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Bioactive Metabolites from Marine Organisms of Okinawan Waters

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1 INTRODUCTION

The islands of Okinawa are located in the subtropical waters between Taiwan in the southwest and Kyushu in the northeast. Because of warm Kuroshio current, the water temperature rarely falls below 20 °C even in winter time when atmospheric temperature is typically around 15 °C. Accordingly, coral reefs are well developed along the islands. Marine fauna and flora of Okinawan waters are therefore not much different from those of the tropical Indo-Pacific waters.

One of the most characteristic features of a coral reef as a biological community is the variety of species and their high population density. There are numerous inter- and intra-species interactions. Many seemingly defenseless organisms are equipped with chemical weapons or defensive chemicals as their survival strategy. It is mainly such chemicals that organic chemists are searching for as bioactive metabolites. Due to the variety of species and interactions one finds more opportunity with coral-reef organisms in the discovery of new bioactive compounds. This is reflected in the high incidence of bioactivity observed with coral-reef organisms in the screenings as discussed in the next section and also in the fact that more compounds have been discovered from organisms of coral-reef areas than from other areas in the past two decades.

Chemical study of marine organisms of Okinawan waters was first initiated in 1960s by Hashimoto's group who aimed at finding toxins associated with food intoxication (ref. 1). Fujisawa Pharmaceautical Company started drug screening for the organisms of this areas in early 1970s. Today, a number of research groups throughout the country are actively engaged in the quest of bioactive substances from Okinawan waters. In collaboration with Harbor Branch Oceanographic Institution of the United States, we have recently carried out screening for potential drugs. Our main objective was to discover new antitumor and antiviral agents. A number of active compounds have been isolated. In this Chapter I will describe the result of the screening and some selected compounds having cytotoxicity, antiviral, and other biological activity.

2 SCREENING

From 1985 to 1987 we collected more than 300 species of marine organisms on the coral reefs of Okinawa and its vicinity islands. They were mostly sponges (53%), coelenterates (23%), and algae (16%). Sample sizes varied from a few hundred grams to several kilograms. Each of these organisms was extracted by steeping in either methanol or acetone for 2-5 days. The extract was concentrated to an aqueous suspension which was then partitioned between ethyl acetate and water. Concentration of the ethyl acetate layer gave a lipophilic extract. The aqueous layer was concentrated to dryness and extracted with methanol to afford methanol-soluble extract. Both extracts were submitted for screening of cytotoxic, antiviral, and antifungal activity. The in vitro cytotoxicity test was carried out using P388 murine leukemia cells. For antiviral test herpes simplex virus type 1 (HSV-1) and vesicular stomatitis virus (VSV) were used. Antifungal activity was evaluated by inhibition of the growth of Candida albicans. The results of cytotoxicity and antifungal activity are summarized in Table 1. More than 60% of sponges and tunicates showed

TABLE 1
Incidences of marine organisms showing cytotoxicity and antifungal activity

Organism	Number of species tested	Species showing cytotoxicity[a]		Species showing antifungal activity[b]	
		No.	%	No.	%
Sponge	173	111	62.4	55	30.9
Coelenterate	76	27	35.5	10	13.2
Alga	52	7	13.5	2	3.8
Tunicate	19	12	63.2	2	10.5
Mollusk	6	3	50.0	0	0
Total	331	160	48.3	69	20.8

[a] $IC_{50} \leq 10$ μg/ml. [b] MIC ≤300 μg/ml against Candida albicans.

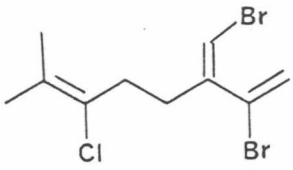
significant level (IC_{50} <10 μg/ml) of cytotoxicity. These figures are substantially higher than those of reported incidences for cytotoxicity or antitumor activity of marine organisms (refs. 2-5). For example, Nemanich et al. (ref. 2) reported that 31% of sponges were active against KB cells. However, such direct comparison is meaningless because of the differences in assay systems, extraction solvents, evaluation criteria, etc. A factor contributed to the higher values of our results, however, is that we collected organisms selectively.

A number of extracts also showed antiviral activity, but for many of them it was associated with strong cytotoxicity. Only several species showed interesting activity. Some active compounds isolated from them are described in Section 4.

3 CYTOTOXIC SUBSTANCES

3.1 Cyclohexadienones

The red alga Desmia (Syn. Chondrococcus) hornemanni is widely distributed in the tropical and subtropical waters of the Pacific. It has been the subject of several chemical investigations and shown to be a rich source of halogenated monoterpenes. The monoterpenes fall into two structural types, myrcenes (e.g. 1) from Japanese (ref. 6), Hawaiian (ref. 7), and Australian samples (ref. 9) or ochtodenes (e.g. 2) from Hawaiian (refs. 7,8), Australian (ref. 9), and Tahitian samples (ref. 10).



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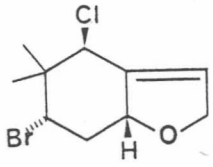

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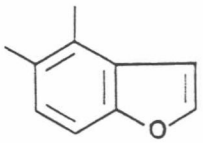

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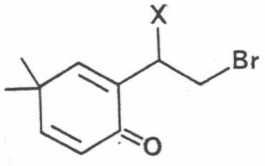
When we examined the alga from Okinawa, we noticed the presence of both acyclic and cyclic monoterpenes. In our preliminary work we isolated 4,5-dimethylbenzo[b]furan (3) which has also been isolated from a Hawaiian sample of the alga (ref. 11). In the subsequent work, an acetone extract of the alga was rapidly separated by flash chromatography on silica gel to afford fractions containing cyclic monoterpenes. Purification of the fractions on the same column gave compounds 4-9. Each pair of the compounds 4, 7, and 8 was obtained



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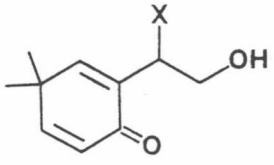
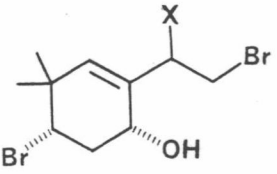
4b X=Br



5 X=Cl
6 X=OH



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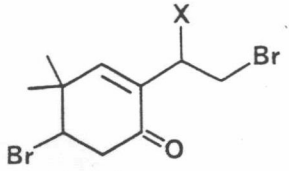

