Journal of Natural Products
Vol. 52, No. 2, pp. 225-238, Mar-Apr 1989

PHARMACOLOGICALLY ACTIVE METABOLITES FROM SYMBIOTIC MICROALGAE IN OKINAWAN MARINE INVERTEBRATES[1]

JUN'ICHI KOBAYASHI

Mitsubishi Kasei Institute of Life Sciences, Minamiooya, Machida, Tokyo 194, Japan

ABSTRACT.—Amphidinolides A [**1**], B [**2**], C [**4**], and D [**5**], novel macrolides with potent antineoplastic activity, have been isolated from the cultured cells of the marine dinoflagellate *Amphidinium* sp., a symbiotic microalga in an Okinawan flatworm. The structures have been assigned mainly on the basis of 2D nmr. Some compounds with interesting pharmacological activities have been also obtained from the cultured cells of other marine microalgae, symbionts of coral or bivalve. Cultivation of microalgae and isolation and structure elucidation of the bioactive compounds are described.

Marine organisms have proved to be a rich source of compounds with diverse structural features and interesting biological activities (1–6). Some of the marine natural products isolated have not only served as potential lead compounds for clinically useful drugs (7,8) but actually used as chemical probes useful for basic studies in the fields of life sciences such as biochemistry, pharmacology, or physiology (9–12). However, it is often difficult to supply an adequate amount of interesting natural products from marine organisms such as sponges due to limitation of collections.

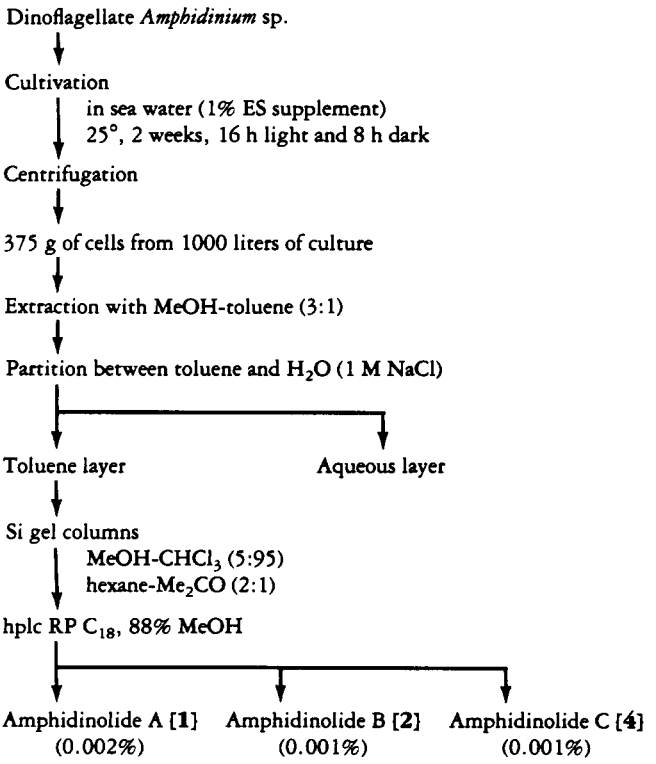
Increasing attention has been recently paid to marine microorganisms as another hopeful source of bioactive compounds. In early studies, marine microorganisms have been investigated mainly to clarify causes of seafood toxins. For example, it has been found that a pufferfish toxin, tetrodotoxin, is produced by Gram-positive bacteria (*Alteromonas* spp.) (13), and a Japanese shellfish toxin, neosurugatoxin, is also a product of bacteria (14). Furthermore, it has been demonstrated that aplysiatoxin isolated from the blue-green algae of *Oscillatoriacea* is a dermatitis toxin responsible for swimmer's itch in Hawaii (15), while saxitoxin (a paralytic shellfish toxin), brevetoxins (red tide toxins), or maitotoxin (a ciguatera toxin) are produced by the dinoflagellates *Gonyaulax catenella* (16), *Gimnodinium breve* (17,18), and *Gambierdiscus toxicus* (19), respectively.

Recently, some bioactive compounds isolated from marine invertebrates such as sponges have been found to originate from symbiotic microorganisms (20,21). Of symbiotic microorganisms, especially microalgae in marine invertebrates were expected to be a hopeful source of interesting bioactive compounds, if large-scale cultivation was possible in the laboratory. Therefore, we have tried to cultivate some symbiotic microalgae separated from host marine invertebrates such as flatworm, bivalve, or coral in an attempt to isolate novel bioactive compounds. This presentation deals with the cultivation of symbiotic microalgae (dinoflagellates and haptophytes) and the isolation and structure elucidation of pharmacologically active compounds from them.

GENERAL PROCEDURE OF MICROALGAL CULTIVATION.—Symbiotic microalgae (dinoflagellates, blue-green algae, haptophytes, etc.) are isolated from the inner tissue of host marine animals (coelentrates, molluscs, flatworms, etc.). The isolated microalgae are pre-cultivated in sea water medium (20 ml–2 liters) under various conditions with some factors (supplement, temperature, light, etc.) varied, to determine optimal culture conditions. Finally, microalgae showing acceptable growth rate and interesting biological activities are selected and cultured on a larger scale (400–1000 liters).

[1]Presented as a plenary lecture at the "Microalgae as a Source of New Biologically Active Natural Products" Symposium of the International Congress on Natural Products Research at Park City, Utah, July 17–21, 1988.

AMPHIDINOLIDES A [**1**], B [**2**], C [**4**], AND D [**5**].—The marine dinoflagellate *Amphidinium* sp. was isolated from the inner tissue of the Okinawan flatworm *Amphiscolops* sp. and grown unialgally in a sea water medium (Scheme 1) enriched with ES sup-



SCHEME 1. Purification procedure for amphidinolides A [**1**], B [**2**], and C [**4**] from cultured dinoflagellate *Amphidinium* sp.

plement (Table 1). The toluene-soluble materials of the harvested cells were subjected to repeated chromatographies on Si gel columns followed by reversed-phase hplc to give three antineoplastic compounds, named amphidinolides A [**1**] (22), B [**2**] (23), and C [**4**] (24), as shown in Scheme 1.

