, but juvenile individuals of this soapfish are still conspicuous with large white spots on a purple background. It was once considered as a ciguateric fish in the Ryukyus. Ciguatera is an endemic mass fish poisoning, where outbreaks of a toxigenic benthic dinoflagellate turn fishes of a region toxic one after another through biological concentration of the toxin along the food chain [5, 11, 12]. Large fishes in the late stages of the food chain are common causes of human intoxication, and commonly their liver and other viscera are particularly toxic. Hashimoto and Kamiya, who first examined this soapfish as a possible ciguateric fish, noted strong ichthyotoxicity and hemolyticity in the extract of its skin while the viscera were not toxic [13]. Mucous secretion obtained from its skin indeed caused ciguateric symptoms in cats when administered orally, thereby confirming its local reputation at that time.

Ichthyotoxicity of this secretion, extracted into *n*-butanol from the aqueous suspension, as in the case of pahutoxin (see above), displayed three maxima in its countercurrent distribution between 2-butanol and 1% aqueous acetic acid, and the respective toxic principles, named grammistins, were purified by gel filtration on Sephadex LH-20 [14]. The major principle among the three, grammistin A, was still shown to separate further into two toxic constituents by cation exchange chromatography [11]. Respective grammistins were shown to be peptidic compounds of molecular weights around 4 kDa, being composed predominantly of hydrophobic amino acid residues. Based on incomplete recovery of amino acids in the hydrolytic analysis, approximately 80% in each case, and the positive coloration with Dragendorff reagent, these molecules were considered to contain also nonpeptidic moieties with tertiary or quarternary amines [14].

Prior to the isolation of grammistins in Tokyo, Maretzki and del Castillo at the University of Puerto Rico reported properties of the mucus toxin from an Atlantic soapfish *Rypticus saponaceus*, or *pez jabón* as it is locally called [15]. The soapfish, similar in appearance to *Pogonoperca punctata*, was also known among Puerto Rican fishermen as it kills other fishes when placed in the same tank. Its mucous secretion was not only ichthyotoxic, but also lethal to mice in intraperitoneal injection. The toxicity of the secretion was gradually lost in neutral media presumably due to enzymatic decomposition as in the case of the boxfish secre-

tion, since it was found fairly stable in acidic media of *p*H 3–4. The authors obtained a toxic gummy preparation through extraction with *n*-butanol from sea water, into which the secretion was exuded; removal of inorganic salts as a precipitate resulted in an air-dried extract. This gummy preparation was soluble in aqueous hydrochloric acid but yielded a toxic precipitate when *p*H was raised above 8. The hydrolytic amino acid analysis showed it also to be largely peptidic and rich in hydrophobic amino acid residues, but total recovery of amino acid therein and the nitrogen content in Kjeldahl elemental analysis were both too low for the preparation to be free from nonpeptidic components. Rationalizing these results and those from gel filtration and dialysis, they concluded that a polypeptide or a conjugated protein was responsible for, or at least associated with, the toxicity of the secretion. This toxin was subsequently further purified through gel filtration by Toro Goyco et al. who named it rypticin, which was shown to be similar to grammistins from *P. punctata* in size as well as in the amino acid composition [16].

The hemolytic factor in the secretion of the golden striped bass, *G. sexlineatus*, was also analogous to grammistins based on its behavior in gel filtration and countercurrent distribution [17]. On the other hand, while the secretion was originally reported to be antibacterial against *Escherichia coli* [18], grammistins were shown to be neither antibacterial nor antiviral [11]. This discrepancy implies that secretions of grammistid fishes contain other bioactive components in addition to grammistins or grammistin-like substances. In practice, a lipophilic factor of low molecular weight has been isolated from the secretion of *Diploprion bifasciatum* in addition to the hydrophilic hemolysin [11]. Structural determination of this factor, later found to be strongly cytotoxic in the assay on fertilized echinoderm eggs, besides being hemolytic and ichthyotoxic, is underway at the present using modern spectroscopic techniques [19]. The compound was shown to be of molecular weight 590 Da in electron-impact and field-desorption mass spectrometry and to possess a fatty chain moiety from the NMR data, and released no amino acids in conventional hydrolysis, though several nitrogen atoms have been indicated to be present. It should be noted that no oxygen atom was indicated aside from amide and possibly carboxyl functionalities in its ^{13}C NMR spectrum. The hemolytic factor in the secretion of *Aulacocephalus temmincki* has been indicated by gel filtration to be of a higher molecular weight than grammistins. In addition, countercurrent distribution of this secretion exhibited the presence of large quantities of lipophilic components of peptidic or phenolic nature, but without hemolytic activity, which is atypical among grammistid secretions [17].

4 Coral-Gobies and Clingfishes

Unlike other members of the goby family, Gobiidae, which are generally somber-hued and hidden in sandy bottoms or camouflaged by their color, coral-gobies, *Gobiodon* spp., are relatively showy with golden yellow coloration and are usually sheltered among stony coral branches. They were discovered to exude toxic secre-

tions on occasions in which other small fishes, damselfish for instance, were liable to die when placed in the same container with them or scooped with the same net where they had been handled. Their intact secretion rapidly lost its toxicity, like the others, and even the toxicity of its frozen sea water solution gradually faded [11, 20]. Hashimoto and Shiomi, aware of this instability from their experience in soapfishes, treated the scraped epidermis of these small fishes with ethanol in reflux prior to further extraction with aqueous ethanol and isolation of the toxin from the extract [20]. The collective extract from some 500 individuals of a coral-goby *G. quinquestrigatus*, weighing 500 milligram on average, collected around Ishigaki Island in the Ryukyus was defatted with diethyl ether from its aqueous suspension and then salted out to yield a bioactive precipitate. The major hemolytic fraction was obtained in the same way as grammistins, namely, by countercurrent distribution and subsequent gel filtration. This fraction, shown to be a mixture by its thin layer chromatograms, was separated through cation exchange to yield two active constituents, the major and more basic of which was intensively examined with regard to its chemical structure and shown to be a very similar substance to grammistins, as was indicated by its chromatography behavior, with a small difference in its amino acid composition [11].

