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Pharmacologically active substances from southern Pacific marine invertebrates

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Abstract - Crude extracts prepared from marine organisms, which were collected in southern Pacific waters and classified into 10 groups, were applied to the pharmacological screening system which consists of 7 assays. The activity index which reflects both the probability and the potency of each group for each assay was derived from the statistical analysis of the screening result. Pharmacologically active constituents, i.e. cytotoxic compounds, coronary vasodilative compounds, and hypotensive compounds were isolated and identified.

INTRODUCTION

We carried out a random screening of a wide variety of marine organisms collected in southern Pacific waters, in order to accumulate information regarding their pharmacological activities. The 583 specimens were lyophilized and first extracted with ethanol to prepare the lipid soluble extracts and then with 40 % aqueous ethanol to prepare water soluble extracts. The crude extracts were applied to the pharmacological screening system which consists of antimicrobial assay, cytotoxicity assay, coronary vasodilation assay, cardiotonic assay, antiulcer assay, angiotensin converting enzyme inhibition assay, and platelet aggregation inhibition assay. The crude extracts which showed strong pharmacological activities were selected and submitted to the purification procedures; the fractionations were followed by monitoring the activities.

PHARMACOLOGICAL SCREENING

Antimicrobial assay. Paper disk method was applied to the following microorganisms; Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Mycobacterium smegmatis, Escherichia coli, Pseudomonas aeruginosa, Aspergillus flavus, and Candida albicans. The potencies of antimicrobial activities were evaluated based on the diameters of the inhibition zones at the test concentration of 5,000 μg/ml/disk; - (diameter = 0 mm), ± (0 mm < diameter ≦ 10 mm), + (10 mm < diameter ≦ 14 mm), ++ (14 mm < diameter ≦ 20 mm), +++ (20 mm < diameter).

Cytotoxicity assay. The lethal effects on PV_4 cultured cells transformed with polyoma virus were evaluated by T/C %; T/C = number of living cells in the test culture / number of living cells in the control culture; - (at 100 μg/ml test concentration, on 8th day: 60 % < T/C), ± (at 100 μg/ml test concentration, on 8th day: T/C ≦ 60% and at 10 μg/ml test concentration, on 8th day: 60 % ≦ T/C), + (at 10 μg/ml test concentration, on 8th day: 40 % < T/C < 60 %), ++ (at 10 μg/ml test concentration, in 24 hours: 0% < T/C ≦ 40 %), +++ (at 10 μg/ml test concentration, in 24 hours: T/C = 0 %).

Coronary vasodilation assay. The test extracts were screened on isolated guinea pig hearts with use of Langendorf's perfusion technique. The potencies of the activity were evaluated comparing with the standard activity (100 %) of papaverine (33 μg/heart); - (effect < 50 % at 500 μg/heart of water soluble extracts and effect ≦ 20 % at 100 μg/heart of lipid soluble extracts), + (50 % ≦ effect at 500 μg/heart and effect < 50 % at 100 μg/heart of water soluble extracts and 20 % ≦ effect < 50 % at 100 μg/heart of lipid soluble extracts), ++ (50 % ≦ effect at 100 μg/heart and effect < 50 % at 10 μg/heart), +++ (50 % ≦ effect at 10 μg/heart).

Cardiotonic assay. The test extracts were screened on isolated guinea pig hearts with use of Langendorf's perfusion technique. The potencies of the activity were evaluated by comparing with the standard activity of papaverine (33 μg/heart); - (effect at 500 μg/heart < standard), + (standard ≦ effect at 500 μg/heart and effect at 100 μg/heart < standard), ++ (standard ≦ effect at 100 μg/heart).

