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BIOACTIVE MARINE NATURAL PRODUCTS, WITH EMPHASIS ON HANDLING OF WATER-SOLUBLE COMPOUNDS[1]

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ABSTRACT.—Recent progress in separation techniques has enabled us to work with water-soluble components which, more often than not, are responsible for biological activities found in marine organisms. Isolation techniques and chemical and biological aspects of water-soluble marine natural products will be discussed with some representative examples.

Marine organisms have proven to be rich sources of interesting organic molecules. A great number of compounds with diverse structural features and interesting biological activities has been reported and reviewed by several authors (1-3). However, one might notice immediately that the current list of isolated compounds is heavily in favor of lipid-soluble compounds or, at most, compounds with medium polarity. For example, Faulkner (3) has listed over three-hundred marine natural products, of which a mere six were *bona fide* water-soluble compounds. One of the reasons for such an imbalance may be due to the seemingly unlimited abundance of interesting lipid-soluble compounds in marine organisms. The real reason lies, however, in the considerable difficulties associated with the isolation and purification of water-soluble compounds, which discourage most traditional natural products chemists. The majority of reported marine compounds has been isolated by simple extraction partition with organic solvents followed by the rather classic chromatography.

Problems associated with the isolation of water-soluble compounds are by no means unique to marine natural products, but such inherent problems as the scarcity of starting organisms—many investigations were done with only a few animals—and the abundance of salts carried over from seawater into the water extracts, make the isolation of water-soluble compounds from marine organisms more perplexing. Nevertheless, we cannot ignore the water-soluble fractions so long as the objective is to isolate compounds with certain biological activity. This article is intended to assist those traditional natural products chemists who wish to venture into working with water-soluble compounds. Because many excellent compilations of marine natural products are already in the literature, this presentation is limited to typical molecules along with the isolation methods and some basic strategies.

PREPARATION OF EXTRACTS.—Because target compounds are extremely polar, aqueous media or strongly polar solvents such as MeOH must be used for extraction. In the case of aqueous solutions, an inevitable problem is bacterial and fungal growth, which often degrades the active components or gives false results in bioassays due to endotoxins produced by the microorganisms. The latter case is particularly disturbing in antitumor activity screening because many endotoxins (e.g., lipopolysaccharides, LPS) show distinct antitumor activities. Addition of alcohols and, if permissible, such fungicides as sodium azide will help prevent the microbial growth. If small amounts of immiscible organic solvents do not interfere with further processing, covering the extract with *n*-BuOH or toluene will also be helpful. Enzymes such as glycosidases, sulfatase, proteinase, and various oxidases are usually activated upon homogenization or

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freezing-thawing causing the transformation of compounds and a loss of desired activity. If heat does not destroy the activity, brief heating or autoclaving may alleviate the problem considerably. For example, boiling the starting organisms prior to extraction, was used in the case of the natural insecticide nereistoxin from sea worms (4) and antitumor glycoproteins from scallops (5).

The concentration of aqueous extracts also creates problems because of the great heat of evaporation of water. Prolonged, the evaporation process often leads to the destruction of activity and to microbial growth. In order to circumvent such problems, freeze-drying should be utilized extensively.

DESALTING.—Desalting is probably the most important, and often the most difficult, process. The presence of large amounts of inorganic salts causes interference in all chromatography systems, including gel-filtration. It also gives rise to false results in bioassays.

Various desalting methods used in biochemistry are, in most cases, not applicable for the isolation of low-molecular-weight compounds. With the molecular sizes not so different from inorganic ions, salts will appear almost at the same position as the desired compounds on the general desalting gels or membranes widely used in biochemical preparations. If the target molecules are soluble and stable in MeOH, crude desalting can be done by dissolving thoroughly dried residues—preferably freeze-dried—in absolute MeOH. Repetition of this procedure can remove the majority of salts to make further desalting much easier. For desalting small molecular compounds, gels with small matrices such as Sephadex G-10 or Bio-Gel P-2 are recommended. Careful fractionation on these gels often results in the separation of the desired compounds from the major salt fraction. If the desired compound is reasonably hydrophobic, one may try other nonionic resins, such as XAD-2, XAD-7, polyethylene or polypropylene powder, and porous polyether-type resins. Those resins often retain or retard the elution of organic molecules. Filtration through small pore membranes usually gives imperfect separation of salts. Adsorption on active charcoal is also sometimes effective for rough desalting.

FRACTIONATION.—Often, bioactive components constitute a very minor part of the entire extract. The isolation of a target molecule from an overwhelming amount of other water-soluble organic ingredients and inorganic salts is, indeed, a formidable task. Because there is no established standard fractionation for water-soluble compounds, such as there is for lipid-soluble compounds, one must often rely on trial and error. However, some basic principles relevant to the isolation will be described: (a) Separation by molecular sizes—Various gel filtration and ultrafiltration processes through membranes (e.g., Amicon) are effective for both rough and fine fractionation according to molecular sizes. (b) Ion-exchange chromatography—If the ionic character of the compounds and their stability on the resin and in buffer solutions are known, ion-exchange resins will be the most effective means of separating water-soluble compounds. Choice of resins depends on the ionic character and stabilities of target compounds on the resins. It should be noted, however, that many compounds decompose on the H^+ form of strongly acidic resins or the OH^- form of strongly basic resins. Similarly, the extreme pH of solutions used for elution often causes decomposition. The use of weakly acidic or basic resins or properly buffered resins is preferred in such cases. Fortunately, various forms of resins with medium acidity or basicity are now available. (c) Reverse-phase (RP) columns—RP columns with various hydrophobic stationary phases are now being used for the separation of compounds with a wide range of polarity. With the proper combination of organic solvents, such as MeOH and acetonitrile, and buffers, successes are reported in biochemical analysis with almost all compounds. How-