Hemolytic factors from secretions of other congeneric species (*G. rivulatus, G. okinawae*, and an unidentified species) collected in the same region were also investigated, all resulting in analogous compounds which were indicated to comprise peptidic regions, being rich in hydrophobic amino acid residues, and hydrophobic moieties responding positively to Dragendorff reagent [20]. Moreover, a coral-goby of a different genus, *Paragobiodon echinocephalus*, which still retains a bright yellow color and is sheltered among coral branches like *Gobiodon* spp., was examined as a control. In concert with the histological difference observed in the epidermis of the two genera, no grammistin-like component was present in the extract of the epidermis of this control goby [20]. The contrast between the developed squamae possessed by it and the atrophied ones in *Gobiodon* spp. agreed well with the hypothesis of Cameron and Endean [2] mentioned in the Introduction.

Clingfishes are small and slender fishes, several centimeters in length, which set up their sheltered habitats among coral branches or spines of sea urchins, and constitute their own family, Gobiesocidae. Randall noticed that two species of them commonly seen among spines of the needle spine sea urchin *Diadema setosum* along the southern coasts of Japan, *Diademichthys lineatus* and *Lepadichthys frenatus*, release considerable amounts of mucous secretion when disturbed [21]. While they appear to be completely protected by the long pointed spines, they also have the common characteristics with the other fishes possessing the defense secretion, such as their lack of squamation. Isolation of ichthyotoxic components from the secretion of *D. lineatus* was attempted by Hori et al., giving an ichthyotoxic and hemolytic grammistin-like preparation with chromatographic homogeneity through the *n*-butanol extraction from the secretion, gel filtration, and subsequent column and thin-layer silica gel chromatography [21]. Phenylalanine, lysine, and histidine were predominant amino acids in its hydrolyzate, and the total recovery of amino acids was 44%, much less than the case in grammistins and the similar substances from the secretion of coral-gobies. While this may well

be attributed to a larger nonpeptidic portion of the molecule, it may also reflect the possibly lower purity of this preparation [22]. Although further investigation of this substance has been discontinued due to difficulty in collection of the fish, a name, gobiesocin, was conditionally proposed for the factor on its ubiquity among members of this family. Minor hemolyticity of the secretion was partitioned into diethyl ether, and was concentrated by silica gel chromatography to a fraction being rich in fatty acids.

5 Soles of the Genus *Pardachirus*

Juvenile fishes of the order Pleuronectiformes, or flatfishes, are nectonic with their eyes still separated on both sides of their bodies as in many other fishes, but one eye migrates via the brow to the other side as the fish grows, and at the same time the body lies on its side with the blind side down. While popular food flatfishes, halibuts and flounders, belong to a suborder Pleuronectoidei, soles and tonguefishes belong to another suborder Soleoidei; many of them resembling bovine tongues and thus are so called in Japanese, *ushi-no-shita*. Classifying the latter further, the popular soles in French restaurants are lefteye and belong to the tonguefish family, Cynoglossidae, and righteye soles, *Pardachirus* spp., belong to the sole family, Soleidae. Even though flatfishes have adopted the prostrate posture, their undulant swimming motion persists in the same direction as other fishes, that is, vertical. With this undulation they ingeniously bury themselves in the sand, and thus successfully evade detection by most predators. Sharks, skates, and rays, however, possess aggregate sensory organs located at their snouts and called Lorenzini's ampullae with which they manage to receive even minute electromagnetic waves emanating from live vertebrates [23]. This sensory structure is also suspected to function as a compass through detection of electric fields which are generated by the induced water current in their own swimming and by terrestrial magnetism. It has been shown experimentally that sharks attack electrodes buried in the sand even preferentially to food placed nearby. It

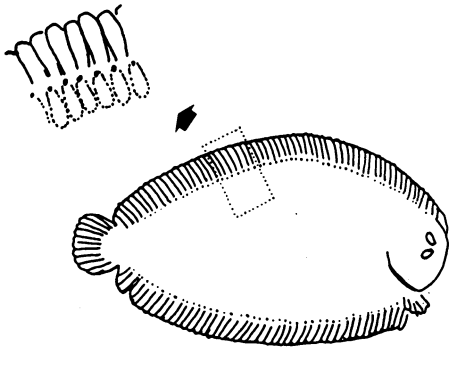


Fig. 1. Location of the toxin glands in *Pardachirus* spp.

is for this reason that oceanic cables often suffer from shark attacks. Flatfishes are a favorite food of elasmobranch fishes; therefore chemical defense of *Pardachirus* spp., as described in the following, can reasonably be expected to have developed against predatory sharks.

