Developments in Marine Natural Products. Receptor-Specific Bioactive Compounds

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DEVELOPMENTS IN MARINE NATURAL PRODUCTS. RECEPTOR-SPECIFIC BIOACTIVE COMPOUNDS[1]

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ABSTRACT.—The discovery of thousands of new marine natural products over the past two decades has been spurred by findings of potent bioactivity. In recent years it has become apparent that many such compounds have affinities for certain cellular receptors in the mammalian cell, functional properties that may also play a part in the largely unknown roles of these compounds in their respective parent organisms.

Our work in marine natural products has led to the discovery of compounds with significant activity in several assays with importance in understanding fundamental cellular processes and treatment of human disease states. We present recent work on the isolation of new bioactive compounds from marine invertebrates with profound activity on mammalian and non-mammalian receptors.

Natural product chemistry has grown in stature during this quarter century to a sophisticated science which now engages modern biology and biochemistry. Biologically active natural products, or secondary metabolites, have become fine tools for pharmacologists and biochemists. As ligands for cellular receptors, natural products are used to explore fundamental processes that elicit behavioral responses in living systems, both in homeostasis and in disease states. As we further explore the structure and activity of secondary metabolites and their potential for treatment of disease, we learn much about the biochemistry and pharmacology of their respective cellular receptors, the targets ultimately responsible for physiological response. Our interest in the chemistry of marine natural products embraces new compounds active in two arenas: as modulators of the ligand-gated calcium ion-channel of the endoplasmic reticulum and as antifungals with a marked dependence to sterol, in particular ergosterol, the predominant sterol found in fungal cell membranes. Although only the former is a receptor according to conventionally accepted definitions, both, we shall see, share properties of ion-channel conductance. In this presentation, I describe our recent work in these two areas.

ION-CHANNELS AND MARINE NATURAL PRODUCTS.—In order to understand the nature of such ion-channel receptors, researchers have exploited natural products as specific receptor ligands. These xenobiotic ion-channel modulators often have superior binding properties which, for various reasons, make them well suited as probes of ion-conductance, receptor structure, and conformation. For example, tetrodotoxin, a well-known marine natural product from Fugu fish and other sources, has proved invaluable to neuropharmacologists in the study of the voltage-dependent sodium ion channels and the mediation of neuromuscular excitation potential (1,2). A number of marine dinoflagellate toxins such as the saxitoxins (produced by various dinoflagellates linked with paralytic shellfish poisonings) (3,4), brevetoxins ("red-tide" toxins from *Ptychodiscus brevis*) (5,6), and more recently ciguatoxins (involved with ciguatera poisoning) (7–9) have also been employed in the study of sodium channel action. A major challenge in molecular and cell biology is understanding the structure and function of ligand-gated Ca^{2+} channels of endoplasmic reticulum (ER), and natural products as channel probes have made possible significant advances in this area. Ryanodine, a plant alkaloid from

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Ryania speciosa, binds tightly to this ion-channel resulting in a permanently "open" state (10,11). The conus toxins from marine cone shell molluscs (*Conus* spp.) (12) and maitotoxin (13), a potent Ca^{2+} channel agonist, also obtained from the ciguatera-related dinoflagellate *Gambierdiscus toxicus*, are two examples of new classes of calcium ion-channel modulators with profiles of activity distinct from those of ryanodine or other modulators.

The known sodium and calcium ion-channel modulators have been isolated with disproportionate frequency from the marine environment. Public health concerns brought these marine ion-channel modulators to the attention of scientists in the first place; however, this may be more than a coincidence. Their frequent occurrence begs the question of why they are there. Many natural products are presumed to act as forms of chemical defense, yet it is not easy to see how compounds which in some cases are not deployed could act in this fashion. For whatever reason, could it be that the marine environment has made the evolution of ion-channel modulation and ion-channel modulators in marine organisms a favorable adaptation? If so, then would a search for calcium ion-channel modulators from less well known marine invertebrates be fruitful in discovering modulators with the properties of useful biochemical probes? Indeed, Ohizumi and co-workers (14,15) have reported several marine natural products with significant calcium channel activity (for example, bromoeudistomin D from the marine ascidian *Eudistoma* sp.). How many other such ion-channel modulators await discovery from, say, marine sponges or coelenterates?