ever, for preparative purposes, one may encounter several problems. First, the sample size is very limited, and the injection of large amounts of crude material results in the incapacitation of an expensive column. For that reason, the separation on RP columns is usually reserved for the final purification or fine separation after sufficient purification. The second problem with RP columns is the use of buffer solutions. For most polar or ionic compounds, to effect good separation and recoveries, the use of buffers with appropriate pH's and ionic strengths is often unavoidable. Thus, the separation of minute components from the buffer may become a major problem. One solution to this problem may be the use of volatile buffers, which can be removed by vacuum evaporation or freeze-drying. Some examples of volatile buffer formulae are listed in Table 1. It

TABLE 1. Examples of Volatile Buffers

Buffer	pH
Ammonium bicarbonate	5-7
Ammonium acetate	7-8
Pyridine-HOAc (16.1:278.5)[a]	3.1
Pyridine-HOAc (161.2:143.2)	5.0
Pyridine-HOAc-α-Picoline (11.8:0.1:28.2)	8.0
Pyridine-HOAc-2,4,6-Collidine (10:0.4:10)	8.3
Pyridine-HOAc-*N*-ethylmorpholine (7.5:0.1-0.5:12.5)	9.3

[a]Amounts in 1000 ml.

should be noted that they are also useful in regular ion-exchange chromatography. (d) High- or medium-pressure chromatography on porous material—Recently various new types of stationary phases have been developed and found to be more effective for preparative purposes than the traditional C_{18} or C_8 columns. They are mostly porous matrices, which possess both molecular filtration and adsorption capabilities and withstand high pressure. One example is bonded silica with various pore sizes for separation of various molecular sizes (e.g., TSK-125, TSK-250, TSK-400). Another increasingly popular material is rigid organic matrices such as styrene-divinylbenzene copolymers with adsorptive properties and pore characteristics. Examples are TSK Type H gels with different pore sizes, sold by several companies, and Hitachi Gel 3000 series. Even though these packing materials are not yet very familiar to ordinary natural products chemists, they will unquestionably become powerful tools for the separation of water-soluble substances. (e) Combination of ion-exchange and size-exclusion chromatography—Attachment of ion-exchange capabilities of matrices of various pore sizes provides a very powerful separation capability. Classic examples are DEAE Sephadex and carboxymethylcellulose, which have been extensively used in biochemical experiments. Now, supports with functional groups on a variety of matrices are available on the market. In most cases, the actual separation is due to the combination of three principles: ion-exchange, size-exclusion, or hydrophilic/hydrophobic interactions. Thus again, the selection of a proper matrix may be the key for a successful separation.

Table 2 lists some chromatography supports often used to separate water-soluble substances. Traditionally, compounds with basic characters are separated on cation exchange resins, and those with acidic functional groups on anion exchange resins. Strongly acidic or basic resins are also widely used to separate neutral and amphoteric compounds.

TABLE 2. Selected Examples of Chromatography Supports[a] and Applicable Water-Soluble Compounds

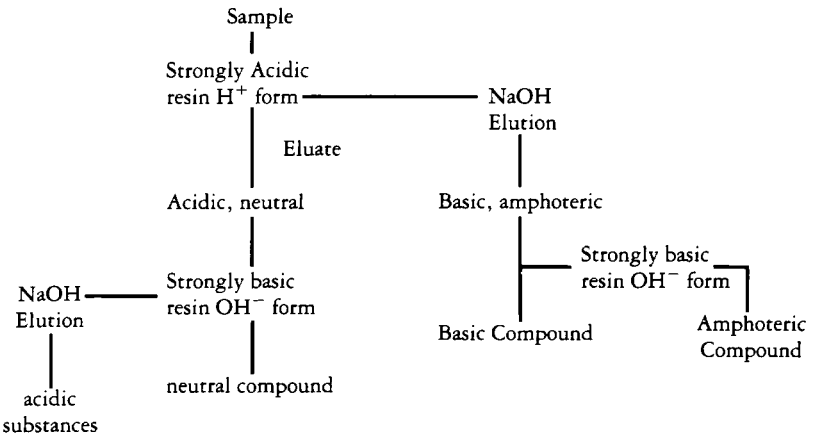
Neutral Compounds	Support
Mono- and oligo-saccharides	Sephadex G-10, G-15, Bio-Gel P-2, strong cation exchange (-SO_3H) resins, weakly basic anion exchange, e.g. -$(CH_2)NH_2$ resins
Polysaccharides	Sephadex G-50, G-100, G-200, Bio-Gel P
Glycoproteins and Proteins	Sephadex G-50-200, Bio-Gel P-2, Hydroxylappatite, DEAE bonded gels
Oligopeptides	Sephadex G-10, -15, Bio-Gel P-2, P-10, Sephadex LH-20, RP hplc (C_8, C_{18})
Amine, guanidine, amino acids derivatives	Strong cation exchange resins (-SO_3-) weak cation exchange resins (-COOH) RP hplc (C_8, C_{18} CN etc)
Nucleic Acids	Anion exchange resins, RP (C_8, C_{18})
Polar carboxylic acids	Strong or weak anion exchange resins, RP hplc (C_{18})
Glycosides	Sephadex G-10, LH-20, RP (C_{18}, C_8), XAD-2, XAD-7

[a]Sephadex—Pharmacia Fine Chemicals; BioGel—Bio Rad Laboratories; TSK—Toyo Soda Co.