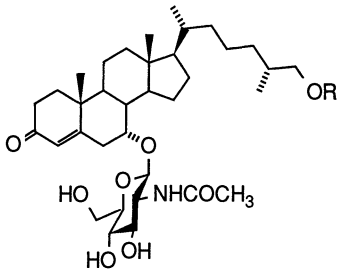
Moses sole, *P. marmoratus*, had been reputed as a shark repellent fish in the Red Sea; it was named after Moses, who led the Jewish people safely across the Red Sea from Egypt back to Israel, as described in Exodus in the Old Testament [24]. The fish became known to the rest of the world, when Clark reported her observation on a reef white-tip shark *Triaenodon obesus*, which attempted to attack the sole suspended on a string in an observatorial aquarium, but was repelled by the sole at the moment when its jaws opened widely [25]. The sole was recovered without a single scar. A congeneric *ushi-no-shita (P. pavoninus)*, distributed along tropic coasts of the eastern Indian and western Pacific Oceans, was also known in the Ryukyus to secrete ichthyotoxic mucus from its "fin organs" [26]. Clark and George intensively investigated the morphology of the two species of sole and verified the presence of sacciform secretory glands along the base of their dorsal and anal fins (pelvic fins of flatfishes are only remnant at the front of abdomen; the fin on the ventral side, i.e. the right edge of the body in the case of righteye flatfishes, is the anal fin) [27]. Small pores are visible to the naked eye, corresponding to individual secretory glands along both edges of the body, a pair of which from both sides of the body in turn corresponds to each fin spine (Fig. 1). These pores form a characteristic of this genus. A milky secretion comes out of these pores when the fishes are disturbed by being taken out of the water, for instance, or when the base of the fins is squeezed. These investigators considered the soles to be unique in their highly developed multicellular toxin glands and therefore to have evolved independently of the other fishes with toxic mucous secretion. These glands, however, may have resulted merely from localization of secretory cells with mucous toxins, as are shown in other examples such as the labial glands in trunkfishes.

Chemical studies of the Moses sole secretion started at the Hebrew University in Jerusalem along with biological studies. The secretion possessed strong ichthyotoxic and hemolytic activity, which was, as is usual, so unstable in aqueous suspension that only one seventh survived lyophilization of the secretion [28]. Gel filtration of the lyophilizate furnished an ichthyotoxic component, which was homogeneous in electrophoresis and ultracentrifugation and was named pardaxin [29]. This toxin was at first reported to be a protein of approximately 17 kDa based on its behavior in gel filtration, and was believed to be so until the recent revision described later. Diverse bioactivity has been reported on the crude Moses sole secretion or pardaxin prepared therefrom, apart from ichthyotoxicity and hemolyticity; this is in contrast to rather tardy progress made in its chemistry. Activities include contractility on smooth muscles [30, 31], inhibition of a $(Na^+\text{-}K^+)$-ATPase in homogenized fish gills [32], multiple activity on virions such as vesicular stomatitis virus [33], and so forth, all of which, however, can be attributed to the destruction or disturbance of plasma membranes resulting from the detergent-like action of pardaxin [34]. Accordingly, bioactivity of pardaxin has been compared [28, 35] to the well studied hemolytic factor of bee venom from *Apis mellifera*, melittin [36]. Shark repellency of this secretion as its lyophilizate

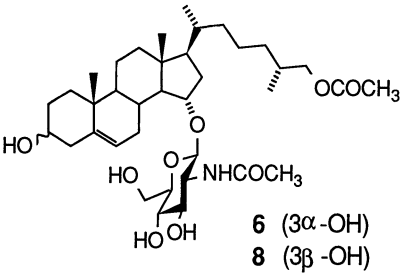
has been corroborated in an assay method invented by Gruber at the University of Miami, using immobilized lemon sharks, *Negaprion brevirostris* [37]. Persuaded by the strong surfactant property of this secretion, Gruber and Zlotkin screened a variety of commercial detergents in order to pinpoint a potential shark repellent agent; this led to sodium lauryl sulfate (SDS) as the most potent shark repellent tested to date [38]. SDS has been shown to be effective also in a field test on wild man-attacking blue sharks *Prionace glauca* [39].

In contrast to the conspicuous markings of the fishes mentioned earlier, *Pardachirus* spp. have protective coloration as do the other flatfishes. In the author's own experience while collecting *P. pavoninus* near Ishigaki Island, the Ryukyus, the sole had the camouflage color of the white sandy bottom, and thus required intense attention to spot it. In fact, the white ground is unnecessary for them to be camouflaged since they could blacken substantially to match the bottom of a concrete tank when placed there. It was so much the worse when the sole was buried in the sand as usually was the case. Nevertheless, the shape of its rim was just visible on close scrutiny of the sand with only its eyes sticking out. Once it had been spotted, it was much easier to capture it by holding up a hand net in front of its head, because it leaped into the net when prodded from behind. Even in the event of failure, it swam only for a meter or so before burying itself again. Its swimming speed was slow enough for one to chase it easily with swim fins. Soles of this genus are the most sluggish of all the flatfishes according to Clark and George [27]. *P. pavoninus* is considered to be too bitter to eat and therefore does not appear in Japanese markets even though it is occasionally found in trawls in the Ryukyus. According to a local fisherman at Ishigaki, however, it is delicious as *sashimi* with its skin sliced off. Being tasteful is not a disadvantage despite its sluggishness, because it possesses a chemical defense.