TABLE 1. Contents of Provasoli's ES Supplement.[a]

Distilled H_2O	100 ml
$NaNO_3$	350 mg
Na_2-glycerophosphate	50 mg
Fe (as EDTA[b]; 1:1 mol)	2.5 mg
PII solution of metals[c]	25 ml
Vitamin B_{12}	10 μg
Vitamin B_1	0.5 mg
Biotin	5 μg
TRIS[d]	500 mg
pH	7.8

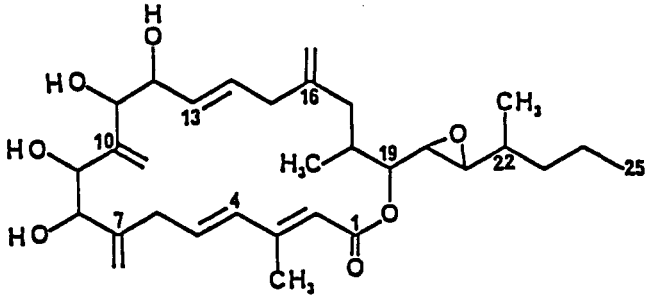
[a]This was filtered with sterile membrane filter and added to sterile sea water to make a 1% solution.

[b]Ethylenediaminetetraacetic acid.

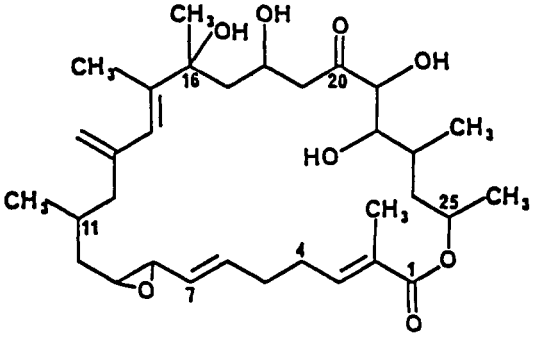
[c]The solution consists of Fe (as Cl^-, 1 mg), B (H_3BO_3, 20 mg), Mn (as Cl^-, 4 mg), Zn (as Cl^-, 500 μg), Co (as Cl^-, 100 μg), Na_2-EDTA (100 mg), and distilled H_2O (100 ml).

[d]Tris(hydroxymethyl)aminomethane.

Amphidinolide A [**1**] is a novel 20-membered macrocyclic lactone (22). The ^{1}H-^{1}H COSY (Figure 1) data were sufficient to elucidate the structure of **1**, because most of carbons were protonated. The assignment of the methyl group at C-22 was made from comparison of the carbon chemical shifts at C-23, C-24, and C-25 between **1** and the corresponding chemical shifts for pectinatone (25). Amphidinolide A [**1**] contains several interesting structural features. Three *exo*-methylene groups on the macrolide ring appear unique and four hydroxy groups are located closely to each other (C-8–C-12) to form a hydrophilic part in the molecule (25).



1



2 and **5** (stereoisomers at C-21)

Amphidinolide B [**2**] is a novel 26-membered macrocyclic lactone without an alkyl side chain (23). The structure of amphidinolide B was assigned originally as **3** on the basis of the ^{1}H-^{1}H COSY (Figure 2) and nOe data (23), but it has been more recently revised to be **2** from detailed analysis of the ^{1}H-detected heteronuclear multiple-bond correlation (HMBC) spectra as will be described below.

Amphidinolide C [**4**] is the first 25-membered macrocyclic lactone from a natural source (24). This compound includes two tetrahydrofuran rings, two ketone carbonyls, four hydroxy groups, and two exomethylenes. Three partial structures of C-2–C-14, C-16–C-17, and C-19–C-34 were elucidated by the ^{1}H-^{1}H COSY (Figure 3), ^{1}H-^{13}C COSY, RCT-COSY, and NOESY data. The connectivities of the three segments separated through three carbonyls (C-1, C-15, and C-18) were established by ^{13}C-^{1}H long-range couplings ($^2J_{C\text{-}H}$ and $^3J_{C\text{-}H}$) observed in the HMBC spectrum (Figure 4). The diene moiety (C-9–C-10 and C-36–C-37) was slowly oxidized in solution to afford a [4 + 2] cycloaddition product **A**, supporting the structure **4** (24).

The structure of the diene part (C-13–C-15) of amphidinolide B was reexamined by analysis of the HMBC spectrum, because the diene structure **3** previously proposed (23) was different from the corresponding one of amphidinolide C [**4**]. In the HMBC