TABLE 1. Classification of marine specimens

classification	number of specimens
sponges	270 (142)
algae	146 (134)
corals	84 (66)
sea cucumbers	9 (9)
higher plants	7 (6)
snails	6 (6)
sea urchins	7 (6)
tunicates	18 (12)
sea anemones	5 (5)
others	31 (29)
total	583 (415)

( ): Numbers of the specimens submitted to the statistical analysis

TABLE 3. Calculation of activity index of sponge specimens for cytotoxicity

activity	+++	++	+	±	-
	factor 4	factor 3	factor 2	factor 1	factor 0
percentage	2.1 %	2.8 %	13.4 %	33.8 %	47.9 %

Activity Index =

$(4 \times 2.1) + (3 \times 2.8) + (2 \times 13.4) + (1 \times 33.8) + (0 \times 47.9) = 77$

TABLE 2. Activity index

	antimicrobial*								cytotoxicity	coronary vasodilation	cardiotonic	antiulcer	ACE inhibition	platelet aggregation inhibition
	S.a.	B.s.	M.l.	M.s.	E.c.	P.a.	A.f.	C.a.						
sponges	27	55	27	19	0	0	3	8	77	191	116	1	48	6
algae	6	13	6	3	0	0	0	0	37	227	149	0	17	4
corals	9	10	4	4	0	0	0	0	83	212	150	3	9	5
sea cucumbers	0	0	0	0	0	0	113	75	144	178	33	22	22	0
higher plants	0	0	0	0	0	0	0	0	0	200	50	0	33	0
snails	0	0	0	0	0	0	0	0	33	300	150	0	0	50
sea urchins	0	0	0	0	0	0	0	0	17	333	250	0	0	0
tunicates	0	0	0	0	0	0	0	0	75	142	108	0	50	0
sea anemones	0	40	0	0	0	0	0	0	80	180	120	0	60	0
others	16	12	0	0	0	0	12	4	41	228	134	0	14	9

* Abbreviations of microorganisms; S.a. = *Staphylococcus aureus*, B.s. = *Bacillus subtilis* M.l. = *Micrococcus luteus*, M.s. = *Mycobacterium smegmatis*, E.c. = *Escherichia coli*, P.a. = *Pseudomonas aeruginosa*, A.f. = *Aspergillus flavus*, C.a. = *Candida albicans*.

TABLE 4. ^1H n.m.r. data of clavularin A; ⌐ : protons correlated by decoupling studies.

	$CDCl_3$		C_6D_6	
	chemical shift δ p.p.m.	coupling constant J Hz	chemical shift δ p.p.m.	coupling constant J Hz
2-H	6.02	dd, 11.7, 2.7	5.97	ddd, 11.7, 1.6, 1.6
3-H	6.76	ddd, 11.7, 7.2, 4.1	6.27	ddd, 11.7, 4.5, 4.5
4α-H	ca. 2.42	m	ca. 1.93	m
4β-H	ca. 2.42	m	ca. 1.93	m
5α-H	ca. 2.1	m	1.68	m
5β-H	1.26	m	0.98	m
6α-H	ca. 2.1	m	1.76	m
6β-Me	0.83	d, 7.2	0.70	d, 6.8
7α-H	2.81	ddd, 9.7, 5.4, 4.1	2.58	ddd, 9.8, 5.5, 3.4
8-H	1.61	m	1.50	m
8-H	ca. 2.1	m	ca. 2.15	m
9-H	2.31	ddd, 17.1, 8.2, 6.6	ca. 2.15	m
9-H	2.49	ddd, 17.1, 9.0, 5.4	ca. 1.93	m
9-COMe	2.12	s	1.70	s

Antiulcer assay. Mice were kept immersed in water at 23° C for 5 hours in order to form stressed ulcers on their stomachs. Antiulcer activity was evaluated based on the extent of inhibition effect on the ulcer formation at 500 mg/kg dose of a test extract; - (inhibition < 50 %), + (50 % ≦ inhibition < 70 %), ++ (70 % ≦ inhibition < 90 %), +++ (90 % ≦ inhibition).