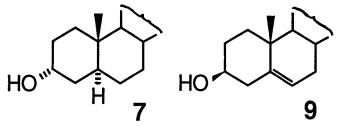
The secretion was milked from captured *P. pavoninus* on the spot, frozen immediately, and transported to the laboratory, where it was lyophilized. Ichthyotoxic and hemolytic activity of the lyophilized secretion, being too unstable, as expected, to be quantified reproducibly, was found distributed between the acetone precipitate and the supernatant. While the bioactivity of the precipitate was still unstable and considered to arise from pardaxin-like material, that of the supernatant was extracted from water by ethyl acetate. The organic extract was fractioned through a silica gel column to furnish an active fraction which still contained 80% of the mass applied to the column. This fraction was further separated by reversed-phase chromatography into its six components, all of which turned out to be ichthyotoxic. That is, these active components constituted 80% of the total lipophilic contents in the secretion. ^{1}H-NMR and other spectroscopic data on these active components, named pavoninin-1 to -6, showed them all to be steroid monoglycosides of analogous structures (**4** to **9**, respectively), which were established by the aid of chemical conversions [40, 41]. Speculation by Thompson that the boxfish toxin might be a steroidal saponin [4], based on the surfactant properties of the secretion rather than chemical evidence, had come true. The most abundant constituent was pavoninin-5 in agreement with its closest chemical structure to cholesterol, followed by its epimer, pavoninin-3, and then pavoninin-1. Although the least abundant, pavoninin-2, was possibly an artifact produced from pavoninin-1, it retained ichthyotoxicity and had already existed in the



4 (R = $COCH_3$)
5 (R = H)



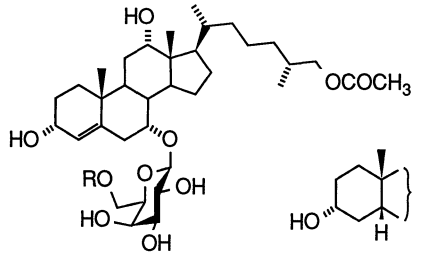
6 (3α-OH)
8 (3β -OH)



7 **9**

crude lyophilizate of the secretion based on its thin-layer chromatogram. Shark repellency of the mixture of pavoninins was indicated in a behavioral assay on a dog shark *Mustelus griseus*, which had been attracted by a bait [41].

In view of this discovery it seemed reasonable that a similar lipophilic shark repellent factor may be present in the Moses sole secretion in addition to pardaxin, which had been considered to be the only factor until then. Gruber, who also observed the shark repellency of pavoninins in his own assay system [37], prepared a lipophilic extract from the lyophilized Moses sole secretion following the procedures employed in isolation of pavoninins, and verified its ichthyotoxicity [39]. Further purification was achieved by the present author and resulted in the isolation of another series of steroid monoglycosides, which were named mosesin-1 to -5 [42]. Their structures, **10** to **14**, were determined by spectroscopic correlation with pavoninins and cholic acid. The two series differ in their sugar moieties; *N*-acetylglucosamine in the pavoninins is replaced by galactose or its monoacetate in the mosesins. Cholic acid may be involved in the biosynthesis of these compounds. While the ichthyotoxicity of the mosesins was only comparable to that of pavoninins in the Japanese killifish assay (*Oryzias latipes*), the mixture of the mosesins exerted several times greater shark repellency in Gruber's assay methods, where commercial saponin was shown to be a weaker shark repellent than these glycosides (Table 1) despite their equivalent hemolytic potencies. The



10 (R = CH_3CO)
14 (R = H)

12 (R = CH_3CO)
13 (R = H)

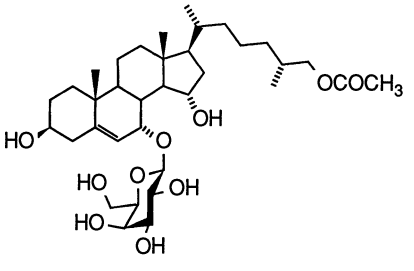


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Table 1. Shark repellent activity against lemon sharks *N. brevirostris* in the methods by Gruber [37–39]

Repellent samples	Threshold concentrations ($mg\ mL^{-1}$)	
	Feeding assay[a]	TI test[b]
Pavoninins	~5	1.0~5.0
Mosesins	2.0~5.0	0.1~1.0
The lyophilized Moses sole secretion	0.8~3.0	0.66[c]
Commercial saponin	~50	100[c]
SDS	0.2~2.0	0.45[c]

[a] Repellency against hungry sharks attracted by a bait.
[b] Termination of tonic immobility.
[c] ED_{50} values.

point of attachment of the monoglycoside to the hydrophobic steroid nucleus and its orientation, or the resulting dipole moment of the molecule, are perhaps important for these molecules to manifest shark repellency. At any rate, repellent activity was clearly shown not to parallel ichthyotoxicity or hemolytic activity.