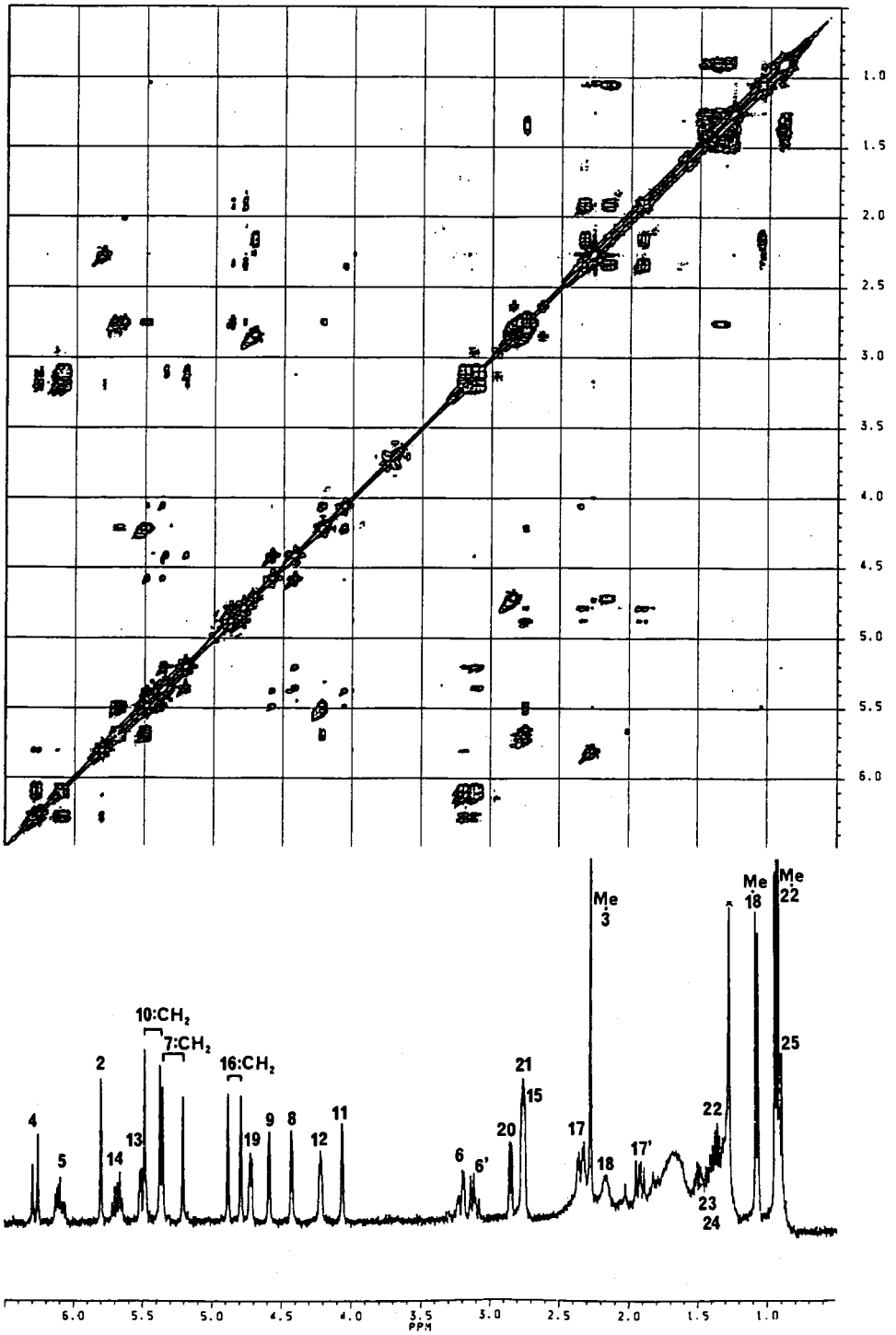


FIGURE 1. The ^1H-^1H COSY spectrum of amphidinolide A [**1**] (400 MHz, $CDCl_3$).

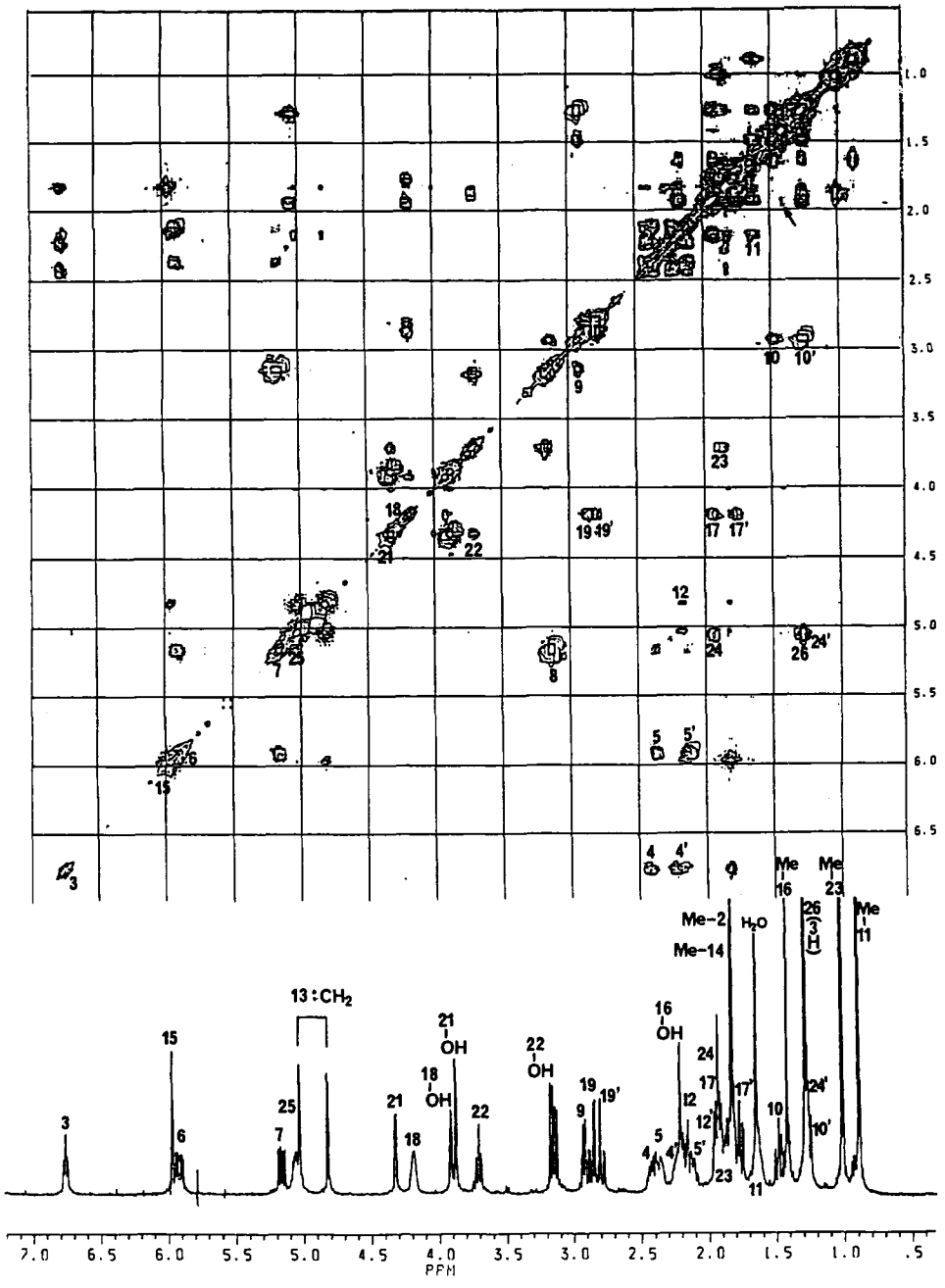


FIGURE 2. The ^1H-^1H COSY spectrum of amphidinolide B [**2**] (400 MHz, $CDCl_3$).

spectra, the methyl proton at C-16 was coupled to a quaternary carbon (C-15, s) but no to an olefinic methine carbon (C-14, d). Thus the structure of amphidinolide B was re vised to be **2**[2].

The fourth component, amphidinolide D [**5**], was isolated together with am phidinolide B [**2**] from another batch of the cultured *Amphidinium* sp. Amphidinolid D [**5**] showed uv, ir, and ms spectra identical to those of amphidinolide B [**2**]. Com

[2]J. Kobayashi, M. Ishibashi, H. Nakamura, Y. Ohizumi, Y. Hirata, T. Sasaki, T. Ohta, and Nozoe, unpublished.