In order to answer such questions we began by screening our recent collection of marine invertebrates—sponges, ascidians, coelenterates, and molluscs—from the Pacific Ocean (Great Barrier Reef, Australia, 1990–91). Aqueous MeOH extracts of each of the collected specimens were prepared and, in collaboration with Dr. Isaac Pessah of the Department of Veterinary Toxicology and Pharmacology, we tested for their ability to stimulate Ca^{2+} channel specific ^{3}H-ryanodine binding to ER preparations from rat skeletal muscle (16). Of 147 extracts tested, 10% showed high binding activity (>500% increase over the control). One extract, that of unidentified sponge 90-020, enhanced the binding of ^{3}H-ryanodine (1nM) by 2600% at 20 μg/ml, representing an unprecedented level of receptor activation by a natural product. In order to test the ability of the extract to stimulate release of Ca^{2+}, the extract was added to reconstituted vesicles pre-loaded with Ca^{2+}. Rapid release of Ca^{2+} was observed when the extract was added. This release was completely inhibited by the addition of ruthenium red, a known inhibitor of the Ca^{2+} ion-channel. The activity of the extract is specific for the Ca^{2+} channel and not due to ionophoric properties, because the extract showed no tendency to depolarize the ER membrane.

The next step was purification of the active component(s) of sponge 90-020. The isolation and characterization of the active Ca^{2+} ion-channel agonist(s) was carried out using bioassay-guided purification. Initial purification work was carried out on a small sample (less than 5 g dry wt) collected in 1990 and resulted in the isolation of less than 1 mg of active compound, sufficient to confirm the extraordinary activity but insufficient for structural elucidation. Additional sponge material was obtained in 1991, and this was subjected to the same isolation protocol. The MeOH extract of the entire sample was separated using a modified Kupchan scheme, followed by size exclusion chromatography (Sephadex LH20), monitored by ^{3}H-ryanodine receptor binding. The active fractions were pooled and further purified by reversed-phase flash chromatography and hplc (C_{18} 71–80% MeOH, aqueous 0.1% TFA followed by C_{18} 60–80% MeCN, aqueous 0.1% TFA) to obtain two major active compounds, F5 (5 mg) and F6 (8 mg), shown to be homogeneous by ^{1}H-nmr and ^{13}C-nmr. Preliminary analysis of spectroscopic data suggested that the active compounds are medium mol wt alkaloids;

however, structural characterization of these compounds is yet to be completed. The complete structures, together with pharmacological properties, will be reported in due course. In addition, we plan to investigate the Ca^{2+} ion-channel activity, not only in the skeletal ER but also in intracellular Ca^{2+} release and signal transduction.

The large number of active specimens in this pre-screen was somewhat surprising. We expected that some of this activity could be explained by the presence of endogenous ATP, which also has a binding site on the Ca^{2+} channel. However, examination of some of these active extracts by ^1H nmr showed no evidence of the presence of unusually large concentrations of nucleotides. It is expected that additional Ca^{2+} ion-channel activators will be found after bioassay-guided fraction of the remaining samples. We anticipate that the outcome of this work will be the discovery of marine natural products that are useful biochemical probes for different classes of Ca^{2+} ion-channels. In turn, the application of new modulators to studies of intracellular Ca^{2+} release, the least understood component of signal transduction, and the second messenger cascade, may afford a greater understanding of intracellular signalling.

STEROL-SENSITIVE ANTIFUNGAL ACTIVITY.—The polyene macrolides are a group of approximately 200 compounds containing a macrocyclic lactone ring and up to nine conjugated trans double bonds (17). Amphotericin B is the only polyene approved for internal use and is effective in life-threatening disseminated mycoses. Like other polyenes, amphotericin B elicits antifungal activity by interacting with the fungal cell membrane. Ergosterol, the major sterol present in fungal cell membranes, is the membrane-bound receptor for polyenes. Although complete details of the mode of action are still to be resolved, it appears that amphotericin B binds noncovalently to ergosterol present in fungal cell membranes followed by spontaneous formation of an ordered molecular assembly (18). This assembly has the dimensions and physio-chemical properties of a pore, or ion-channel, permeable to potassium. The activity of amphotericin B is attributed, in part, to induced leakage of potassium ions, followed by cell lysis and death. Certain advantages are associated with this mode of activity that are lacking in other classes of antifungal drugs. Amphotericin is believed to be selective for yeast cells because its affinity for ergosterol is much higher than for cholesterol, present in mammalian cell membranes. However, other factors are almost certainly involved. The presence of ergosterol is essential for fungal cell integrity and function, so the development of fungal strains resistant to polyenes is rare. This appealing mode of activity is tempered by side effects occurring with the clinical intravenous administration of amphotericin B where significant toxicity precludes more general use. Clearly, new compounds with a similar mode of activity but better clinical profiles may be useful leads in the development of antifungal drugs to be used, for example, in the treatment of disseminated mycoses that are frequently found in AIDS patients.