The shark repellency of the mosesins does not exceed that of the original crude secretion. Therefore, pardaxin must still be significantly involved in the repellent property of the secretion. Isolation of the pardaxin-like component from the acetone precipitate of the *P. pavoninus* secretion by Thompson et al. was hampered at the beginning by instability of its toxicity in neutral aqueous media. This problem was eventually solved by gel filtration of the acetone precipitate through Sephadex G-150 directly without desalting, thus yielding a stable ichthyotoxic preparation, which also showed moderate shark repellency to reef white-tip sharks *Triaenodon obesus*. The elution volume of this preparation corresponded to what had been reported for pardaxin [29], the apparent molecular weight being estimated around 10 kDa by fine calibration. The material was further separated by anion-exchange chromatography into two active fractions containing the major components of different isoelectric points. They were purified by reversed-phase HPLC, where the long retention of these components indicated their strong hydrophobicity. The two major components thus isolated, together with a minor one from the major and more acidic anion-exchange fraction, were named pardaxins P-1 to P-3 in accordance with pardaxin in their chromatographic behavior, and the species name of the sole, *pavoninus*. All were shown by analysis of their acid hydrolyzates to be peptides of 33 amino acid residues each; this result is compatible with their electrophoretic movement in an SDS/polyacrylamide system. The apparent molecular sizes shown in gel filtration are accounted for by their oligomeric association. Automated Edman degradation of each peptide revealed their amino acid sequences as shown in Fig. 2 [43]. The major peptides, pardaxins P-1 and P-2, were subsequently synthesized by the solid-phase method, and their chemical structures were validated by identical HPLC retention, spectroscopic data, and hemolytic activity [44]. These peptides did not surpass pavoninins in ichthyotoxicity and hemolyticity, when compared by effective median molar concentration.

```
                                        5                    10
Pardaxin  P-1  Gly-Phe-Phe-Ala-Leu-Ile-Pro-Lys-Ile-Ile-Ser-
      "   P-2   -   -   -   -   -   -   -   -   -   -   -
      "   P-3   -   -   -   - -Phe-  -   -   -   -   -   -
      "   M-1   -   -   -   -   -   -   -   -   -   -   -

                                   15                   20
Pardaxin  P-1  -Ser-Pro-Leu-Phe-Lys-Thr-Leu-Leu-Ser-Ala-Val-
      "   P-2   -   - -Ile-  -   -   -   -   -   -   -   -
      "   P-3   -   -   -   -   -   -   -   -   -   -   -
      "   M-1   -   -   -   -   -   -   -   -   -   -   -

                              25                   30
Pardaxin  P-1  -Gly-Ser-Ala-Leu-Ser-Ser-Ser-Gly-Glu-Gln-Glu
      "   P-2   -   -   -   -   -   -   -   - -Gly-  -   -
      "   P-3   -   -   -   -   -   -   -   -   -   -   -
      "   M-1   -   -   -   -   -   -   -   - -Asp-  -   -
```

Fig. 2. Amino acid sequences of pardaxins. "–" represents the same residue as pardaxin P-1

The amino acid sequence of pardaxin from the Moses sole secretion was first reported for the ten residues nearest its amino terminus [35], which coincided with that seen in pardaxins P-1 and P-2. It was then revised by Lazarovici et al., who also reported the presence of a minor pardaxin, at the eighth and tenth residues, while reporting the molecular sizes of the two pardaxins (pardaxins I and II) to be similar to pardaxins P based on SDS electrophoresis [45]. Thompson et al. independently isolated the major peptide, which chromatographically corresponded to pardaxin P-1, from the acetone precipitate of the Moses sole secretion. Contrary to the revision by Lazarovici et al., its amino acid sequence obtained by automated Edman degradation and by the aid of thermolytic digestion showed its difference from pardaxin P-1 to be only at the third residue from the carboxyl terminus, where the glutamic residue in pardaxin P-1 was replaced by an aspartic acid residue (Fig. 2) [46]. This peptide, which they believe to be the originally reported pardaxin, has been named pardaxin M-1, reserving "pardaxin" as a general term.

These amino acid sequences, where the carboxyl-terminus regions are totally hydrophilic while the remainder is fairly hydrophobic, make one think of detergents, and from this point of view the pardaxins resemble the bee venom, melittin (**15**). Therefore, an early suggestion by Primor et al. that pardaxin is a substance with melittin-like bioactivity was structurally supported. While it is improbable for these two kinds of bioactive peptides to be genetically related across a long phylogenetic distance between insects and fishes, it is worth noting that such distant animals use similar substances for the same purpose, defense, in different ecological environments. These peptides also share common physical characteristics, such as strong surfactant properties and oligomer formation in buffer solutions giving the appearance of larger molecules. These common features arise not only from the similarity in their amphiphilic amino acid sequences (primary struc-

```
                    5                   10
Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-

  15                  20                  25
-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln-NH2

                    15
```

tures), but also from resemblance in their estimated conformations (secondary structures). It was suggested that pardaxins P adopt α-helical structures in the middle regions of their sequences on the basis of their circular dichroic spectra and as a result of an estimate by an empirical predictive method, where the probability of each secondary structure is calculated for a local sequence based on statistics in proteins of known crystalline structures. Axial projection of the predicted helix along its axis exposes clear polarization of hydrophobic and hydrophilic residues in an equatorial direction (Fig. 3). Their extremely strong surfactant property is believed to originate rather from this equatorially amphiphilic helix, and the oligomerization in buffer solutions or interaction with lipid bilayer membranes are presumably brought about through its large hydrophobic surface [44].

The respective roles of the steroid glycosides and of the peptidic pardaxins in the defense mechanism still remain unclear. The stronger shark repellency shown by the crude Moses sole secretion than either of those by mosesins or pardaxins P commands either some kind of synergistic effect between two factors or existence of another overlooked (therefore not ichthyotoxic) or unstable factor. Otherwise the Moses sole pardaxin(s), whose shark repellency has not been assayed yet in stabilized form, must have a stronger shark repellency than the other tested factors. Also yet to be found in the future is the receptor site in the shark.