Angiotensin converting enzyme (ACE) inhibition assay. ACE inhibition assay was carried out by the quantitative analysis of hippuric acid produced from hippuril-L-hystidil-L-leucine with ACE. The crude enzyme was prepared from pig lung extract. The activity was evaluated based on 100 % inhibition caused by adding hydrochloric acid; - (inhibition < 30 %), + (30 % ≦ inhibition < 50 %), ++ (50 % ≦ inhibition < 80 %), +++ (80 % ≦ inhibition). The test concentrations were 400 μg/ml for the water soluble extracts and 600 μg/ml for the lipid soluble extracts.

Platelet aggregation inhibition assay. The inhibition effects on the platelet aggregations with adenosine 5'-diphosphate (ADP), collagen, or arachidonic acid were evaluated; - (no inhibition), ± (200 μg/ml < IC_{50}), + (100 μg/ml < IC_{50} < 200 μg/ml), ++ (10 μg/ml < IC_{50} ≦ 100 μg/ml), +++ (IC_{50} ≦ 10 μg/ml).

The 415 specimens out of the 583 specimens were submitted to the statistical analysis; the marine specimens were classified into 10 groups; sponges, algae, corals, sea cucumbers, higher plants, snails, sea urchins, tunicates, sea anemones, and others (Table 1). The activity index which varies from 0 to 400 was introduced in order to find the classification-activity relationship (Table 2). As an example, the calculation of the activity index of the sponge group for cytotoxicity is shown here; 2.1 %, 2.8 %, 13.4 %, 33.8 %, and 47.9 % of the test extracts showed the activities +++, ++, +, ±, -. The factors 4, 3, 2, 1, 0 were given to the activities +++, ++, +, ±, -, and the activity index was calculated as follows; (4 × 2.1) + (3 × 2.8) + (2 × 13.4) + (1 × 33.8) + (0 × 47.9) = 77 (Table 3).

Classification - activity relationship. The result of the first screening showed the following profile.

1) Sponge extracts showed moderate values of antimicrobial activity indices for Gram-positive bacteria.
2) Sea cucumber extracts showed large values of antimicrobial activity indices for a fungus and a yeast, and also a large value of cytotoxicity index.
3) Sea anemone extracts showed a moderate value of antimicrobial activity index for *Bacillus subtilis*.
4) Most of the groups showed large values of activity indices for coronary vasodilation activity and cardiotonic activity; these are partly due to free fatty acids and/or amines contained in many of the test extracts.

CYTOTOXIC SUBSTANCES

Clavularins from a soft coral, *Clavularia koellikeri*. A new class of cytotoxic compounds, clavularin A 1a and clavularin B 1b were isolated from a soft coral, Clavularia koellikeri collected in Okinawa, Japan. We proposed the structures 2a and 2b for clavularin A and B based on the spectroscopic studies (ref. 1). However, in the subsequent Corrigendum (ref. 2), we revised the original proposals and assigned the seven-membered ring structures 1a and 1b based on the sodium borohydride reduction of clavularin A. The carbon sequence from C-1 to C-9 in clavularin A was fully established by the intensive decoupling studies of its ^1H n.m.r. spectra (Table 4). There remained