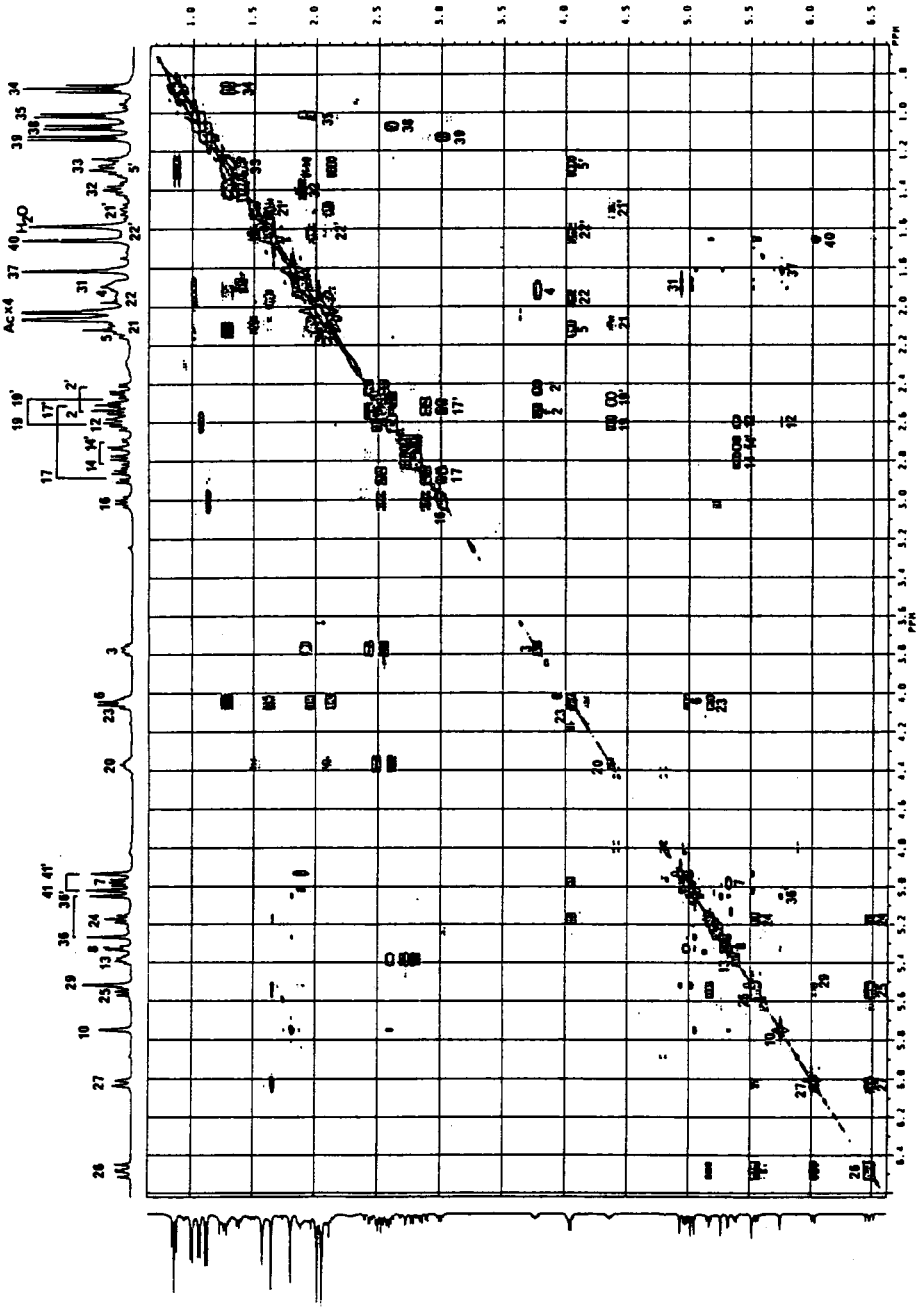


FIGURE 3. The ^1H-^1H COSY spectrum of the acetate of amphidinolide C [4] (400 MHz, $CDCl_3$).

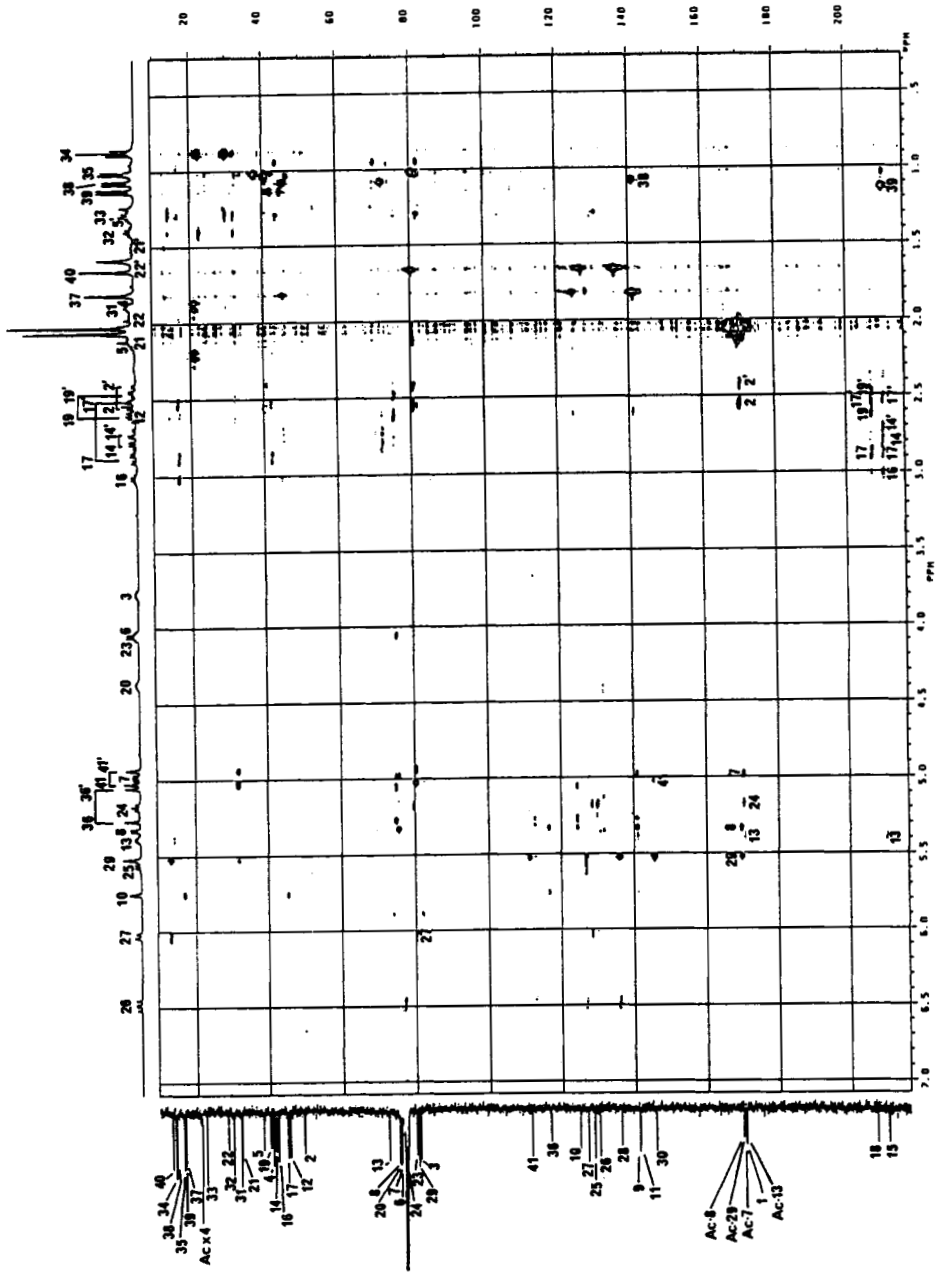
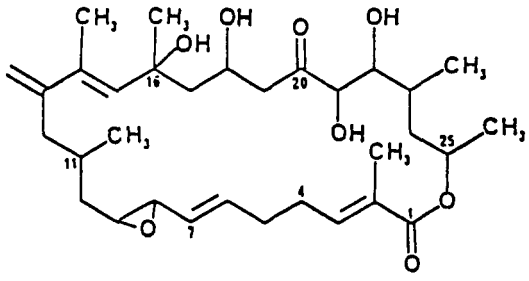
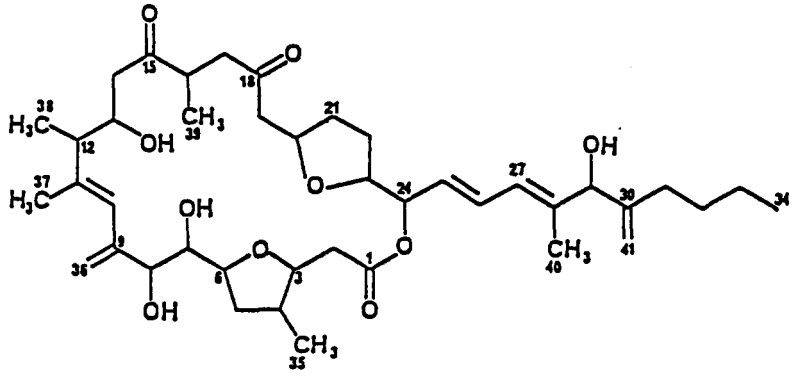