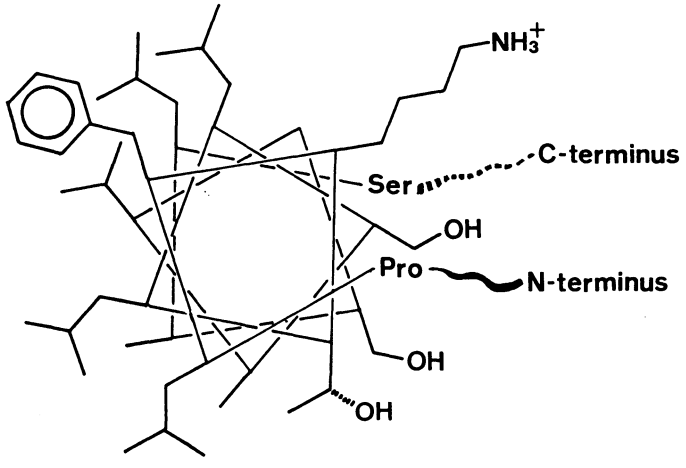

Fig. 3. Axial projection of the α-helix estimated in pardaxins. The isobutyl at the bottom is replaced by (*S*)-*s*-butyl in pardaxin P-2

The immobilized shark reacted against repellents whether the sample solution was dosed into the oral cavity or the nasal sac. This means little in reality because all probable target organs are connected to the oral cavity by sea water, through which chemical solutes are diffused quickly. Primor et al. suspected the gills, where inhaled water at the mouth is exhaled in respiration, to be the place where sharks sense these repellents on the basis of the toxic action of pardaxin on the gills of sharks [47] as well as of teleost fishes [32, 48]. In supporting evidence, a reef white-tip shark, to which was administered a preparation containing pardaxins P into its oral cavity, flapped its gill opercula in rapid succession while this usually sluggish shark was actively swimming. Thompson speculated that the repellents act on pain receptors in certain mucous membranes of sharks, as melittin is suspected to act as a pain-inducing factor [49].

6 Puffers

It is hardly necessary to mention that puffers, belonging to the family Tetraodontidae, possess a potent poison, tetrodotoxin; from recent evidence it appears that they use this poison for their own defense. Puffers, which belong to the same order Tetraodontiformes as trunkfishes and boxfishes, would have an advantage from a chemical defense, as they are relatively conspicuous, solitary, and yet sluggish. However, their chemical defense has been considered a passive one at the species or family level, where the predators of puffers or their eggs have diminished in number or have become extinct due to poisoning, or where predators have learned that agony ensues on eating them. On the other hand, chemical defense of puffers as individuals had also been suggested for a fairly long time. Eger at University of Hawaii described in his M.S. thesis back in 1963, when the structure of tetrodotoxin was yet to be solved, that a puffer *Arothron hispidus* released a toxin resembling tetrodotoxin when it was placed in dilute hydrochloric acid. He also described that the puffer possessed secretory cells in its epidermis which he called serous glands [5, 11]. Another puffer *Canthigaster rivulatus* and a porcupinefish *Diodon hystrix*, a member of a different family, Diodontidae, were also described as releasing the toxin. When Randall [21] fed a small puffer, *C. solandri*, to a red snapper *Lutjanus bohar* in captivity, the latter took the puffer into its mouth but immediately ejected it forcefully. Scientific identification of the toxin as tetrodotoxin, however, has been a recent event resulting from the advent of modern analytical technology, which has led to a tetrodotoxin analyzer [50].

Kodama et al., who applied electric shock to many species of puffers in an aquarium, noted the release of tetrodotoxin from all the species whose skin is known to be poisonous [51, 52]. Their histological investigation of the skin of toxin-releasing puffers such as *Takifugu pardalis* confirmed the existence of peculiar secretory cells, which were not stained by any common fixative, and which they called TTX cells [52]. Some of these cells were observed to agglutinate and invaginate to form "TTX glands." These cells were not found in the skin of *T. xanthopterum* which did not release the toxin, but some were seen in that of *T. rubripes*, though not in sufficient abundance to form glands, despite no release

of the toxin from it. The skin of these two species is considered to be safe to eat. In a few species less toxic tetrodotoxin analogs are known to coexist in poisonous organs, including the skin [53]. Tetrodotoxin detected in TTX glands, however, was free from them while the analogs were also detected in the extract of the skin, indicating that these cells specifically store tetrodotoxin. As an explanation of the specific storage of the toxin in the skin, the possibility of a detoxification mechanism was suggested in addition to defense, on the basis that the phenomenon is limited to some species. It is also possible on the other hand that some species merely take advantage of resembling the poisonous species in order not to be attacked.

Instead of electric shock, Saito et al. stimulated a poisonous puffer, *T. niphobles*, by wrapping it in gauze and prodding it while held, which also resulted in the release of a large amount of tetrodotoxin [54]. Little toxin was found left in the skin of the treated puffer. This observation supported the suspected property of individual variation in toxicity, which has been known for the puffer skin due to different handling following capture [11]. When this stimulation was applied to cultured *T. rubripes*, which had been made poisonous by feeding it livers of toxic species, the puffer released tetrodotoxin. This species is considered not to have tetrodotoxin in the skin, and is, in fact, the major source of the skin served in Japanese *fugu* restaurants.

As is well known, puffers inflate like a ball when they sense danger, as indicated by their name, in order to intimidate an enemy. Eger inferred that this action presses their secretory glands on the body surface, thus resulting in the release of their content, but inflation is not necessary for them to release the toxin according to Kodama et al. [52]. Puffers became apparently insensitive to the electric stimulation or too tired to inflate after repeated application for two hours, but the shock still continued to induce the release of the toxin thereafter.