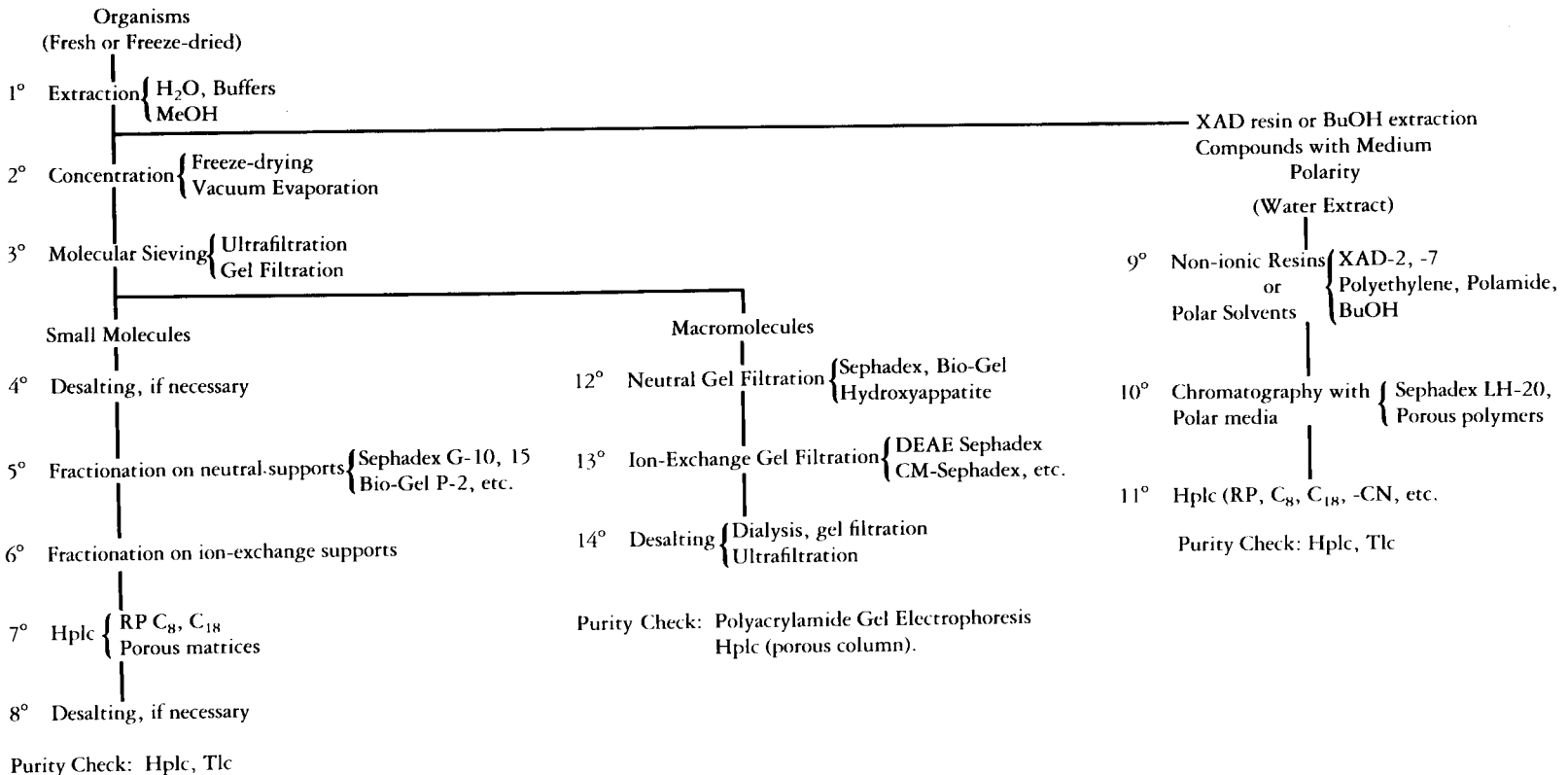
Scheme 1 shows the classic fractionation method for water-soluble compounds. Usually the final products are separated as picrates or other less soluble crystalline salts. However, the scheme is not applicable for compounds labile to strong acids and bases. As a general procedure for compounds with unknown properties, this author recommends a fractionation shown in Scheme 2. There, the use of drastic conditions is avoided in the initial step, and compounds are roughly fractionated by molecular sizes under a near-neutral condition. If the activity is found in a macromolecular range, the well-established method in biochemistry for various biopolymers should be followed. The fractionation of small molecular compounds may involve trial and error steps. However, drastic conditions should be avoided as much as possible in the early steps.

Some interesting water-soluble marine compounds, along with their isolation methods, are reviewed below.