FIGURE 4. The HMBC spectrum of the acetate of amphidinolide C [4] in $CDCl_3$.



3

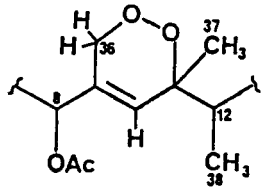


4

parison of the ^{1}H- and ^{13}C-nmr data including the ^{1}H-^{1}H COSY and NOESY spectra indicated that amphidinolide D [**5**] was a stereoisomer at C-21 of amphidinolide B [**2**][2].

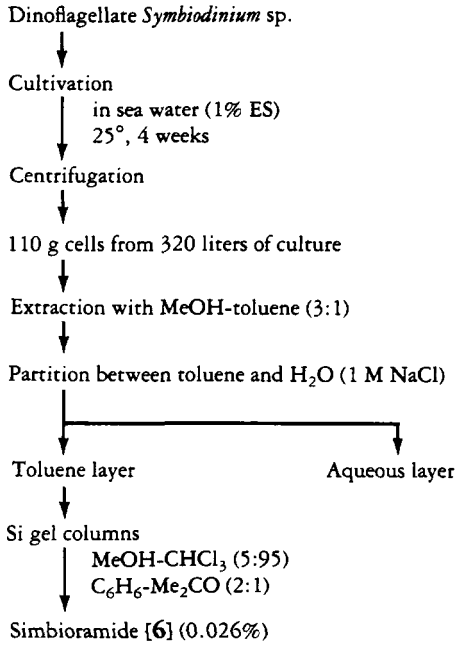
Amphidinolides A [**1**], B [**2**], C [**4**], and D [**5**] exhibited potent antineoplastic activity against L1210 murine leukemia cells in vitro with IC_{50} values of 2.4, 0.00014, 0.0058, and 0.019 μg/ml, respectively (24). Amphidinolide B [**2**] is the most active and 10,000 times more potent than amphidinolide A [**1**]. It is noted that these macrolides isolated from the same dinoflagellate are quite different from one another in substitution patterns and activities. Recently, a number of macrolides with structural similarities have been isolated from blue-green algae, sponges, and nudibranchs (2,5,6). There remain questions as to whether these macrolides were produced by the host animals or by symbiotic microorganisms (24).

SYMBIORAMIDE [**6**].—A novel sphingosine derivative, symbioramide [**6**], was isolated from the cultured marine dinoflagellate *Symbiodinium* sp. as a sarcoplasmic reticulum (SR) Ca^{2+}-ATPase activator (26). The dinoflagellate, which was obtained from the gill cells of the Okinawan bivalve *Fragum* sp., was cultured under the conditions shown in Scheme 2. The toluene-soluble fraction of the harvested cells was chromato-



A

[2]J. Kobayashi, M. Ishibashi, H. Nakamura, Y. Ohizumi, Y. Hirata, T. Sasaki, T. Ohta, and S. Nozoe, unpublished.

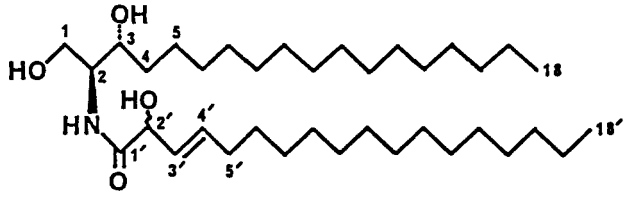


SCHEME 2. Separation of symbioramide [**6**] from cultured dinoflagellate *Symbiodinium* sp.