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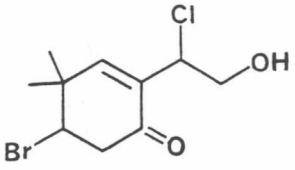
8b X=Br



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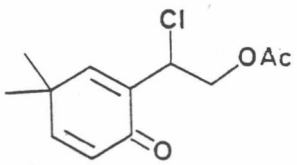
as a mixture and characterized without further separation. Compound **5** is extremely labile in the presence of a trace of an acid. It undergoes spontaneous trasnsformation into benzofuran **3** at room temperature in several hours (ref. 12). It is tempting to speculate that **5** is first converted to a phenol (**10**) by the dienone-phenol rearrangement (ref. 13) and then undergoes cyclization to form the furan ring. However, the acetyl derivative **11** and the



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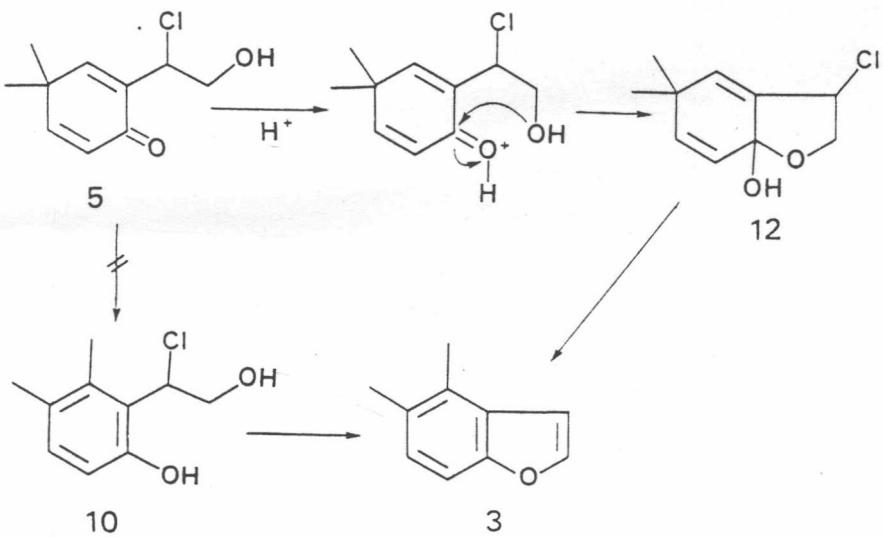
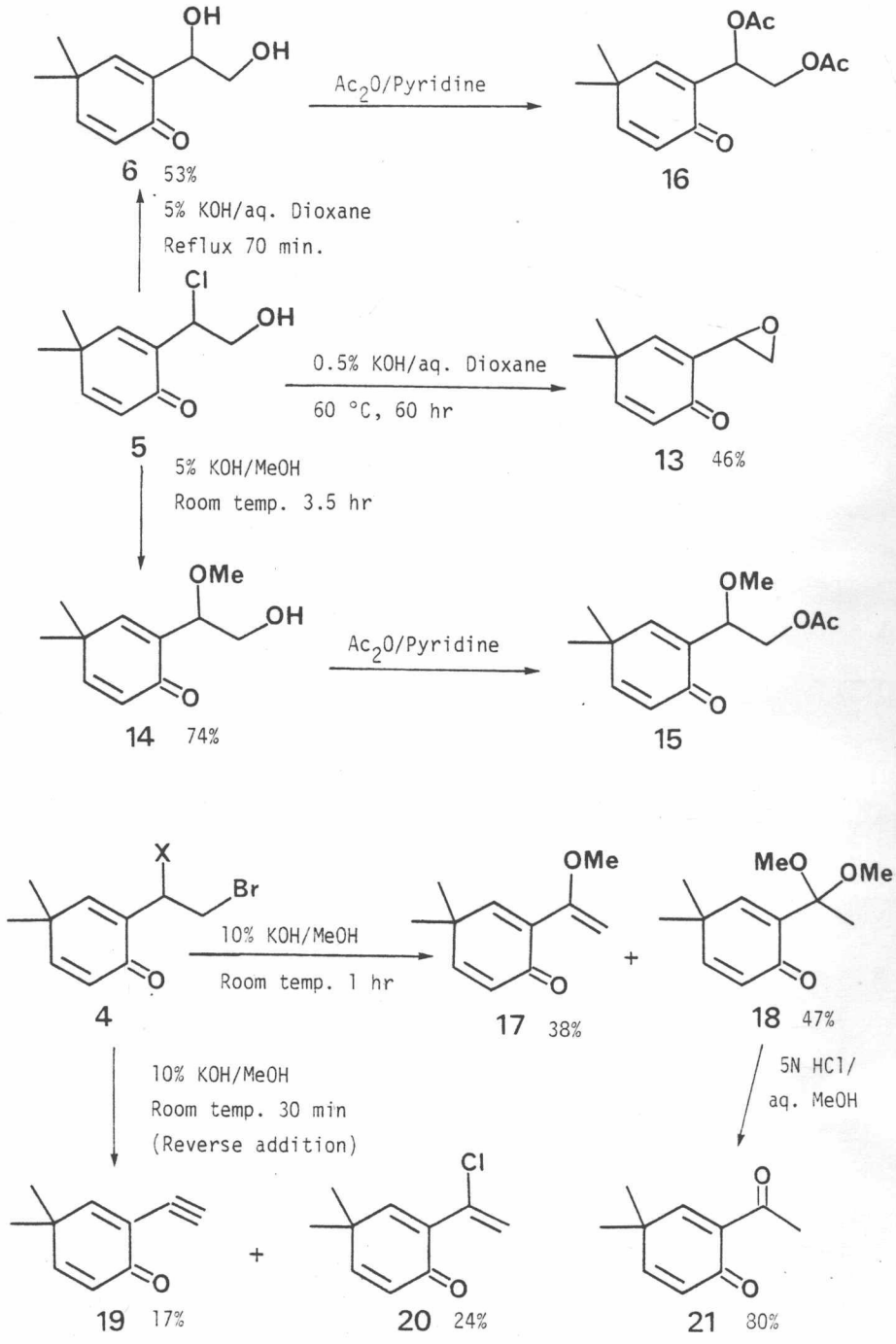
mixture of **4a** and **4b** are quite stable and are not converted to **3** at all in similar conditions. This fact suggests that a plausible mechanism is to involve the hemiketal **12** as an intermediate which can be easily converted to **3** by the cynchronized aromatization of the two rings (Scheme 1).