PARALYTIC SHELLFISH POISONING (PSP) TOXINS.—PSP toxins are originally produced by dinoflagellates, *Gonyaulax* spp. and *Pyrodinium bahmense* var. *compressa* but accumulate in shellfish, causing fatal poisoning (6,7). One of the toxins, saxitoxin, was isolated with relative ease because it can be tightly adsorbed by the weakly acidic resin, Amberlite IRC-50 (8). However, the isolation of the toxins with less basicity creates

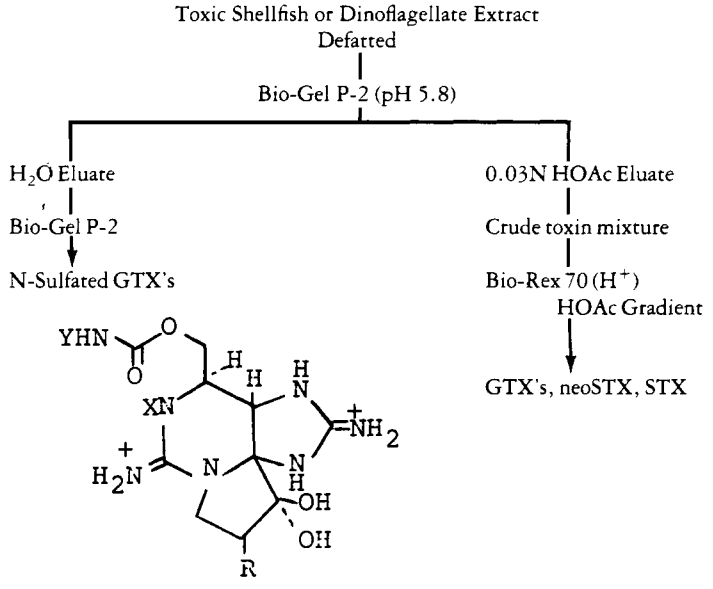


SCHEME 1. Classic fractionation scheme for water-soluble compounds.



SCHEME 2. An example of general fractionation procedure for unknown water-soluble compounds.

enormous difficulties. After many trials, it was discovered that most of the toxins could be adsorbed on Sephadex G-15 or Bio-Gel P-2 gels and eluted with a dilute HOAc solution (9). There is no real rationale for this rather selective adsorption of the toxins on the particular gels, but the discovery opened a way to purify the toxins (Scheme 3) (10). After the crude separation, fine fractionation was accomplished on medium-pressure chromatography on the carboxylate type resin, Bio-Rex 70, using HOAc gradient as an eluting buffer. So far, more than ten new compounds have been isolated and used as important probes to investigate the mechanism of action of sodium channels (11).

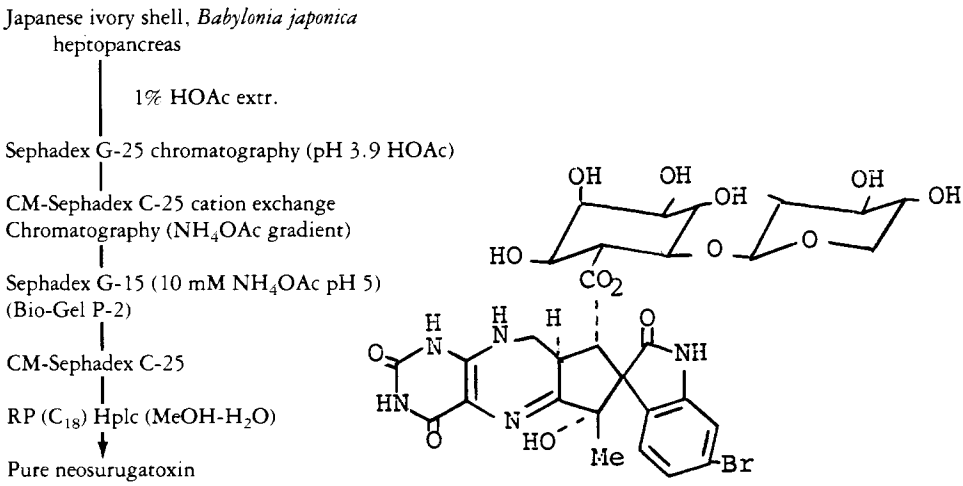


Gonyautoxin (GTX) R=OSO_3^-, Y=H or OH
Y=H, SO_3^-
Neosaxitoxin (neoSTX) R=H, X=OH, Y=H
Saxitoxin (STX) R=H, X=H, Y=H

SCHEME 3. Isolation of paralytic shellfish poisons (10).

SURUGATOXIN AND NEOSURUGATOXIN.—The Japanese edible marine snail, *Babylonia japonica*, sometimes turns toxic in certain areas. Hashimoto and his co-workers discovered that the toxin(s) has atropine-like activity and could be purified by Sephadex G-25 chromatography to a mixture of two closely related compounds (12). Kosuge's group obtained a crystalline compound and named it surugatoxin after Suruga Bay whence the toxic shellfish were derived (13). However, almost ten years later, another toxin, named neosurugatoxin, was isolated, and its structure was determined by X-ray (14). The purification procedure (Scheme 4) is a typical one, consisting of gel filtration, ion-exchange chromatography, and final purification by RP hplc (15). Interestingly, neosurugatoxin was proven to be highly toxic, whereas surugatoxin itself, which was first isolated as the major toxin, is nontoxic or only slightly toxic at most.