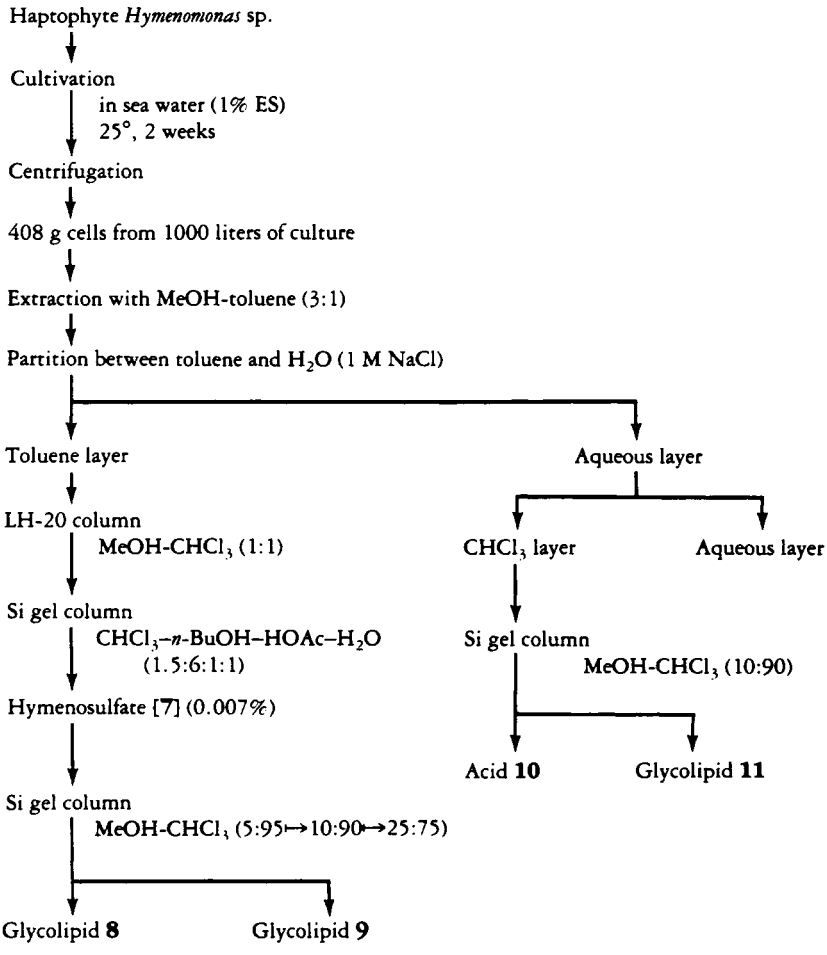
graphed on Si gel columns to yield simbioramide [**6**]. Acid hydrolysis furnished methyl 2-hydroxyoctadec-3(*E*)-enoate and 2(*S*)-amino-3(*R*)-hydroxyoctadecan-1-ol, confirming the structure **6** for symbioramide (26).

The Ca^{2+}-ATPase in SR membrane plays a key role in muscle relaxation by energizing Ca^{2+} pumping from the cytoplasm into the lumen of SR (27). Symbioramide [**6**] (10^{-4} M) activated SR Ca^{2+}-ATPase activity by 30%. This is the first example of SR Ca^{2+}-ATPase activator of marine origin. Symbioramide [**6**] also exhibited antileukemic activity against L-1210 murine leukemia cells in vitro, with an IC_{50} value of 9.5 μg/ml. The α-hydroxy-β,γ-dehydro fatty acid contained in **6** is seldom found from natural sources (26).

HYMENOSULFATE [**7**].—The Haptophyceae are microscopic, unicellar algae, which are widely distributed in the oceans and often constitute a major proportion of marine phytoplankton. A novel sterol sulfate, hymenosulfate [**7**], with potent Ca^{2+}-releasing activity in SR (10), was isolated from the cultured marine haptophyte *Hymenomonas* sp. (28). The haptophyte was isolated from an unidentified Okinawan stony coral and cultured under the conditions shown in Scheme 3. The toluene-soluble portion of the harvested cells was chromatographed on an LH-20 and a Si gel column to give hymenosulfate [**7**] (28). The major components of the toluene layer proved to be



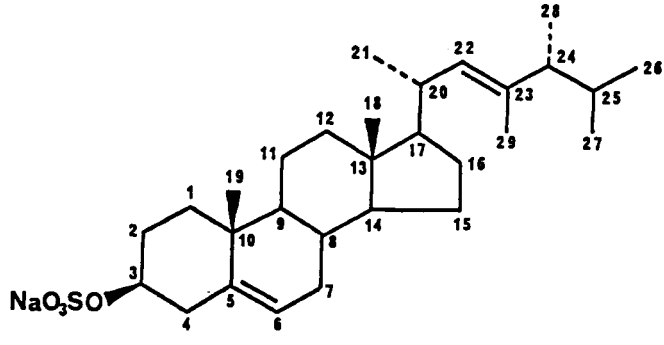
6

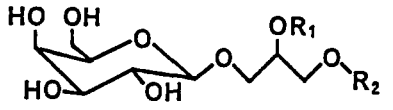


SCHEME 3. Isolation of hymenosulfate [7], glycolipids **8**, **9**, and **11**, and fatty acid **10** from cultured haptophyte *Hymenomonas* sp.

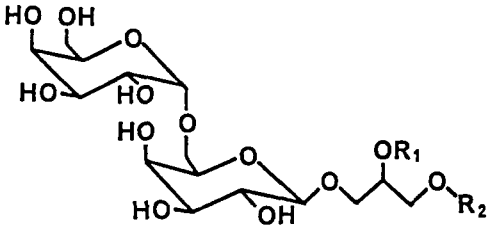
glycolipids such as mono- and di-galactosyldiacylglycerols **8** and **9**, respectively, while the $CHCl_3$-soluble materials of the aqueous layer were found to contain mainly octadecatetraenoic acid [**10**] together with a small amount of monogalactosylmonoacylglycerol [**11**] (28).

The structure of the sterol part of **7** obtained from acid hydrolysis was identified as (24*R*)-23,24-dimethylcholesta-(22*E*)-5,22-dien-3β-ol by comparison of the ^1H nmr





8 $R_1=R_2=X$
11 $R_1=H$, $R_2=X$



9 $R_1=R_2=X$

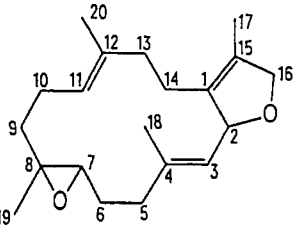
10 X-OH

data with those of Withers *et al.* (29). The presence of a sulfate group in **7** was confirmed by ion chromatography of sulfate ions liberated by solvolysis (28).