Scheme 1. Mechanism for the formation of benzofuran **3**

Compounds **6–9** could be related to compounds **4** and **5** by simple treatment. Reaction of **5** with 5% KOH in wet dioxane under reflux gave **6** in 53% yield. Oxidation of the mixture of **7a** and **7b** with Jones reagent furnished a mixture of **8a** and **8b** which in turn was treated with 1% KOH in methanol to give a mixture of **4a** and **4b** (ref. 14).



Scheme 2. Preparation of cyclohexadienone derivatives

Treatment of compounds **4** and **5** with potassium hydroxide under various conditions gave several derivatives as shown in Scheme 2 (ref. 14). The vinyl ether **17** and ketal **18** were obtained by addition of **4** to a solution of 10% KOH in dry methanol at room temperature and allowing the mixture to stand for 1 hr. Conversely, when the alkaline solution was added to a neat oil of **4** and the mixture was allowed to stand for 30 min, the reaction products were the acetylene **19** and vinyl chloride **20**. Cytotoxicity of these compounds was evaluated using the cells of P388, A549 human lung carcinoma, and HCT-8 human colon adenocarcinoma. The results are shown in Table 2. Among them compounds **4**, **13**, **19**, **20**, and **21** exhibited relatively high activity. The acetate **11** showed in vitro antiviral activity, but it was too toxic in in vivo assay.

Besides cytotoxicity, the major constituents of the alga, **4** and **5**, were also shown to have ichthyotoxicity and feeding-deterrent properties. Ichthyotoxicity of **4** and **5** against guppies was observed at 1 and 10 ppm, respectively, while feeding deterrency of **4** was demonstrated at 0.1% in feed.

TABLE 2

Cytotoxicity (IC_{50} in μg/ml) of cyclohexadienones[a]

Compound No.	**4**	**6**	**11**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
P388	0.5	>200	5	10	>200	200	50	5	10	1	5	5
A549	5	>100	20	<1	100	>100	>100	30	30	3	5	5
HCT-8	5	100	10	5	5	70	70	20	30	0.3	5	5

[a]The test was conducted by Dr. May Lui at Harbor Branch Oceanographic Institution/SeaPharm Project in 1985.

3.2 Manzamines

A sponge of the genus Haliclona gave an extract which displayed strong cytotoxicity in our screening. In a preliminary work the major active principle was isolated by a rather elaborate procedure and named as manzamine A (**22**) after Manzamo, the place where the sponge was collected. By securing more of the sponge minor active constituents manzamines B, C, and D were also isolated as shown in Fig. 1. In this isolation scheme manzamine A was obtained in a large amount by a simple procedure. Ethyl acetate-soluble oil of the acetone extract was first partitioned between two layers of heptane-methylene chloride-acetonitrile (50:15:35). The residue of the bottom layer was redissolved in methanol and kept in a refrigerator overnight to crystallize out most of manzamine A. The mother liquor was first separated by centrifugal partition chromatography (CPC), and the mobile and stationary phases were further processed to give manzamines B, C, and D as shown in Fig. 1.

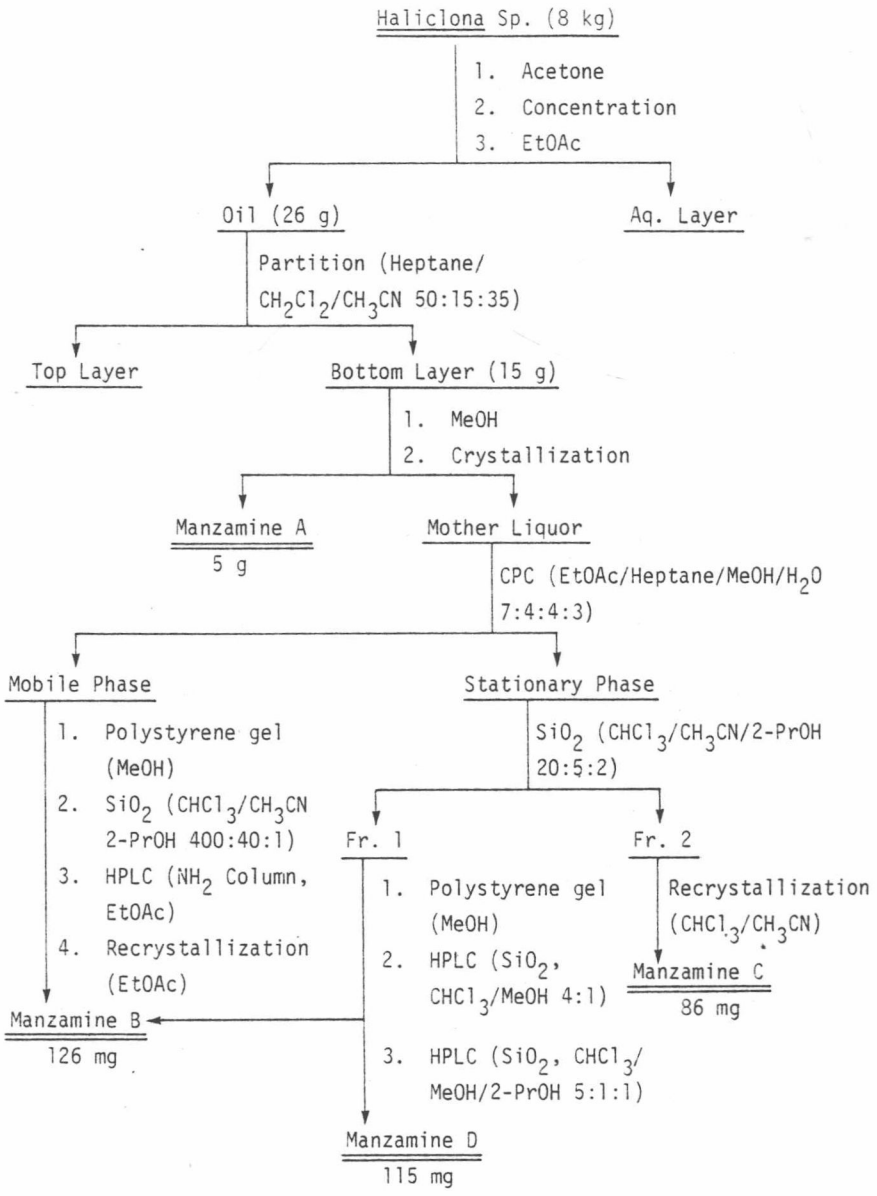
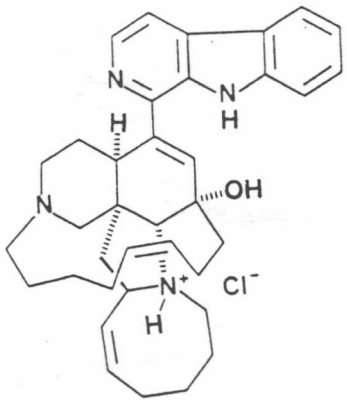