Approximately 4000 new natural products have been reported from marine organisms (19). Many of these compounds possess antifungal activity; however, no polyenes are included in this list (20). In our initial survey of our own marine invertebrate samples (collected from in Australia, 1990, $N = 116$), approximately 8% showed activity against *Candida albicans*. Intrigued by the high incidence of antifungal activity and lack of polyene structures among known marine compounds, we asked the question, "How common is the sterolsensitive polyene mode of activity in marine natural products with non-polyene structure?" By applying an assay for polyene antifungal activity to marine extract (Figure 1) (21), we were able to select for extracts exhibiting competitive binding to added ergosterol, a key property of polyene activity. The anti-*Candida albicans* extracts were segregated into ergosterol-sensitive and ergosterol-insensitive extracts (Figure 2). The surprising finding that approximately half of our anti-

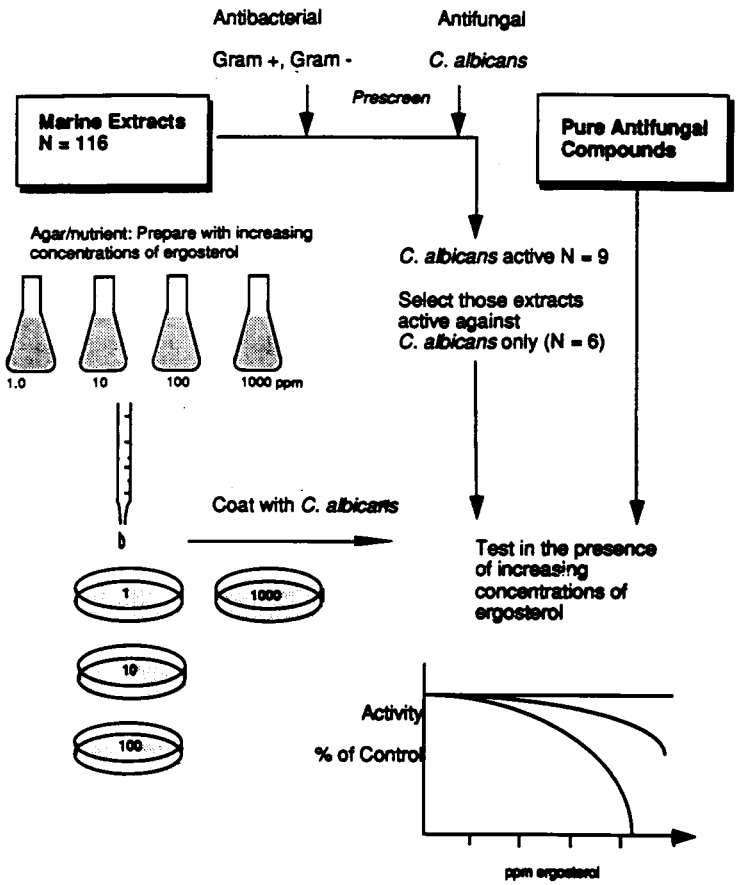


FIGURE 1. Screening of marine extracts for sterol-sensitive antifungal activity.

Candida extracts exhibited ergosterol sensitivity suggests that polyene-like activity is common among marine antifungal compounds.

We next selected two of the extracts for isolation of the responsible bioactive compounds. Extract 90-026, a *Jaspis* sp. from the Great Barrier Reef, showed strong ergosterol sensitivity, while the anti-*C. albicans* activity of 90-123, an extract from the temperate water sponge, *Latrunculia*, from southern Australia, was insensitive to ergosterol. In each case, isolation was carried out by standard methods involving bioassay-guided solvent partition, flash chromatography, and hplc. Structure elucidation of the active compounds was carried out by spectroscopic methods, relying heavily upon 2D nmr. This work provided the first knowledge of chemotypes from marine natural products responsible for both ergosterol-sensitive and ergosterol-insensitive behavior.