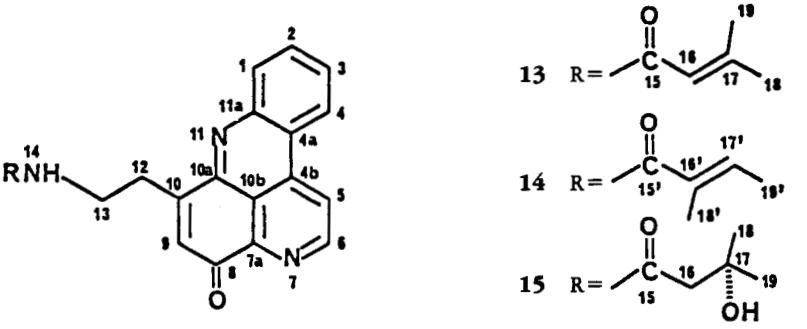
Hymenosulfate [**7**] is the first sterol sulfate from marine microalgae. In the SR, the Ca^{2+}-releasing activity of **7** was ten times more potent than caffeine, a well-known Ca^{2+}-releaser (10). The glycolipids **8**, **9**, and **11** exhibited inhibition of Na^+,K^+-ATPase activity (30) with IC_{50} values of 2×10^{-5} M each (28).

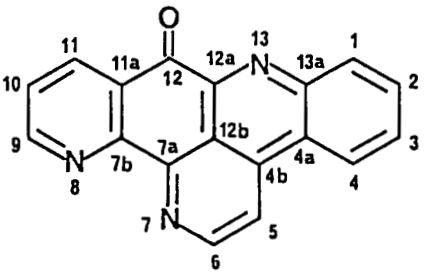
16-DEOXYSARCOPHINE [**12**].—It is well known that soft corals possess symbiotic dinoflagellates belonging to the genus *Symbiodinium*. In our early studies, 16-deoxysarcophine [**12**] with potent Ca-antagonistic activity was isolated from the Okinawan soft coral *Sarcophyton* sp., and the structure was established by X-ray analysis (31). This is the first example of a Ca-antagonistic substance from marine sources and of a nonalkaloid compound with such activity. Recently, 16-deoxysarcophine [**12**] was detected in hplc analysis of the extracts of the symbiotic dinoflagellate *Symbiodinium* sp. collected from the polyps of the soft coral *Sarcophyton* sp. However, it was difficult to culture this dinoflagellate.

OTHER BIOACTIVE MARINE NATURAL PRODUCTS INDICATED AS METABOLITES OF MICROORGANISMS.—Cystodytins A [**13**], B [**14**], and C [**15**] are novel tetracyclic aromatic alkaloids from the Okinawan tunicate *Cystodytes dellechiajei* (32), while asciidemin [**16**] is a novel pentacyclic aromatic alkaloid from another Okinawan tunicate *Didemnum* sp. (33). Prianosins A [**17**], B [**18**], C [**19**], and D [**20**] are also novel polycyclic alkaloids from the Okinawan sponge *Prianos melanos* (34,35). These are all potent antineoplastic agents. Recently, some polycyclic alkaloids with striking struc-



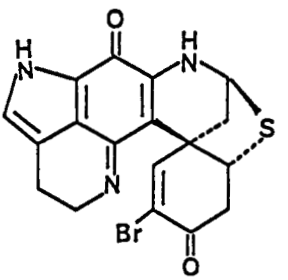
12



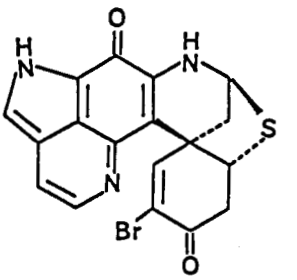


16

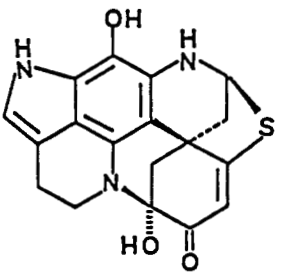
tural similarities to cystodytins **13–15**, ascididemin [**16**] or prianosins **17–20** have been isolated from other species of tunicates (36,37) and sponges (38–40), and even from sea anemone (41). It is well known that sponges possess symbiotic microorganisms such as blue-green algae or dinoflagellates (42). On the other hand, symbiotic microalgae of *Prochloron* spp. belonging to the new phylum Prochlorophyta have been isolated from some tunicates of the family Didemnidae (43), although their cultivation is very difficult. It may be that these polycyclic alkaloids are produced by related microorganisms, which are either part of the diets of these filter feeders or are present as symbionts.



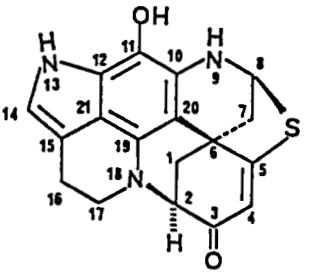
17



18



19



20

CONCLUSION.—Marine microorganisms have been studied mainly to clarify causes of seafood toxins, such as pufferfish, red tide, or ciguatera toxins. Some of these toxins (e.g., tetrodotoxin and maitotoxin) are used as pharmacological tools essential for basic studies on Na^+ or Ca^{2+} ion channels because of their specific activities on ion channels. We have focused on symbiotic microalgae in marine animals to obtain useful bioactive compounds other than toxins, because most marine toxins have been reported from free-living microalgae or microalgae attaching on the surface of seaweeds. As described in this presentation, eventually symbiotic microalgae such as dinoflagellates or haptophytes in marine animals (flatworm, mollusc, or coelenterate) have proven to be promising biomass for novel pharmacologically active substances if their large-scale cultivations are achieved. Studies on bioactive compounds from symbiotic marine microorganisms are now expected as a developing theme of great interest.

ACKNOWLEDGMENTS

The studies of amphidinolides B and C were performed through Special Coordination Funds of the Science and Technology Agency of the Japanese and Technology Agency of the Japanese Government. The studies of prianosins were supported in part by Grant-in-Aid (62010049) for Cancer Research from Ministry of Education, Science, and Culture, Japan. The author thanks all the co-workers involved in this presentation.