Fig. 1. Isolation of manzamines A-D from a sponge, Haliclona sp.

The molecular formula of manzamine A was deduced as $C_{36}H_{44}N_4O$ from high resolution EIMS. The ^1H NMR spectrum (Fig. 2) showed signals well dispersed over a wide range of the field. The aromatic signals together with UV absorption data suggested the presence of a 1-substituted β-carboline (ref. 15). The presence of two di- and one tri-substituted double bonds was shown by the



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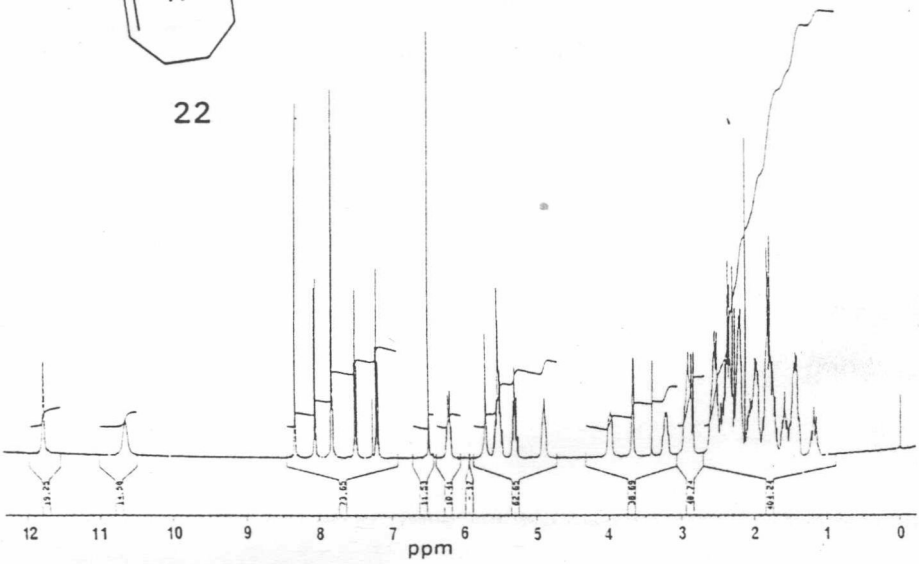
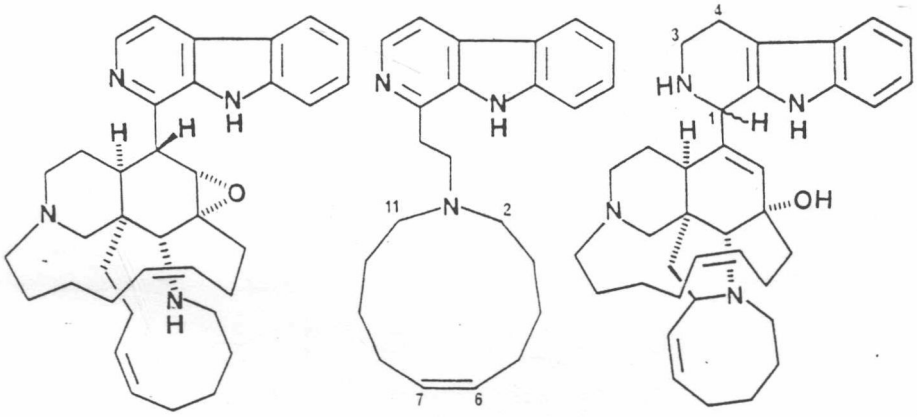


Fig. 2. ^{1}H NMR spectrum (360 MHz) of manzamine A recorded in $CDCl_3$



23 24 25

olefinic proton signals and ^{13}C NMR data. These accounted for 12 of the 17 sites of unsaturation required by the formula. Thus, manzamine A must have five additional rings which include two nitrogen atoms, suggesting a complex structure. Since it deemed impractical to elucidate the structure by spectroscopic method, a crystal of its hydrochloride was submitted for X-ray analysis. The structure (**22**) thus determined was a novel one consisted of a complex pentacyclic array of 5-, 6-, 8-, and 13-membered rings (ref. 16).

Manzamine B exhibited similar ^{1}H and ^{13}C NMR spectra with those of manzamine A. Again, it was unsuccessful to elucidate the full structure by NMR correlation with manzamine A. The structure (**23**) with its absolute configuration was determined by X-ray (ref. 17). In manzamine B (**23**) the tertiary hydroxyl group is cyclized to the vicinal double bond to form an epoxy ring, and the bond bisecting the 5- and 8-membered ring is ruptured to form a