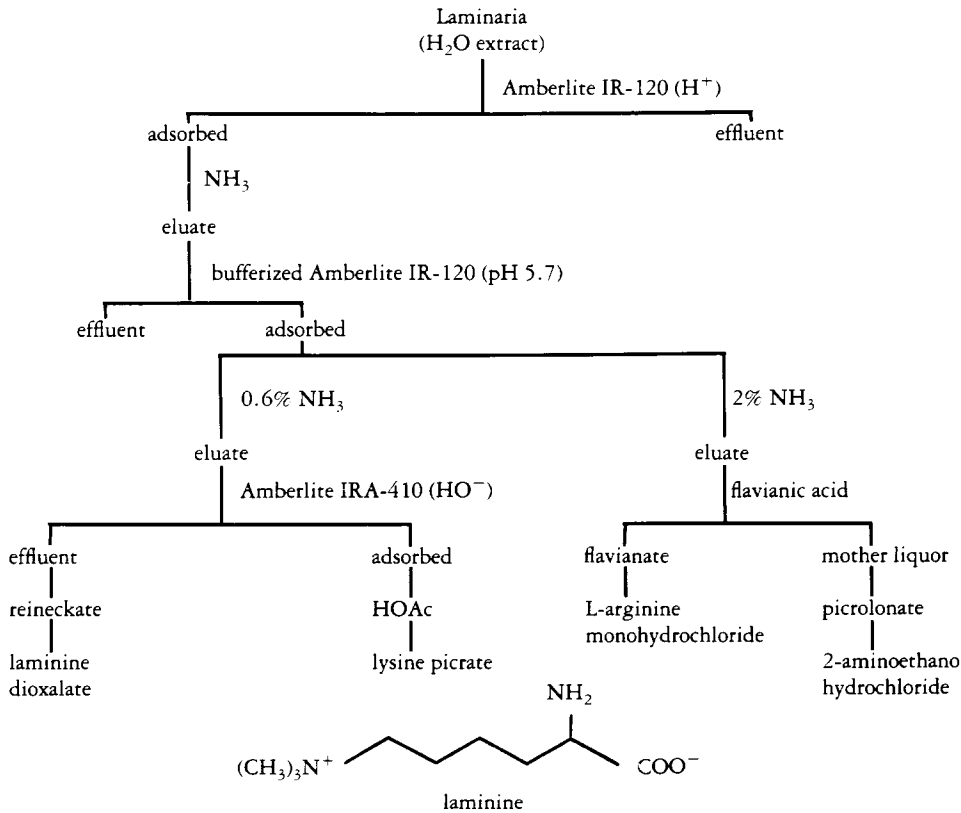
HYPOTENSIVE CONSTITUENT LAMININE FROM "KONBU," *LAMINARIA AUGUSTATA*.—"Konbu" is the Japanese name for several species of brown algae which belong to the genus *Laminaria*. The dried material is not only an important Oriental foodstuff but is also said to be effective in the prevention and treatment of hypertension. Takemoto and his co-workers isolated the hypotensive component according to the classic isolation scheme similar to the one mentioned earlier (Scheme 5) (16). The active component, laminine, proved to be the (5-amino-5-carboxypentyl)-trimethylam-



SCHEME 4. Separation of neosurugatoxin, a toxic principle in Japanese ivory shell (15).

monium salt and is shown to have transitory action that depresses the blood pressure of rabbits and lowers the contraction of smooth muscles (17). Since then, laminine has been found widely distributed and is probably responsible for hypotensive activity found in various algae (18).

CARDIOTOXIC PEPTIDES FROM SEA ANEMONE.—Sea anemone tentacles contain peptides that possess eminent cardiotonic or cardiotoxic activity. The toxin was first isolated from the Mediterranean sea anemone, *Anemonia sulcata*, by fractional precipita-



SCHEME 5. Separation of laminine and the other basic constituents from *Laminaria angustata* (16).

Anemonia sulcata tentacles
Extract
$(NH_4)_2SO_4$ fractional precipitation
DEAE cellulose
Sephadex G-50
Dialysis
Pure Toxins

SCHEME 6. Isolation of sea anemone toxins (19).

tion with $(NH_4)_2SO_4$ and ion-exchange and gel chromatography (Scheme 6) (19). Tanaka and co-workers purified a cardiotonic peptide, anthopleurin-A (Figure 1) from the Californian sea anemone, *Anthopleura xanthogrammica*, by SE-Sephadex C-25, which is a cation exchange gel with sulfoethyl moieties (currently replaced by the sulfopropyl derivative, SP-Sephadex C-25) (20). Marine organisms generally seem to have interesting peptides. One well-known example is eledoisin (Figure 2) from the posterior salivary glands of the octopus, *Eledone meshata*, which was isolated by successive chromatography on alumina and Amberlite CG-50. The undecapeptide shows hypotensive activity in dog at as little as 0.1-0.5 ng/kg (21).

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                    5                   10                  15
H-Gly-Val-Ser-Cys-Leu-Cys-Asp-Ser-Asp-Gly-Pro-Ser-Val-Arg-Gly-Asn-Thr-Leu-Ser-

 20                  25                  30                  35
Gly-Thr-Leu-Trp-Leu-Tyr-Pro-Ser-Gly-Cys-Pro-Ser-Gly-Trp-His-Asn-Cys-Lys-Ala-His-

 40                  45
Gly-Pro-Thr-Ile-Gly-Trp-Cys-Cys-Lys-Gln-OH      A. sulcata toxin II

       Ile          5                   10                  15
H-Gly-Val-Pro-Cys-Leu-Cys-Asp-Ser-Asp-Gly-Pro-Ser-Val-Arg-Gly-Asn-Thr-Leu-Ser-