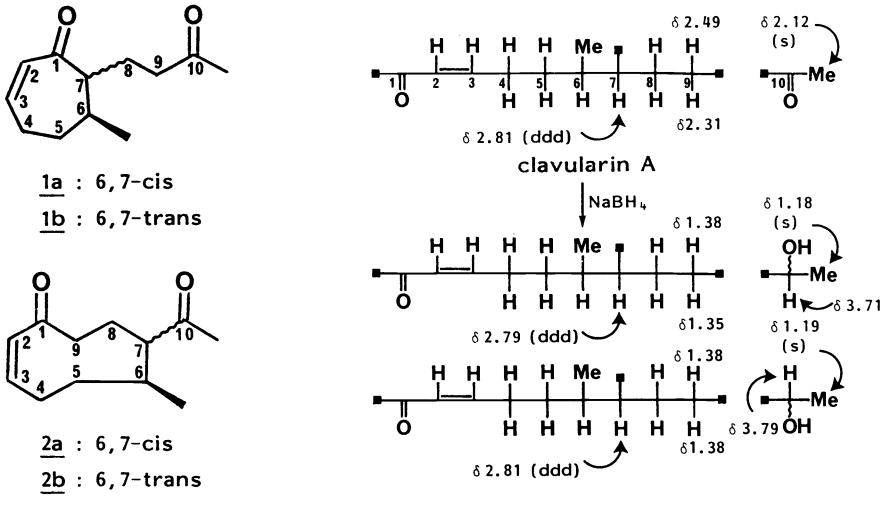


Fig. 1. Sodium borohydride reduction of clavularin A (^1H n.m.r. data in $CDCl_3$)

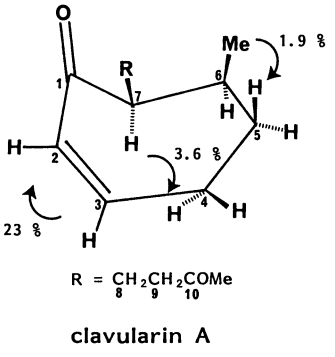


Fig. 2. The conformation and n.O.e. enhancements of clavularin A

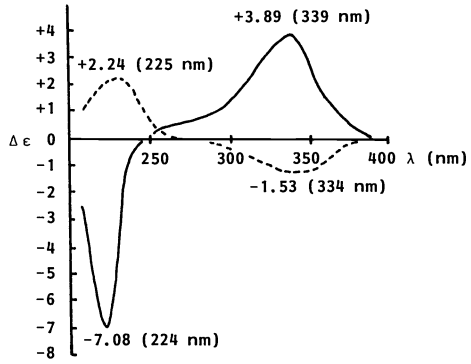
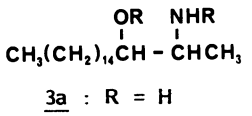


Fig. 3. The c.d. spectra in n-hexane and the skewed α,β-unsaturated ketones of clavularin A and clavularin B.

two possible structures; a) C-1 - C-7 connection to form the seven-membered ring structure 1a and b) C-1 - C-9 connection to form the nine-membered ring structure 2a. In order to determine the connectivity, the acetyl group of clavularin A was reduced (Fig. 1). Two alcohols were isolated; ^{1}H n.m.r. signals (δ 2.49, 2.31) of C-9 methylene protons shifted to higher fields (δ 1.38, 1.35 in one alcohol and δ 1.38, 1.38 in the other), while C-7 methine proton (δ 2.81) remained unaffected; thus the C-1 - C-7 connection to form the seven-membered ring structure 1a was established. The cis-relation of the olefinic protons was confirmed by nuclear Overhauser enhancement (n.O.e.) of 2-H (δ 5.97 in C_6D_6, 23 % increase) caused by the irradiation at 3-H (δ 6.27, Fig. 2). Owing to the shielding effect of the carbonyl group of the α,β-unsaturated ketone, ^{1}H n.m.r. signal of 5β-H in clavularin A appeared at unusually high field (δ 1.26 in $CDCl_3$ and δ 0.98 in C_6D_6) and the c.d. Cotton effect (ref. 3, ref. 4), a negative sign ($\Delta\varepsilon$ = -7.08) for the K-band (224 nm) and a positive sign ($\Delta\varepsilon$ = +3.89) for the R-band (339 nm), showed the negative skew of the α,β-unsaturated ketone (Fig. 3). The β-configuration of 6-Me was indicated by n.O.e. (1.9 % increase) of 5β-H ^{1}H n.m.r. signal (δ 1.26 in $CDCl_3$) caused by the irradiation at 6-Me (δ 0.83) and the α-configuration of 7-H was determined by n.O.e. of 4α-H (δ 2.42 in $CDCl_3$, 3.6 % increase) caused by irradiation at 7-H; (δ 2.81); 7-H can be located in the vicinity of 4-H only when it is in the α-configuration (Fig. 2). On the other hand in the c.d. spectrum of clavularin B, the positive sign ($\Delta\varepsilon$ = +2.24) of the K-band (225 nm) and the negative sign ($\Delta\varepsilon$ = -1.53) of the R-band (334 nm) showed the existence of the positive skew of the α,β-unsaturated ketone. The n.O.e. (3.6 % increase) of 7-H ^{1}H n.m.r. signal (δ 2.21 in C_6D_6) in clavularin B caused by the irradiation at 6-Me (δ 0.83) showed the 7β-H configuration, i.e. clavularin B was shown to be the 7-epimer of clavularin A; this was confirmed by the conversion of clavularin A into clavularin B with hydrochloric acid in methanol. Urech has reported the total synthesis of clavularin B (ref. 5).