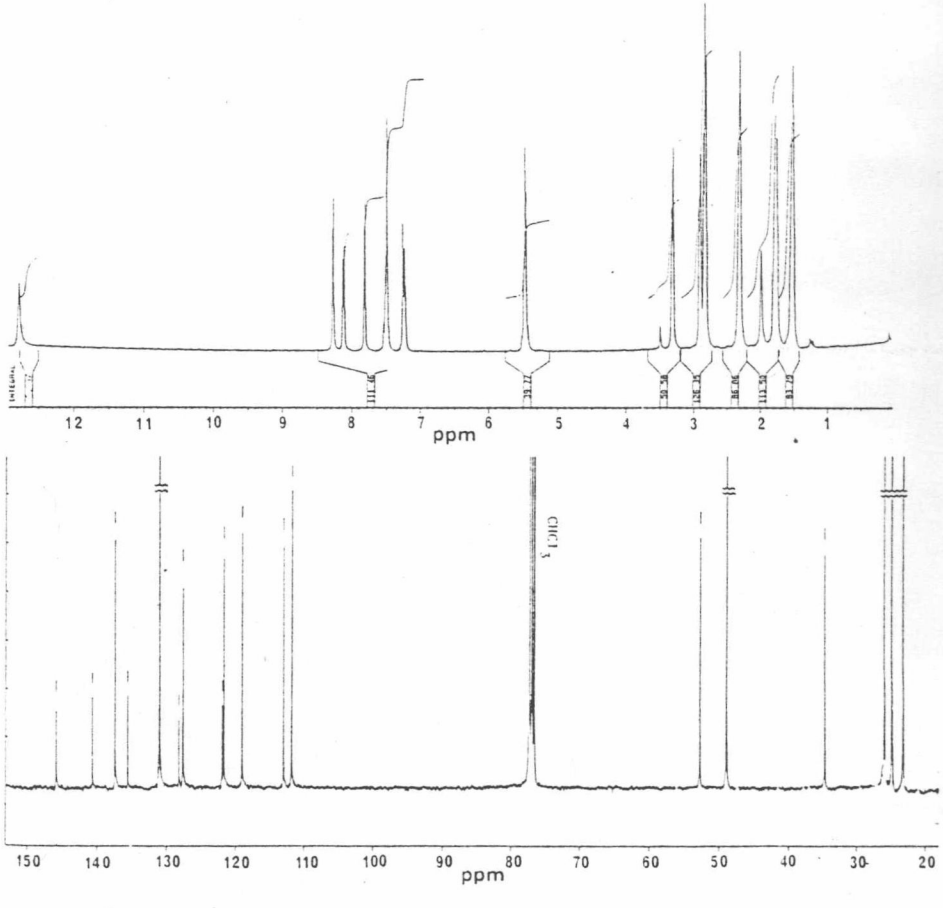


Fig. 3. ^{1}H and ^{13}C NMR spectra of manzamine C recorded in $CDCl_3$

new 11-membered nitrogen heterocycle.

Manzamine C (**24**) revealed relatively simple ^{1}H and ^{13}C NMR spectra as shown in Fig. 3. These data and the unsaturation requirement suggested the presence of only one ring with one nitrogen atom and a double bond besides a 1-substituted β-carboline ring. The spectra show six methylene signals of which four have twice the integration of other two, suggesting a symmetric nature of the ring. Thus, the structure **24** having an 11-membered ring was proposed for manzamine C. It was confirmed by X-ray on a single crystal (ref. 17).

The ^{1}H and ^{13}C NMR spectra of manzamine D was similar to those of manzamine A except for the absence of aromatic proton signals corresponding to 3- and 4-H on the β-carboline ring.This observation together with the molecular ion at m/z 552, four mass units higher than that of **22**, suggested the structure of manzamine D as 1,2,3,4-tetrahydromanzamine A (**25**). An attempt to dehydrogenate **25** with 10% palladium on charcoal was unsuccessful. However, it could be successfully oxidized using DDQ to furnish a product identical with manzamine A. The configuration at C-1 is not known. Some physical properties and cytotoxicity data for manzamines are given in Table 3.

TABLE 3
Molecular formula, melting point, specific rotation, and cytotoxicity (P388) of manzamines A-F (**22-27**).

Compound No.	Molecular formula	MP °C	$[\alpha]_D$	IC_{50} (μg/ml)
22	$C_{36}H_{44}N_4O$	>240 (dec)	+50°	0.07
23	$C_{36}H_{46}N_4O$	198-203	+89°	6.0
24	$C_{23}H_{29}N_3$	77-82	0°	3.0
25	$C_{36}H_{48}N_4O$	165-168	+60.6°	0.5
26	$C_{36}H_{44}N_4O_2$	174-176	+63.7°	5.0
27	$C_{36}H_{44}N_4O_3$	230 (dec)	+59.9°	5.0

Another cytotoxic sponge, Xestospongia sp. was also found to contain manzamine alkaloids as the active constituents. In addition to manzamine A (**22**) two minor components, manzamines E and F were isolated when an extract of the sponge was separated in a similar manner. Some physical data and cytotoxicity of these alkaloids are also given in Table 3. Both metabolites displayed similar NMR spectra to those of manzamines A and B. The spectra for manzamine E are shown in Fig. 4. As seen in the ^{13}C NMR spectrum, both E and F showed signals at δ 214.8 and 216.2, respectively, indicating the presence of a ketonic carbonyl. The IR spectra also showed absorptions for the ketonic carbonyl at 1700 and 1699 cm^{-1} for manzamines E and F, respectively. Extensive 2D-NMR studies (COSY, long range COSY, HETCOSY) enabled us to to determine the