 20                  25                  30                  35
Gly-Ile-Ile-Trp-Leu-Ala-Gly-Cys-Pro-Ser-Gly-Trp-His-Asn-Cys-Lys-His-Gly-Pro-

 40                  45
Thr-Ile-Gly-Trp-Cys-Cys-Lys-Gln-OH      Anthopleurin
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FIGURE 1. Sea anemone toxins

GLYCOSIDIC COMPOUNDS IN ECHINODERMS.—Sea cucumbers and starfish have been known to excrete saponin-like compounds probably as part of the defense mechanism. A number of aglycones have been isolated and their structures determined (22). However, the isolation of native, pure glycosides from mixtures of closely related compounds was not so easy. Kitagawa and his co-workers, who had tremendous expertise in handling terrestrial saponins, successfully purified antifungal saponins, holotoxin A and B, from the Japanese sea cucumber, *Stichopus japonicus*, by medium-pressure partition chromatography on silica gel using $CHCl_3$-MeOH-H_2O mixtures as eluting solvents and determined the structures (23,24). Later, RP chromatography on

Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2

FIGURE 2. Eledoisin (in *Eldone* spp.)

C_{18} columns proved to be a very efficient method to accomplish fine separation of these saponins (25). For example, by successive chromatography on silica and hplc on Bondapek C_{18}, they were able to separate six antifungal triterpenoidal glycosides, stichloside A_1, A_2, B_1, B_2, C_1, and C_2 from the Okinawan sea cucumber, *Stichopus chloronotus*. Similarly, Kitagawa's group succeeded in isolating and determining native saponin thornasteroside A, from the crown-of-thorn starfish, *Acanthaster planci* (26).

While sea cucumbers and starfish contain triterpenoidal and steroidal saponins, respectively, (see Figure 3) closely related echinoderms, sea urchins, have sulfated glycolipids with similar biological activities. Scheme 7 shows the purification procedure that involves droplet-counter-current (DCC) fractionation (27). DCC is a very effective method of fractionating saponins or compounds with similar polarities (28). It takes only small amounts of solvents and can handle fairly large amounts of compounds in comparison with RP chromatography. The drawbacks are, however, in the selection of a proper solvent system (the mobile phase has to form droplets) and the relatively long duration of separation time.

PHEROMONES AND OTHER ALLEROCHEMICAL SUBSTANCES.—Communications using chemicals play big roles in marine life. Many of them are speculated to be water-soluble and difficult to isolate due to their instabilities, but there are a few cases of suc-