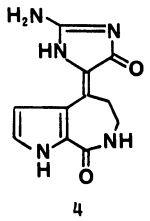
An amino alcohol from a soft coral, *Lemnalia* sp. A cytotoxic amino alcohol 3a was isolated from a soft coral, Lemnalia sp. collected in Gold Reef, Queensland, Australia. Strong cytotoxicity, T/C 0 % at 10 μg/ml was shown. The structure, 2-aminooctadecane-3-ol 3a was assigned based on the spectroscopic studies on the diacetate 3b.

A yellow compound from sponges. Yellow compounds isolated from Phakellia flabellata (ref. 6a) and Hymeniacidon aldis (ref. 6b) have been reported. We also isolated one of the yellow compounds 4 as a cytotoxic constituent in Hymeniaciadon aldis collected in Okinawa, Japan and also in Phakellia flabellata collected in Green Island, Queensland, Australia. A moderate cytotoxicity, (T/C 32.5 % at 100 μg/ml) was shown.



3a : R = H

3b : R = Ac



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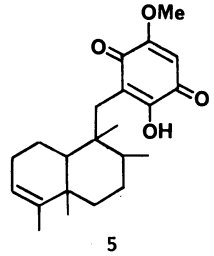

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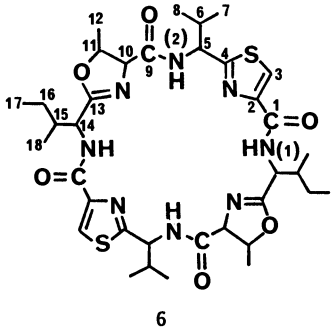
TABLE 5. ^{13}C n.m.r. and ^{1}H n.m.r. spectra of ascidiacyclamide
[: protons correlated by decouplings.

position	^{13}C ($CDCl_3$) δ p.p.m.	^{1}H ($CDCl_3$) δ p.p.m.
1	168.4	
2	160.3	
3	123.0	7.91 (1H, s)
4	149.5	
5	54.7	5.22 (1H, dd, J 6.3, 10.0 Hz)
6	33.4	2.31 (1H, dqq, J 6.3, 6.1, 6.1 Hz)
7	19.1	1.07 (3H, d, J 6.1 Hz)
8	17.9	1.13 (3H, d, J 6.1 Hz)
9	171.2	
10	73.5	4.27 (1H, dd, J 6.3, 1.2 Hz)
11	81.5	4.86 (1H, dq, J 6.3, 6.3 Hz)
12	21.7	1.49 (3H, d, J 6.3 Hz)
13	168.9	
14	52.0	4.83 (1H, ddd, J 8.1, 6.1, 1.2 Hz)
15	37.0	1.95 (1H. m)
16	24.6	1.17 (1H, m)
		1.27 (1H, m)
17	10.7	0.72 (3H, dd, J 6.8, 6.8 Hz)
18	14.9	0.80 (3H, d, J 6.8 Hz)
N(1)		8.01 (1H, d, J 8.1 Hz)
N(2)		7.40 (1H, d, J 10.0 Hz)