LITERATURE CITED

1. P.J. Scheuer, Ed., "Marine Natural Products—Chemical and Biological Perspectives," Academic Press, New York, 1978–1983, Vols. I–V.
2. P.J. Scheuer, Ed., "Bioorganic Marine Chemistry," Springer-Verlag, New York, 1987, Vol. 1.
3. D.J. Faulkner, *Nat. Prod. Rep.*, **1**, 251 (1984).
4. D.J. Faulkner, *Nat. Prod. Rep.*, **1**, 551 (1984).
5. D.J. Faulkner, *Nat. Prod. Rep.*, **3**, 1 (1986).
6. D.J. Faulkner, *Nat. Prod. Rep.*, **4**, 539 (1987).
7. K.L. Rinehart, V. Kishore, K.C. Bible, R. Sakai, D.W. Sullins, and K.-M. Li, *J. Nat. Prod.*, **51**, 1 (1988).
8. K.L. Rinehart Jr., J. Kobayashi, G.C. Harbour, J. Gilmore, M. Mascal, T.G. Holt, L.S. Shield, and F. Lafargue, *J. Am. Chem. Soc.*, **109**, 3378 (1987).
9. T. Narahashi, *Physiol. Rev.*, **54**, 813 (1974).
10. Y. Nakamura, J. Kobayashi, J. Gilmore, M. Mascal, K.L. Rinehart Jr., H. Nakamura, and Y. Ohizumi, *J. Biol. Chem.*, **261**, 4139 (1986).
11. Y. Ohizumi, H. Nakamura, J. Kobayashi, and W.A. Catterall, *J. Biol. Chem.*, **261**, 6149 (1986).
12. J. Takito, H. Nakamura, J. Kobayashi, Y. Ohizumi, K. Ebisawa, and Y. Nonomura, *J. Biol. Chem.*, **26**, 13861 (1986).
13. T. Yasumoto, D. Yasumura, M. Yotsu, T. Michishita, A. Endo, and Y. Kotaki, *Agric. Biol. Chem.*, **50**, 793 (1986).
14. T. Kosuge, K. Tsuji, and K. Hirai, *Chem. Pharm. Bull.*, **30**, 3255 (1982).
15. J.H. Cardellina II, F.-J. Marner, and R.E. Moore, *Science*, **204**, 193 (1979).
16. Y. Shimizu, in: "Handbook of Natural Toxins, Vol. 3. Marine Toxins and Venoms." Ed. by A.T. Tu, Marcel Dekker, New York, 1988, pp. 63–85.
17. Y.-Y. Lin, M. Risk, S.M. Ray, D. van Engen, J. Clardy, J. Golik, J.C. James, and K. Nakanishi, *J. Am. Chem. Soc.*, **103**, 6773 (1981).
18. Y. Shimizu, H.-N. Chou, H. Bando, G. van Duyne, and J. Clardy, *J. Am. Chem. Soc.*, **108**, 514 (1986).
19. A. Yokoyama, M. Murata, Y. Oshima, T. Iwashita, and T. Yasumoto, *J. Biochem.*, **104**, 184 (1988).
20. K. Tachibana, P.J. Scheuer, Y. Tsukitani, H. Kikuchi, D.V. Engen, J. Clardy, Y. Gopichand, and F.J. Schmitz, *J. Am. Chem. Soc.*, **103**, 2469 (1981).
21. Y. Murakami, Y. Oshima, and T. Yasumoto, *Bull. Jpn. Soc. Sci. Fish.*, **48**, 69 (1982).
22. J. Kobayashi, M. Ishibashi, H. Nakamura, Y. Ohizumi, T. Yamasu, T. Sasaki, and Y. Hirata, *Tetrahedron Lett.*, **27**, 5755 (1986).
23. M. Ishibashi, Y. Ohizumi, M. Hamashima, H. Nakamura, Y. Hirata, T. Sasaki, and J. Kobayashi, *J. Chem. Soc., Chem. Commun.*, 1127 (1987).
24. J. Kobayashi, M. Ishibashi, M.R. Wälchli, H. Nakamura, Y. Hirata, T. Sasaki, and Y. Ohizumi, *J. Am. Chem. Soc.*, **110**, 490 (1988).

25. R.J. Capon and D.J. Faulkner, *J. Org. Chem.*, **49**, 2506 (1984).
26. J. Kobayashi, M. Ishibashi, H. Nakamura, Y. Hirata, T. Yamasu, T. Sasaki, and Y. Ohizumi, *Experientia*, **44**, 800 (1988).
27. N. Ikemoto, *Annu. Rev. Physiol.*, **44**, 297 (1982).
28. J. Kobayashi, M. Ishibashi, H. Nakamura, Y. Ohizumi, and Y. Hirata, *J. Chem. Soc., Perkin Trans. 1*, 101 (1989).
29. N.W. Withers, W.C.M.C. Kokke, W. Fenical, and C. Djerassi, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 3764 (1982).
30. Y. Ohizumi, Y. Ishida, and S. Shibata, *J. Pharmacol. Exp. Ther.*, **221**, 748 (1982).
31. J. Kobayashi, Y. Ohizumi, H. Nakamura, T. Yamakado, T. Matsuzaki, and Y. Hirata, *Experientia*, **39**, 67 (1983).
32. J. Kobayashi, J.-F. Cheng, M.R. Wälchli, H. Nakamura, Y. Hirata, T. Sasaki, and Y. Ohizumi, *J. Org. Chem.*, **53**, 1800 (1988).
33. J. Kobayashi, J.-F. Cheng, H. Nakamura, Y. Ohizumi, Y. Hirata, T. Sasaki, T. Ohta, and S. Nozoe, *Tetrahedron Lett.*, **29**, 1177 (1988).
34. J. Kobayashi, J.-F. Cheng, M. Ishibashi, H. Nakamura, Y. Ohizumi, Y. Hiata, T. Sasaki, H. Lu, and J. Clardy, *Tetrahedron Lett.*, **28**, 4939 (1987).
35. J.-F. Cheng, Y. Ohizumi, M.R. Wälchli, H. Nakamura, Y. Hirata, T. Sasaki, and J. Kobayashi, *J. Org. Chem.*, **53**, 4621 (1988).
36. S.J. Bloor and F.J. Schmitz, *J. Am. Chem. Soc.*, **109**, 6134 (1987).
37. A. Rudi, Y. Benayahu, I. Goldberg, and Y. Kashman, *Tetrahedron Lett.*, **29**, 3861 (1988).
38. F.J. Schmitz, S.K. Agarwal, S.P. Gunasekera, P.G. Schmidt, and J.N. Schoolery, *J. Am. Chem. Soc.*, **105**, 4835 (1983).
39. T.F. Molinski, E. Fahy, D.J. Faulkner, G.D. van Duyne, and J. Clardy, *J. Org. Chem.*, **53**, 1341 (1988).
40. N.B. Perry, J.W. Blunt, M.H. Munro, T. Higa, and R. Sakai, *J. Org. Chem.*, **53**, 4127 (1988).
41. G. Cimino, A. Crispino, S. De Rosa, S. De Stefano, M. Gavagnin, and G. Sadano, *Tetrahedron*, **43**, 4023 (1987).
42. T.L. Simpson, "The Cell Biology of Sponges," Springer-Verlag, New York, 1984, pp. 114–123.
43. R.A. Lewin, *Nature*, **261**, 697 (1976).