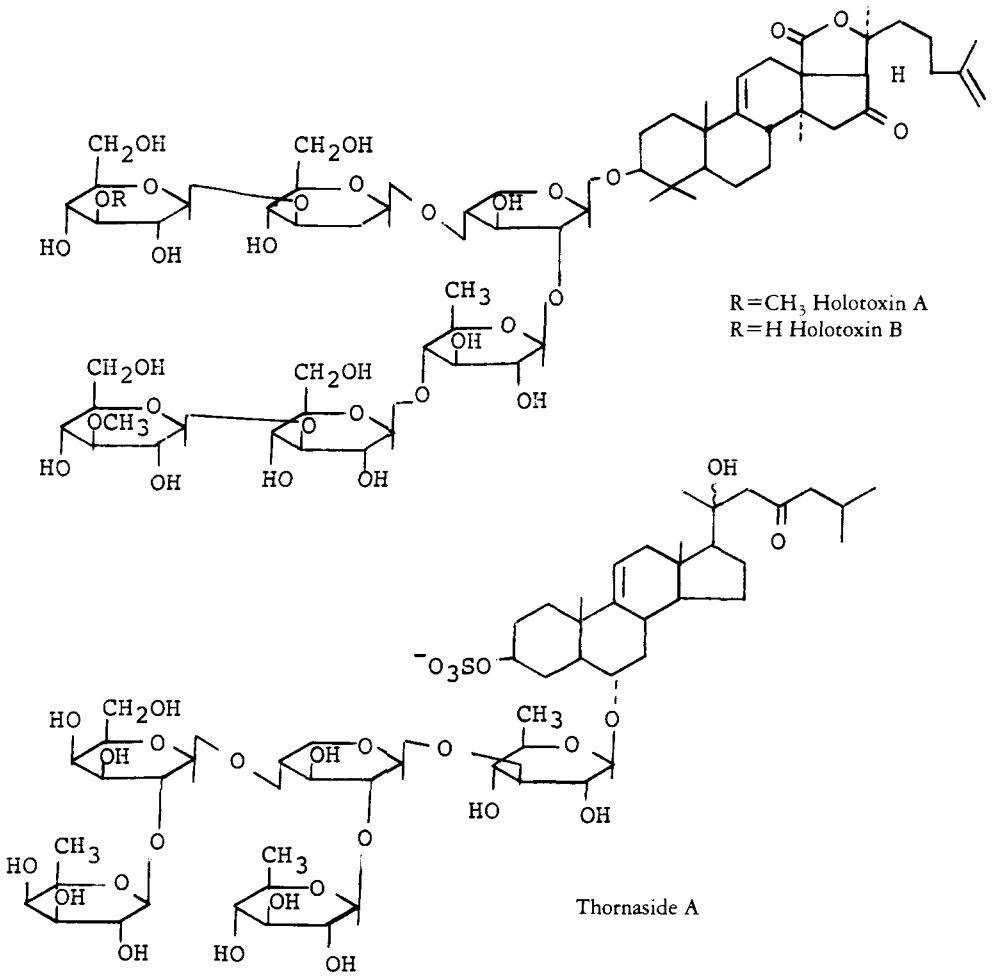
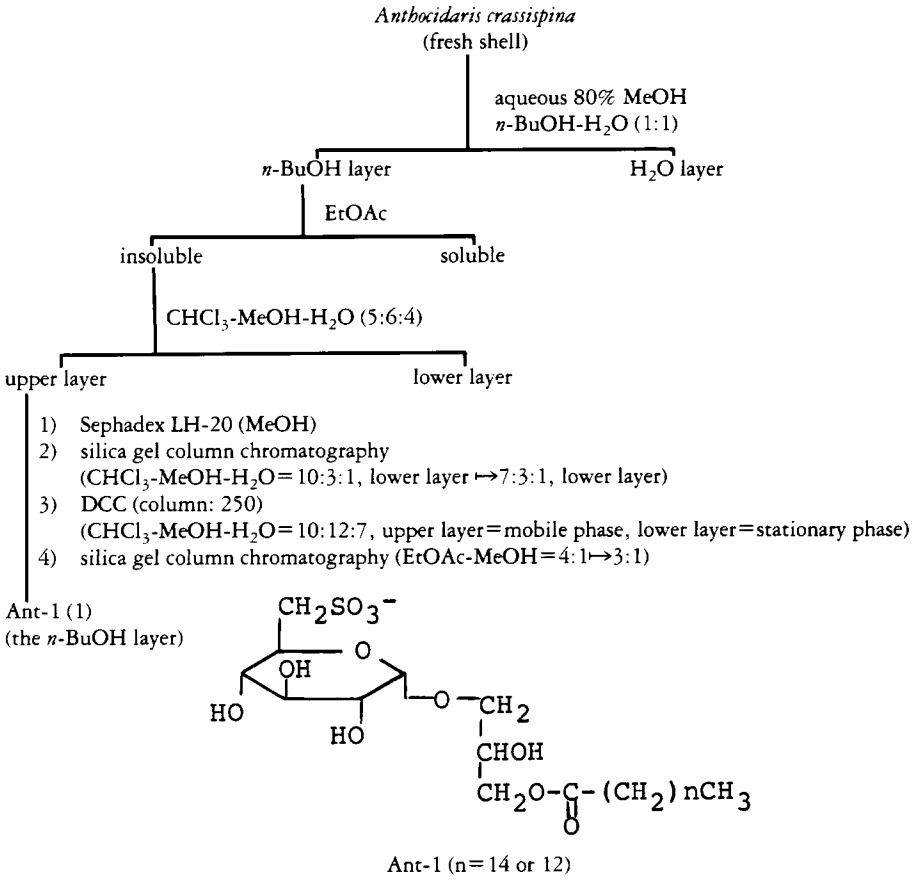
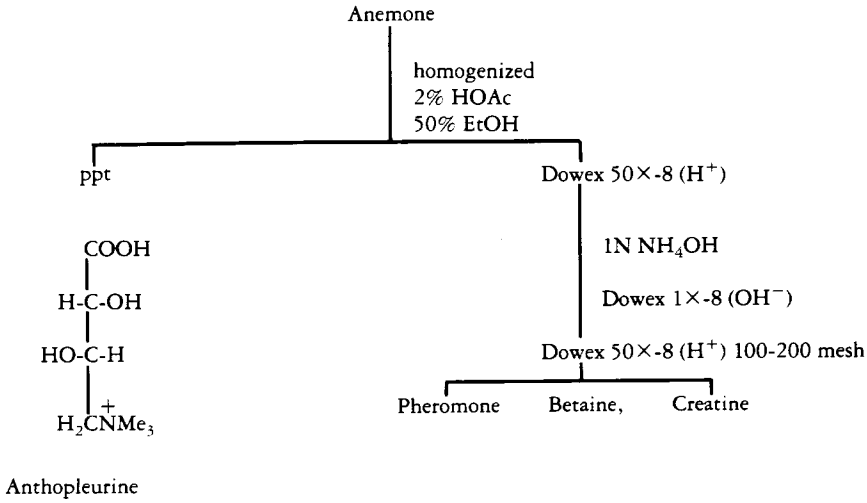


FIGURE 3. Examples of echinoderm saponins.



SCHEME 7. Isolation of sulfoglycolipid Ant-1 from sea urchin (27).

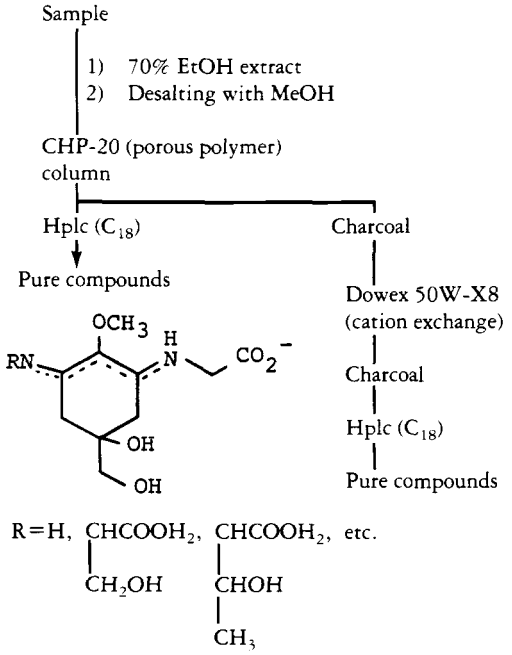
cessful isolation. Howe *et al.* extracted the alarm pheromone of *Anthopleura elegantissima* and determined the structure (29,30). Fortunately, in this case, the target compound was rather stable and withstood the strong ion-exchange chromatography, being eluted prior to such regular constituents as betaine or creatine (Scheme 8) (29). Other exam-



SCHEME 8. Isolation of alarm pheromone, anthopleurine from sea anemone, *Anthopleura elegantissima* (29).

ples of amino acid derivatives in this category are food attractants. Sangster isolated the fish-attracting substance strombine from the shellfish *Strombus gigas* as a Magnesium salt. The compound can attract fish at a concentration of 10 μg/liter (31).