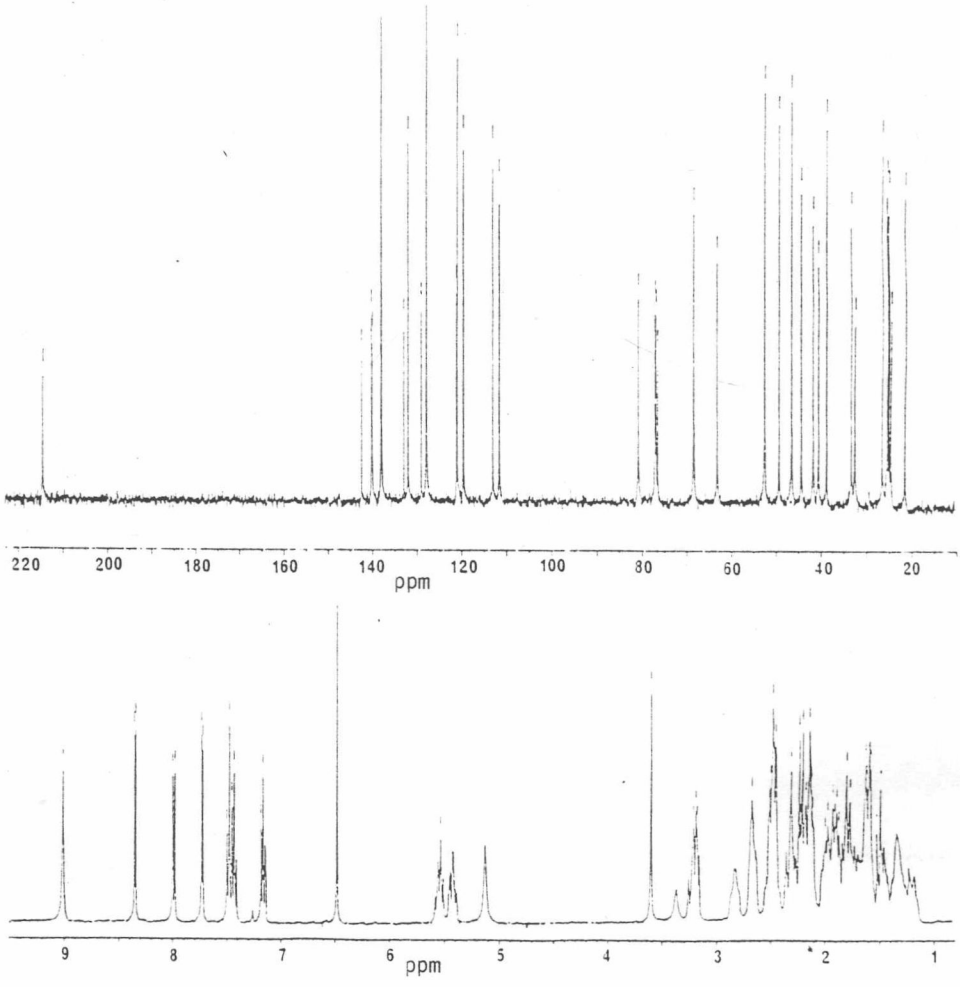
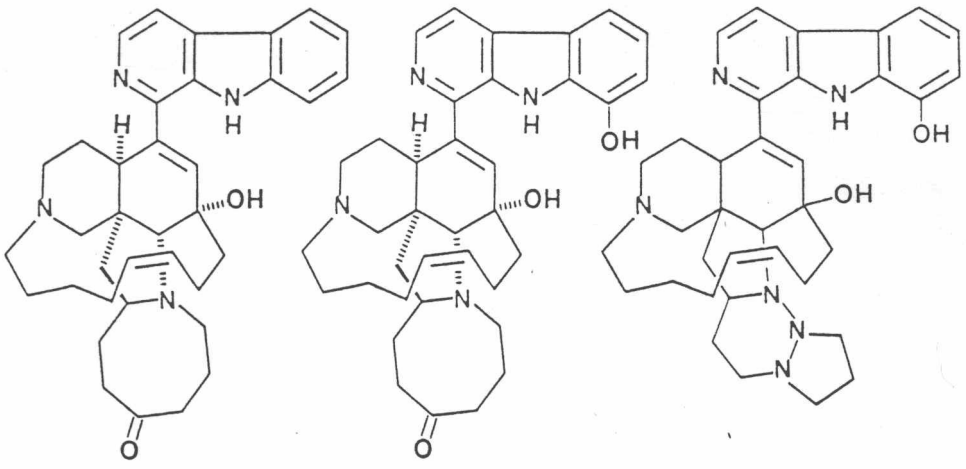


Fig. 4. ^{13}C and ^{1}H NMR spectra of manzamine E recorded in $CDCl_3$

structures as **26** and **27** for manzamines E and F, respectively, and to assign all the NMR signals in comparison with manzamine A (ref. 18).

In the meantime, Nakamura et al. (ref. 19) also reported the isolation of a compound identical with manzamine A and a new alkaloid designated as keramamine B (**28**). When we compared the ^{13}C NMR data of manzamine F (**27**) with those of keramamine B, to our surprise almost all the signals were virtually identical except for the lack of the carbonyl signal in the latter. We then made direct comparison of the two samples by securing a small amount of keramamine B from the authors. They were compared by TLC, UV, and IR and also by TLC of 2,4-dinitrophenyl hydrazone derivatives and of $NaBH_4$ reduction products. As shown by the IR spectra (Fig. 5), all the data indicated that keramamine B contained a



26 27 28

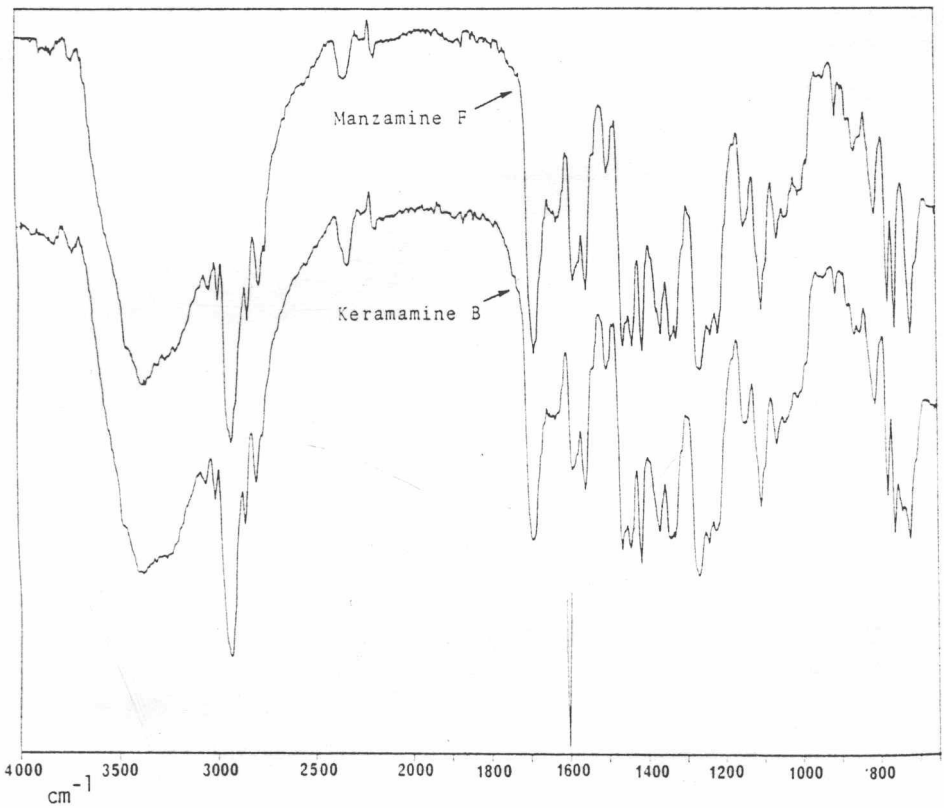
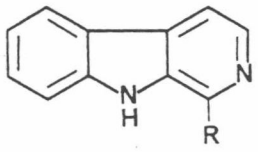


Fig. 5. Infrared spectra (KBr) of manzamine F and keramamine B.