A thinly encrusting sponge *Jaspis* sp., collected from shaded coral habitats on the Northern Great Barrier Reef, provided several alkaloids active against *C. albicans*. The major alkaloids, the known bengazoles A [**1**] and B [**2**] (22) were isolated in yields of 0.056% and 0.037% dry wt of sponge, respectively. Bengazole B exhibited ergosterol-dependent activity similar in profile to amphotericin B; the activity of 10 μg of the alkaloid was reduced to 50% in the presence of 20 ppm of ergosterol. When cholesterol was substituted for ergosterol in the assay, the reduction of bengazole B activity was significantly less. Once again, this sterol structure dependence is reminiscent of the ac-

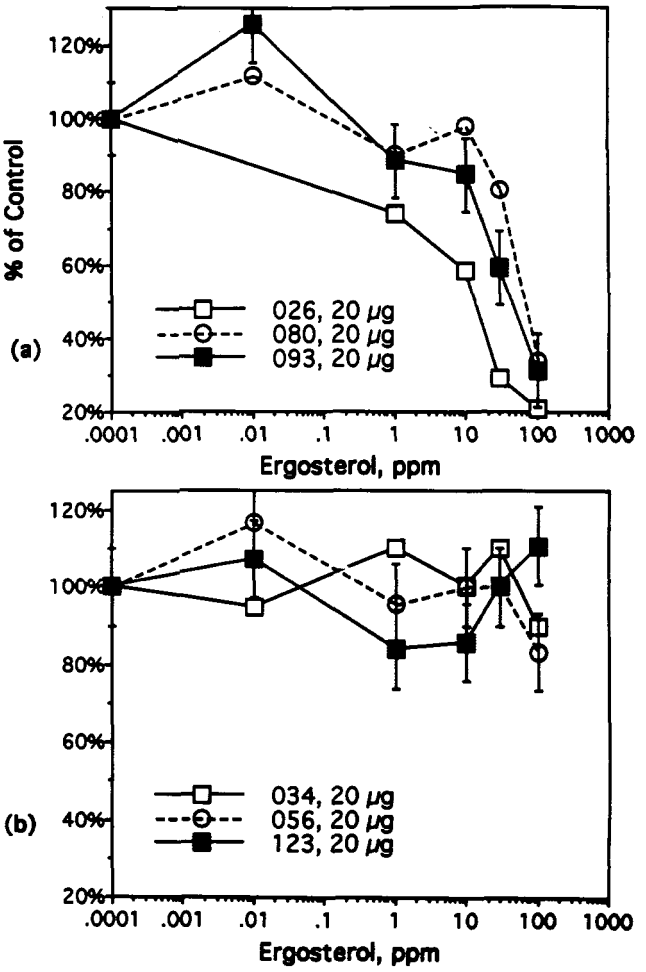
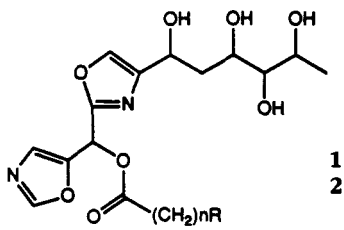


FIGURE 2. Sterol dependence of antifungal marine extracts.

tivity of amphotericin B and suggested that the sterol sensitivity of bengazole B was specific for ergosterol.

In addition to the known compounds, six new bengazoles were isolated as minor components by reversed-phase C_{18} hplc. The retention times of the new compounds suggested compounds both less polar and more polar than **1** and **2**; however, the pure fractions showed almost identical ^{1}H-nmr spectra.

The only differences appeared to be in the ester side chain. This was confirmed by



1, **4**, **6**, **8** n = 8–11; R = —$CH_2CH_2CH_2Me$

2, **5** n = 10,9; R = —CH_2CHMe (Me)

3, **7** n = 10,11; R = —$CHCH_2Me$ (Me)

methanolysis and gc-ms. Treatment of each pure hplc fraction with 5% HCl in MeOH (60°, 1 h) was followed by extraction with *n*-hexane. Capillary gc-ms of each of the *n*-hexane extracts, including single ion monitoring for the prominent *m/z* 74 fatty acid methyl ester fragment (McLafferty rearrangement), and comparison with bacterial fatty acid methyl ester standards (Supelco®), revealed the identity of the side chains. The yields and structures of these compounds are shown in Table 1. The new bengazoles B1 [**3**], C [**4**], D [**5**], E [**6**], F [**7**], and G [**8**] are simply different saturated fatty acid esters of the parent alkaloid present in bengazoles A and B. Their structures range from straight chain fatty esters (bengazoles C [**4**], E [**6**], G [**8**]), to branched chain compounds in the iso series (bengazole D [**5**]) and the anteiso series (bengazoles B1 [**3**] and F [**7**]). Each new bengazole exhibited comparable activity to bengazoles A and B against *C. albicans* in a simple agar disk diffusion assay (9–10.5 mm zone of inhibition at 0.5 μg per disk).