Although tetrodotoxin is toxic to fishes when intramuscularly injected, it must directly act on their nervous system and thus is not an ichthyotoxin in the usual sense, where this term implies stronger toxicity to fishes than to mammals. Besides being different from other repellent defense substances of fishes in this point, this toxin lacks surfactant and hemolytic properties; therefore the mechanism of its repellent action is presumed to be also different. Sea hares and certain insects, such as stink bugs, are known to accumulate their defense substances which originate from their food. It has been experimentally shown that tetrodotoxin in puffers is also exogenous [55] and that the toxin is in fact a bacterial product [56]. This is another difference between this toxin and other repellent substances of fishes, which are probably synthesized by the animals themselves.

7 Venomous Fishes with Skin Toxins

Traumatization of the enemy by venomous spines, through which a toxin is injected directly into its body, is a well-known form of chemical defense in certain fishes, though it is associated with mechanical defense. Halstead separated the toxins in this category of defense, as named them venoms or acanthotoxins, from

crinotoxins, which reach the enemy through the environment [5]. When fishes are involved, they are called ichthyocrinotoxins. While the two apparently differ, the resemblance between melittin and the pardaxins in their biophysical characteristics may well suggest that possible common features exist between them. Cameron and Endean postulated that secretory organs of these two kinds of toxins had evolved from common "proteinaceous cells" in different fashion; one kind agglutinating and becoming conjugated with fin spines to form venomous glands [2]. In contrast to the proteinaceous nature of the fish venoms, as far as known to date, nonpeptidic molecules of low molecular weight have been identified as ichthyocrinotoxins. Provided these findings are confirmed, the two biological types of defense substances must therefore have diverged long ago during evolutionary history, as judged from the difference in their chemical types. On the contrary, it is possible that in the near future further chemical investigation of venoms will lead to discoveries of small molecules similar to those known as ichthyocrinotoxins. As a provisional implication of that, the presence of an ichthyotoxin of a low molecular weight has been reported in the *n*-butanol extract of the venomous spines cut out from live specimens of a lionfish *Pterois volitans* [57]. Except for its ultraviolet absorption at 287 nm and molecular weight of 327 Da as estimated by mass spectrometry, no information of its chemical structure has been available due to the minuscule quantity of isolated material.

Classification of venomous fishes can be ambiguous because many other fishes also sting with their spiny dorsal fins. Many of those possessing distinct venomous spines, however, have toxin-secreting cells in their skin in addition to venomous glands. Stonefishes of the genus *Synanceia*, for instance, possess secretory glands in their epidermis, which are independent of their venomous glands and are called "granular glands." Cameron et al., who separated the secretion from the granular glands of *S. trachynis* by centrifugation, noted strong toxicity of the supernatant to fishes following intramuscular injection, which was accompanied by necrosis at the point of injection [58]. The supernatant was also toxic to invertebrates which had been placed in the solution. Because of its weak ichthyotoxicity, when applied externally, its hemolytic activity, and its bitter taste, the toxin was believed to function as a feeding deterrent which acts on the gustatory senses rather than as a repellent; hence it was believed to resemble tetrodotoxin of puffers as a defensive agent. More powerful centrifugation of the toxic supernatant yielded a weakly hemolytic precipitate which was also neurotoxic to the isolated guinea-pig ileum; the lethal and necrotic toxicity remained in the supernatant, thereby indicating that at least two bioactive substances are present. It is not known if these substances are related to the venom which is injected through the spines of this stonefish.

Many catfishes, or members of the order Siluriformes, have venomous spines and independent secretory cells in the skin [5]. Al-Hassan et al. at Kuwait University observed vasoconstrictory toxicity, as shown on isolated mammalian arteries, in the "gel" obtained from the skin of the Arabian Gulf catfish *Arius thalassinus*, and attributed it to a factor with cholinergic activity and one involved in the release of prostaglandins [59, 60]. The cholinergic bioactivity was resistant to both heat and trypsin, and therefore it was suggested that it arises from a nonproteinaceous substance of low molecular weight. The body surface of this fish, includ-

ing the venomous spines, is covered by the toxic gel, which presumably is injected into the body of a victim along with the venom. Shiomi et al. at the Tokyo University of Fisheries meanwhile reported the presence of a similar toxic secretion as well as peculiar secretory cells in the skin of an oriental marine catfish, *Plotosus lineatus* [61]. This relatively small catfish is famous in that thousands of juvenile individuals school giving the appearance of a huge black fish. The secretion contained toxins of approximate molecular weight 12 kDa, and a hemolytic factor of higher molecular weight, around 180 kDa. The toxin was lethal to mice on intravenous injection and caused edematous inflation at the leg of mice when a sublethal dose was injected subcutaneously. The toxin was separable into two components by anion exchange. Both high and low molecular weight factors of the crude secretion were destroyed by heat, but were moderately stable in neutral media and also resistant to a bacterial protease. Though we have no information on the stability of the purified factors, the resistance to bacterial protease should be noted, since this property is the opposite to that observed in many other ichthyocrinotoxic secretion.

Members of the toadfish family, Batrachoididae, are venomous fishes of somewhat repulsive appearance, with a large depressed head; each individual has two venomous spines on the back and two more at the base of the pectoral fins, but the toxicity of the venom is said to be weak [5]. In the case of the Caribbean oyster toadfish, *Opsanus tau*, it is even uncertain whether its spines are connected to venom glands. However, it possesses secretory glands, called axillary and pectoral glands, which are open to the body surface and considered to emit an ichthyotoxin, in front and behind its pectoral fins. It does not release its toxin as readily as other ichthyocrinotoxic fishes, but rough treatment, such as agitation with a glass rod, can cause release of its ichthyotoxic mucus [3]. Nair et al., who collected this fish at Jamaica Bay, recovered ichthyotoxicity from the crude secretion in a nonpolar fraction obtained through silica gel chromatography [62]. As a result of a GC-MS analysis of the fraction, the major component was shown to be 3-octanone (ethyl pentyl ketone). This simple compound, also known as a minor constituent of an ant alarm pheromone [63], was confirmed to be ichthyotoxic by testing of a commercial sample. Three individuals of a killifish, *Fundulus heteroclitus*, placed in a one part-per-million solution of 3-octanone started gasping immediately and all appeared to become immobile at the bottom within eight minutes. Replacement of water at this moment, however, revived them to their original agility in two minutes. This observation was regarded as an indication that this substance acted on the central nervous system of the fishes. GC-MS analysis of the active fraction also showed 3-decanone and 5-dodecanone to be present as minor constituents.