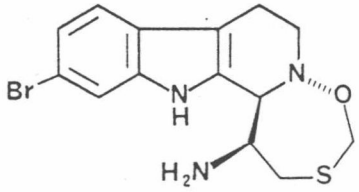
carbonyl group and that the two samples were indistinguishable. Thus, the structure **28** is incorrect, and it should be revised as **27**.

In spite of the widespread occurrence of β-carboline alkaloids in terrestrial plants (ref. 20), the structures of manzamines are unique and unprecedented in nature. Less complex β-carbolines have so far been found from several species of marine organisms. Harman (**29**) and norharman (**30**) have been isolated from the dinoflagellate Noctilluca miliaris (ref. 21). Brominated harman and derivatives have been reported from the hydroid Aglaophenia pluma (ref. 22). A series of bioactive β-carboline, eudistomines (e.g. eudistomin K, **31**) have been isolated from three species of tunicates: Eudistoma olivaceum (ref. 23), E. glaucus (ref. 24), and Ritterella sigillinoides (ref. 25). Eudistomines are reported to have antiviral activity.



29 R = CH_3

30 R = H



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3.3 Cyclic Peroxides

In the screening for cytotoxicity an extract of the sponge Plakortis lita exhibited significant activity. Separation of the extract gave five cyclic peroxides (**32-36**) as active constituents.

A sample of the freeze-dried sponge was extracted with ethyl acetate, and the extract was separated according to the procedure shown in Fig. 6. Since the major active constituent which was identified as known compound, chondrillin (**32**) (ref. 26), was present in more than 70% of the crude extract, separation of the minor active constituents was difficult to achieve by conventional chromatography alone. Thus, a fraction containing minor components was first separated from most of chondrillin by use of CPC. Each of the minor constituents was then separated by HPLC as shown in Fig. 6.

Like chondrillin (**32**) all the minor peroxides were methyl esters of fatty acids and contained a methoxy group at C-6, an epidioxy bridge between C-3 and C-6, and a double bond at C-4 in common. The structures of the new compounds were secured by spectroscopic data (ref. 27). EIMS of these peroxides showed no molecular ions but ions corresponding to $(M - O_2)^+$, resulting from retro-Diels-Alder type fragmentation. All the minor compounds **33-36** have the same stereochemistry and are epimeric to **32** as determined by NOE difference spectroscopy.

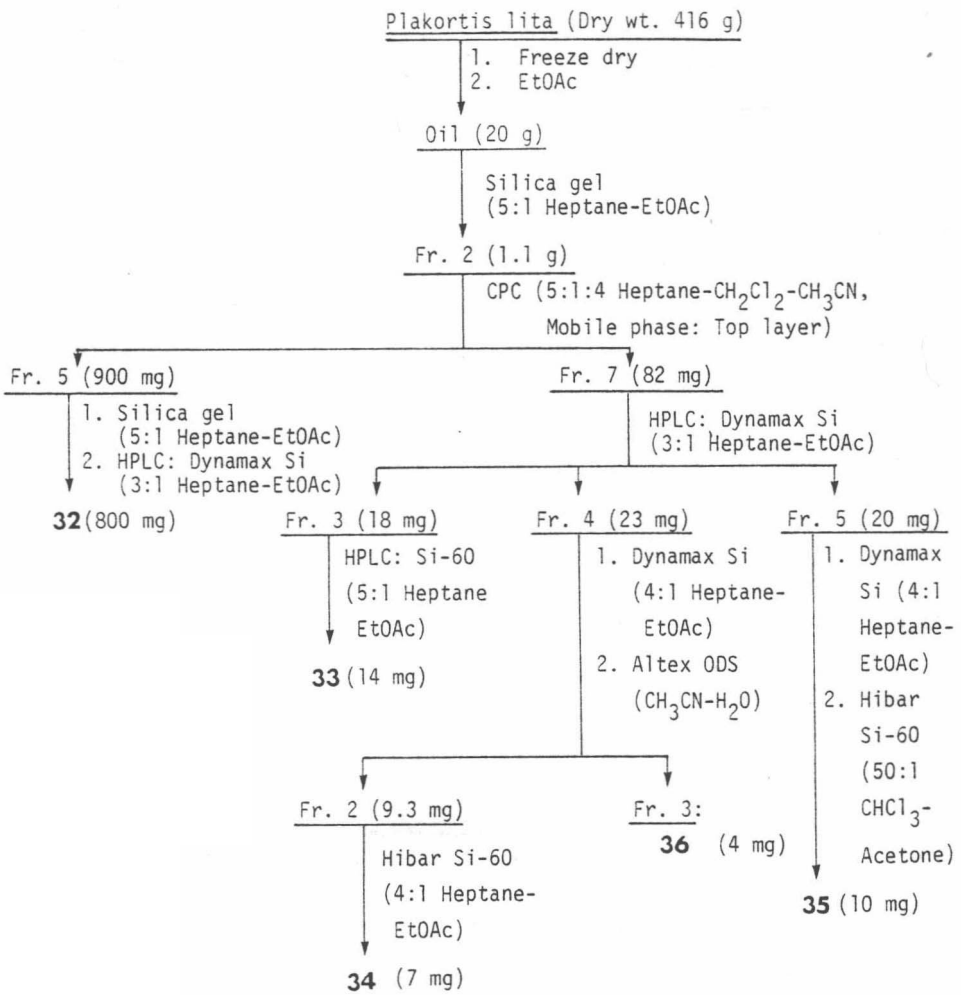
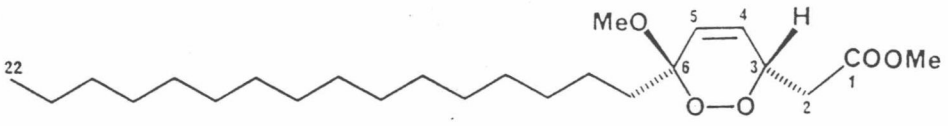


Fig. 6. Isolation of cyclic peroxides from the sponge Plakortis lita.