Isospongiaquinone from a sponge, *Thorecta* sp. Kazlauskas, et al. have reported five quinone compounds from Stelospongia conulata (ref. 7). We isolated one of the quinones, isospongiaquinone 5 as a cytotoxic constituent of Thorecta sp. collected in Port Hacking, New South Wales, Australia. The T/C 10.5 % at 10 μg/ml was shown in in vitro tests and a moderate effect (ILS 25 % at 10 mg/kg dose) in in vivo tests with P388 bearing mice. The ILS % is expressed as follows.

$$\text{ILS \%} = \left(\frac{\text{mean survival time of the test group}}{\text{mean survival time of the control group}} - 1 \right) \times 100$$

Cyclic peptides from a compound ascidian. Two cytotoxic cyclic peptides, ascidiacyclamide 6 and ulithiacyclamide 7 were isolated from an unidentified species of compound ascidian collected in Rodda Reef, Queensland, Australia; T/C 0 % at 10 μg/ml was shown for both compounds. Ulithiacyclamide has been reported by Ireland, et al. (ref. 8). The structure of ascidiacyclamide was determined mainly by a hydrolysis reaction to give isoleucine, threonine, and another hydrolysate, and also by ^{1}H n.m.r. and ^{13}C n.m.r. studies including decoupling studies (Table 4 and ref. 9). The substitutions on the thiazole ring were determined by ^{13}C n.m.r. chemical shifts at δ 160.3 (s), 149.5 (s), and 123.0 (d) and ^{1}H n.m.r. chemical shift at δ 7.91 (s); the chemical shifts were quite similar to those reported for the thiazole ring in ulicyclamide (ref. 8). The existence of the oxazoline ring formed by condensation of isoleucine and threonine was confirmed by homoallylic coupling between 10-H (δ 4.27, 1H, dd, J = 6.3, 1.2 Hz) and 14-H (δ 4.83, 1H, ddd, J = 8.1, 6.1, 1.2 Hz, ref. 8 and 10). The ^{13}C n.m.r. assignments for C-1, C-9, C-13, and the relation between the isoleucine-threonine part and the thiazole ring bearing a valine side-chain were elucidated by the use of the long range selective proton decoupling technique.



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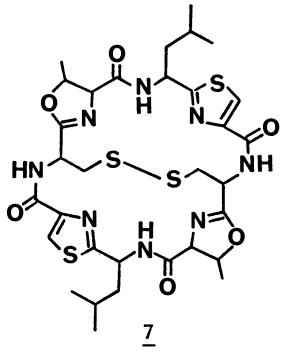

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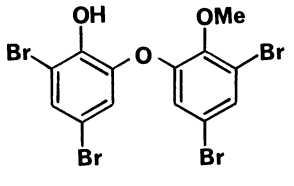
The ^{13}C n.m.r. signal at δ 171.2 (m, C-9) was simplified by irradiation of the ^{1}H n.m.r. signal at δ 5.22 (5-H) or at δ 7.40 (N(2)-H); thus the 5-H - C-9 relation and the N(2)-H - C-9 relation were shown. The ^{13}C n.m.r. signal at δ 168.4 (m, C-1) was simplified by irradiation of the ^{1}H n.m.r. signal at δ 8.01 (N(1)-H); thus the C-1 - N(1)-H relation was shown. The total synthesis of ascidiacyclamide has been reported by Hamada, et al. (ref. 11).

CORONARY VASODILATIVE COMPOUNDS FROM SPONGES

A polybrominated diphenyl ether from *Dysidea herbacea*. Carte, et al. (ref. 12) and Norton, et al. (ref. 13) have isolated many polybrominated diphenyl ethers as antimicrobial substances from Dysidea herbacea. We isolated one of them (8) as a coronary vasodilative constituent of the same spcies collected in Quobba Lagoon, Western Australia; the polybrominated diphenyl ether 8 showed a very strong coronary vasodilative activity (ED_{50} = 0.56 μg/heart).