8 Miscellaneous

A moray eel, *Gymnothorax nudivomer*, family Muraenidae, releases a hemolytic secretion from its body surface, but extreme instability of the activity has prohibited chemical characterization of the factor; it is probably a protein of a high

molecular weight based on its behavior in gel filtration [11]. Peculiar cells have been observed in the epidermis of this and a few other species of moray eel.

Although similar secretory cells of an Atlantic hagfish, *Myxine glutinosa*, family Myxinidae, have been described in detail [5], no chemical study of the secretion has been reported. The ample mucous secretion released from these cells, represented by those called thread cells, is suspected to concern the parasitic nature of this fish [64]. The host fish may not be aware of the parasite because of the secretion. In a similar vein, the buccal secretion of lampreys, which are also parasites of large fishes and belong to the same class as hagfishes, Agnata, being considered to be the most primitive in the phylum Vertebrata, is known to prevent coagulation of the blood which they suck from the host [5, 64]. In addition to defense against predation, more diverse functions of the mucous secretion of fishes will possibly be discovered upon further investigation. Protection from bacterial infection may be inferred from the antibacterial activity of the secretion of the golden striped bass. It is also suspected that the secretion from the blind side of *Pardachirus* soles might be used to paralyze worms, which are their favorite prey [27].

9 Mechanisms of Repellency

In order for a chemical compound to be a repellent defense substance, it needs to be sensed by the predatory fishes; it must act on their sensory receptors, which are usually bound to bilayer phospholipid plasma membranes. The surfactant property seen in many cases must therefore assist the molecule to stick to the membrane. Affinity to the membrane, however, is not the only requirement for the molecule to be sensed. It has to affect the membrane potential of the sensory nerve cells, possibly through the receptor protein existing in their cell membrane; moreover, the action has to be quick in the case of repellents. I would suggest that it is the rapid timing that makes the difference between repellent activity and other bioactivity such as ichthyotoxicity and hemolyticity, which can be achieved by eventual disturbance of the bilayer membranes by bioactive molecules that are deeply buried. As for the surfactant molecules that are likely to form micelles in aqueous media, such as pahutoxins and SDS, the kinetic process from micelles to a bound form on the membrane, for instance monolayers, might be another important requirement to be sensed quickly.

On the other hand, surfactant activity is not always required, though kinetically preferred [65], to be recognized by the chemical sensory receptor as long as the molecule can quickly reach the receptor protein, as is the case with humans who are able to sense sugar and salt. Tetrodotoxin may belong to this group, where the bioactive substance directly attacks the receptor, whether it is the same as the target of surfactant repellents or not. Pungent chemicals are thus expected to repel fishes. In fact, powerful lachrymators were found effective in dispersing schools of a tropical perch, *Kuhla sandvicensis*; among them phenacyl chloride was the most potent [66]. These chemicals, however, are probably nonspecific and act by irritating mucous membranes of animals. Natural mechanisms appear to

be much more sophisticated than some myopic human ideas. Here we see that only the intruder at close range is eliminated without harm to the environment.

Although a number of hypotheses have been suggested regarding the mechanisms of action for the defense substances of fishes, of which only few have been chemically characterized, there is a great deal more to be learned. Many of these substances still remain to be investigated, even for their chemical structures to start with. Not only static chemical structures but dynamic structures, i.e. conformations and forms of association, in sea water and/or in lipid bilayer membranes are indeed needed for further clarification of the structure-activity relationship of the repellent substances. As another crux, knowledge of sensory physiology of fishes is also craved in such mechanistic studies.

10 Concluding Remarks

The author once attempted to transport live soles, *P. pavoninus*, by air from Ishigaki Island, where they were captured, to Okinawa Island, where facilities were available not only to keep them alive but also to examine and treat the secretion chemically. Even though each of seven specimens was placed with sea water in a plastic bag inflated with oxygen during transportation, they were all weak at the end of the journey, and died in a few days. They were probably poisoned by their own toxins, that had presumably been released on vibration caused by transportation. When the fish to be studied cannot be transported alive, certain efforts are required to keep the collected material in its natural state. That is crucial in cases where the substance to be investigated is unstable for a reason such as coexistent catabolic enzymes. This geographic and hence time distance between collection of material and its examination must be the cause of poor reproducibility and disagreement among researchers on the outcome of their investigations. It was fortunate in the above case that an aquarium with flowing water and a freezer were available for use at the site of the collection, thanks to the courtesy of the Yaeyama Branch of Okinawa Prefectural Fishery Experimental Station. While separatory and analytical technology has already made great strides, this problem of distance remains to be overcome in order to study the mechanisms of chemical defense and other chemical communication, not only among fishes but also among other marine organisms. Until a solution has been found, chemical elucidation of their mutual interactions will only proceed slowly to satisfy our curiosity.

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Subject Index