Chondrillin (**32**) exhibited P388 IC_{50} 5 µg/ml, while **33-36** showed IC_{50} 0.05-0.1 µg/ml. This difference in the activity can perhaps be attributed to the difference of stereochemistry of the epidioxy ring.

Two closely related homologues, xestins A and B, have been reported from a sponge of the genus Xestospongia (ref. 28). A number of cyclic peroxides have been known from sponges (ref. 29). Typically they are carboxylic acid derivatives of terpenoids or non-terpenoids. In both cases an epidioxy function bridges between C-3 and C-6 of a carboxylic acid or an ester as in plakortin (**37**) (ref. 30) and trunculin A (**38**) (ref. 31). Many of these peroxides have been reported to have antimicrobial, ichthyotoxic or cytotoxic activity.



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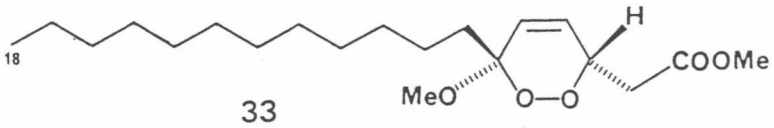

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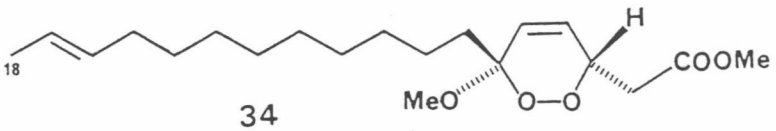

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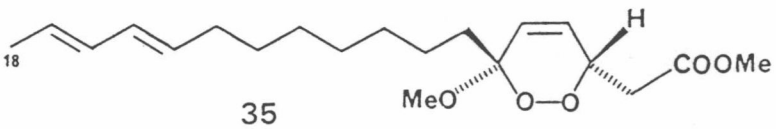

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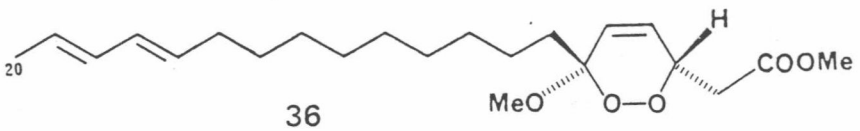

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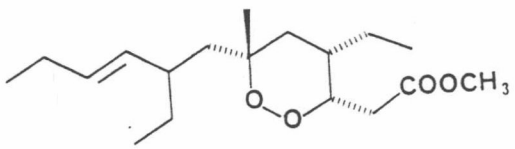

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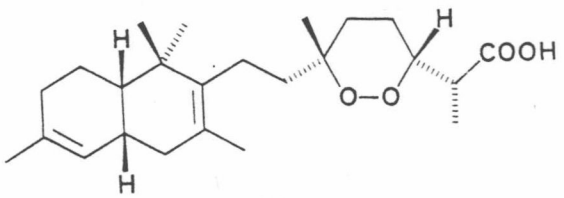

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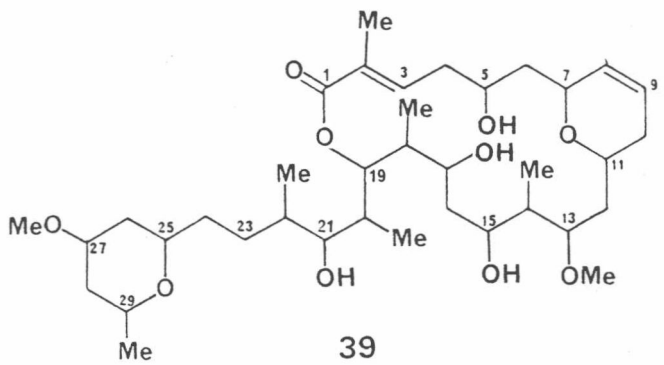
3.4 Misakinolide A

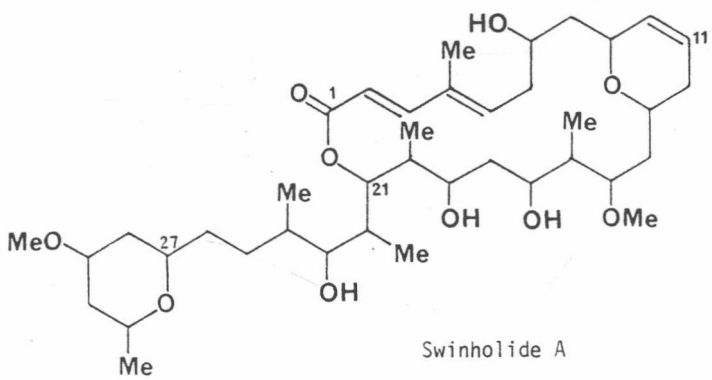
One of the most active extracts in our cytotoxicity screening was that of a sponge, Theonella sp., collected at Maeda-misaki, Okinawa. Bioassay-guided

separation of this extract led to the isolation of an active principle named misakinolide A. Cytotoxicities of this compound were IC_{50} 10, 4, 4, and 0.5 ng/ml against P388, HCT-8, A549, and MDA-MB-231 (human breast adenocarcinoma), respectively. It also showed antifungal activity with MIC 5 µg/ml against Candida albicans. In vivo antitumor test (P388) revealed T/C 145% at a dose of 0.5 mg/kg in mice.

The structure of misakinolide A was first proposed as a 20-membered macrolide (**39**) on the basis of the formula $C_{37}H_{64}O_{10}$ derived from a high resolution FABMS and of extensive 2D-NMR studies (ref. 32). The proposed structure was related to swinholide A, a 22-membered macrolide isolated



39



Swinholide A

recently from Theonella swinhoei (ref. 33). In the meantime, Fusetani's group also isolated a compound showing identical spectral data but having a doubled molecular weight as indicated by low resolution mass spectrometry. Comparison of the two samples by TLC, HPLC, and ^{1}H and ^{13}C NMR spectra demonstrated that they were indistinguishable. Reexamination of our sample by high resolution FABMS and Fusetani's sample by vapor pressure method of molecular weight determination revealed that the compound should best be represented by the