UV-ABSORBING SUBSTANCES IN MARINE ORGANISMS.—A series of uv-absorbing shikimate metabolites condensed with various amino acids are widely distributed among marine plants and animals, including unicellular organisms (32). Despite their rather ubiquitous nature, their origins and functions are not fully understood. Porous phase chromatography, ion-exchange chromatography, and hplc with RP columns proved to be highly efficient in isolating these compounds. Thus, Kobayashi *et al.* used a sequence involving chromatography on a porous polymer, CHP-20 and hplc on C_{18} column to isolate five analogs from the tunicate, *Holocynthis roretzi* (Scheme 9) (33). Okaichi and Tokumura used the sequence: Sephadex G-10—DEAE Sephadex A-25—Hitachi 2616 resin (cation exchange)—Hitachi 2612 resin (cation exchange) to separate five compounds from the dinoflagellate, *Noctiluca miliaris* (34).



SCHEME 9. Isolation of uv-absorbing shikimate metabolites from marine organisms (33).

MARINE BIOPOLYMERS WITH ANTITUMOR ACTIVITY.—Both marine algae and animals are excellent sources of a variety of polysaccharides, glycoproteins, proteins, and lipoglycoproteins. Many of them are compounds with interesting biological activity, such as highly specific agglutinins or antitumor compounds (35). Table 3 shows an example of preliminary screening of macromolecular fractions from marine invertebrates against Sarcoma 180 implanted in mice (36). A striking number of samples demonstrated antitumor activity in this in vivo experiment. The author's group has recently purified one of the active components in the common edible Atlantic clam, *Mercenaria mercenaria,* following the standard purification method (Scheme 10) (37). Throughout the isolation process, disc gel electrophoresis was used to check the purity.

CONCLUSION.—Although grossly neglected, water-soluble fractions from marine organisms contain interesting biologically active compounds, such as antineoplastic compounds, cardiotonic agents, site-specific toxins, hypotensive agents, antimicrobial

TABLE 3. Selected Data of Antitumor Activity[a] of Macromolecular Fractions from Marine Invertebrates

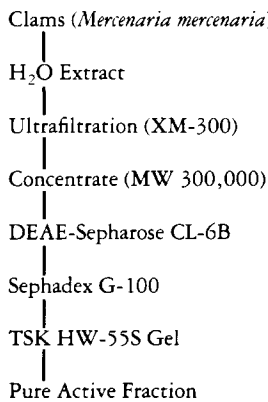
Species[b]	MW × 10^3	Dose (mg/mouse × days)	Tumor inhibition ratio (%)	Complete regression
1. *Ecteinascidia turbinata*	10	10×3	92.5	2/3[c]
2. *Holocynthia hilgendorfi*	10	10×3	74.4	0/5
3. *Styela plicata*	10	10×3	60.7	0/6
4. *Anthocidaris* sp.	300 50	10×3	77.0	1/4
5. *Strongylocentrotus drobachiensis*	300	10×3	72.9	1/6[c]
6. *Mercenaria mercenaria*	300	10×3	61.4	0/5
7. *Mercenaria mercenaria*	300	10×3	85.5	1/4
8. *Mercenaria mercenaria*	300 100	10×3	75.5	0/4[c]
9. *Placopecten magellanicus*	300 10	5×2	81.4	1/5[c]
10. *Aequipecten irradians*	300 10	10×1	76.1	2/4[c]
11. *Crassostrea virginica*	300 100	10×3	82.3	0/4[c]
12. *Mya truncata*	300 100	10×3	77.4	0/3[c]
13. *Nordotis* sp.	50	10×3	79.0	0/6

[a]Antitumor activity was determined using Sarcoma solid form in ICR mice.

[b]Common name 1. Sea squirt, 2. Tunicate, 3. Tunicate, 4. Purple sea urchin, 5. Green sea urchin, 6. Quahog, 7. Little neck clam, 8. Cherry stone clam, 9. Sea scallop, 10. Bay scallop, 11. Oyster, 12. Steamer clam, 13. Abalone.

[c]These fractions were toxic.

compounds, pheromones, hormones, and other allerochemicals. Here, the major problem is in the purification process. Admittedly, it is tedious and time-consuming to work with water-soluble compounds, but recent advances in isolation techniques enables us to isolate labile compounds that might decompose in the traditional isolation scheme. Macromolecular compounds can now be routinely processed. Because most techniques involved are well-established, it is not so difficult for a natural products chemist with little experience in biopolymers to work with the high-molecular compounds.



SCHEME 10. Purification procedure for antitumor biopolymer from the Atlantic clam, *Mercenaria mercenaria* (37).

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