TABLE 1. New Bengazoles from *Jaspis* sp.: Gc-ms Analysis of Hplc Purified Compounds.[a]

Compound	Hplc fraction	Amount (% dry wt)	% of Total active fracts	Fatty acid side chain[b]
4	F6	0.0023	2.0	13:0
5	F8	0.0075	6.3	i-14:0[c]
1[d]	F9	0.052	43.0	14:0
2[d], **3**	F12	0.037	30.4	i-15:0, a-15:0[e]
6	F14	0.008	6.6	15:0
7	F16	0.0085	7.0	a-16:0
8	F18	0.0056	4.7	16:0

[a]Hplc Dynamax RP18, 10 × 300 mm, MeOH-H_2O (90:10), 4.0 ml/min; gc-ms 25 m × 0.25 mm coated capillary (150–150°, 4°/min, He carrier), interfaced to a Hewlett Packard quadrupole mass spectrometer. Retention times were matched with Supelco bacterial FAMEs standards.

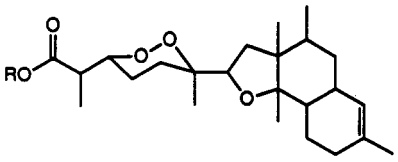
[b]Abbreviations: a-, anteiso; i-, iso-; n:m, where n is the carbon number and m is the number of double bonds.

[c]Assignment confirmed by ^{1}H nmr (δ 0.87 ppm, 6H, isopropyl methyls).

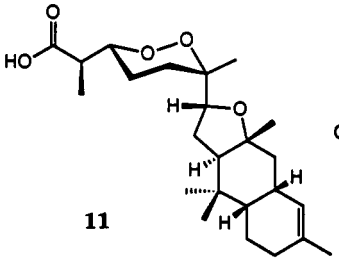
[d]First characterized by Adamczeski *et al.* (22).

[e]5:1 ratio, respectively.

The temperate water marine sponge *Latrunculia* sp. (90-123, Port Phillip Bay, Victoria, Australia, 1990), gave several highly antifungal compounds. The major compound, trunculin F [**9**], was isolated by flash Si gel chromatography of the EtOAc-soluble portion of the MeOH extract. Trunculin F gave a methyl ester **10** upon treatment with CH_2N_2, but this ester was inactive against *C. albicans*. Trunculin F joins a small family of tricyclic norsesterterpene carboxylic acids and their esters bearing cyclic peroxide rings. These compounds have been reported as natural products from *Latrunculia* sp. collected in other parts of Australia, as illustrated by the structures of trunculin B [**11**] (23) and trunculin C methyl ester [**12**] (24) from different *Latrunculia* specimens. The structures of the latter compounds were firmly established by single crystal X-ray diffraction analysis. The assignment of regiochemistry in **9** is based on extensive analysis of COSY and long range HETCOR 2D spectra. However, the stereochemistry of **9** shown is presently undefined. As with other reported trunculins, the relative stereochemistry about the 1,2-dioxane ring in **9** must be considered independently from that of the carbocyclic fragment, and confirmation of the entire relative configuration of trunculin F awaits further spectroscopic studies. The structures of the minor constituents are the subject of ongoing work.



9 R=H
10 R=Me



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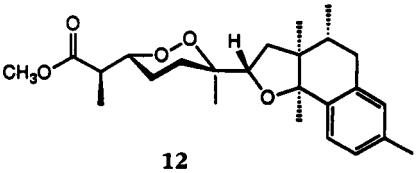

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Ergosterol was without effect on the anti-*C. albicans* activity of trunculin F; no suppression of activity was observed with up to 100 ppm of the sterol in the agar disk diffusion assay. Considering also the lack of activity of the methyl ester **10**, it is clear that the activity of **9** is unrelated to polyene activity and probably involves a separate, but as yet unidentified, mode of action.

In summary, we have embarked on two areas of marine natural products research involving ion-channel conductance and antifungal action. Our early results are encouraging. In answer to our first questions we have found significant activity in new marine natural products. These in turn have posed additional questions of mechanism of activity, de novo biosynthesis, and their innate ecological roles. In time we hope to address these issues.

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