Furano-sesterterpenes from *Ircinia wistari*. Furano-sesterterpenes, ircinianin 9a and its hydrolyzed form 9b were isolated as coronary vasodilative constituents and also as antimicrobial constituents of Ircinia wistari collected in Swain Reef, Queensland, Australia; ircinianin has been reported by Hofheinz, et al. (ref. 14). The hydrolyzed form 9b showed a slightly higher activity (ED_{50} = 11 μg/heart) than the lactone form 9a (ED_{50} = 22 μg/heart).

Macrocyclic 1-oxaquinolizidine compounds from *Xestospongia exigua*. We have reported a new class of macrocyclic 1-oxaquinolizidine compounds, xestospongin A 10, B 11, C 12a, and D 12b as vasodilative constituents of Xestospongia exigua collected in Hook Island, Queensland, Australia (ref. 15). The partial structure of xestospongin C 12a was investigated by spectroscopic studies and the unique structure was fully determined by x-ray crystallography. The location of the hydroxyl group at C-9 in xestospongin D 12b was determined by the comparison of ^1H n.m.r. (in C_6D_6) and ^{13}C n.m.r. (in C_6D_6) data of xestospongin C 12a and D 12b; 10-H signal in xestospongin D 12b was a singlet at δ 4.17, while it was a doublet at δ 4.40 in xestospongin C 12a; C-9 signal of xestospongin D 12b was observed at lower field (δ 70.90) than that of xestospongin C 12a (δ 40.84). The ^{13}C n.m.r. spectrum of xestospongin A 10 showed only 14 signals; thus the point of symmetry structure was determined. Bohlman bands (ref. 16) were observed in i.r. spectra (KBr) of xestospongin A 10 (2758 cm^{-1}), C 12a (2760 cm^{-1}), and D 12b (2762 cm^{-1}). Xestospongin B 11 lacked the Bohlmann band, that is, it lacks the trans-quinolizidine structure; the cis-fused non-steroidal conformations were assigned for both the A,B-rings and C,D-rings by ^1H n.m.r. and ^{13}C n.m.r. spectra. The ^1H n.m.r. spectrum of xestospongin B 11 showed the presence of a secondary methyl at δ 0.51; its position was deduced from the coupling patterns of 4'α-H (δ 2.83, dd, J = 13.4, 4.5 Hz) and 4'β-H (δ 2.72, dd, J = 13.4, 11.0 Hz) signals. The pharmacological effects of the xestospongins were tested by use of anesthetized dogs, and compared with the effects of papaverine. Blood flows of the coronary artery, vertebral artery, and femoral artery were increased; the effects were as strong as those of papaverine. The selectivity of relaxation effects on spiral strips was shown; xestospongins caused relaxation of the basilar artery which was partially contracted with Krebs solution containing 40 mM KCl. The effect was as strong as that of papaverine, while the effects on the coronary artery, femoral artery, and renal artery were much smaller.



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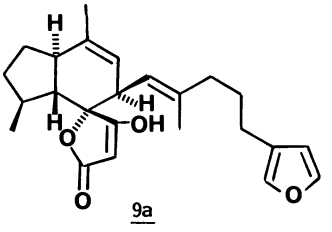

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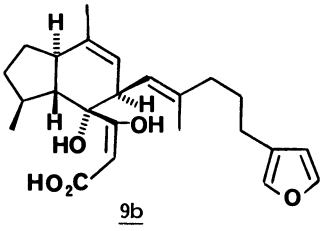

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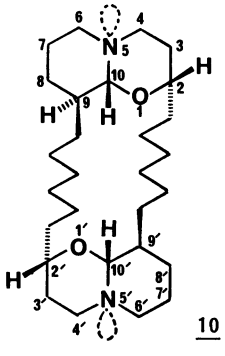

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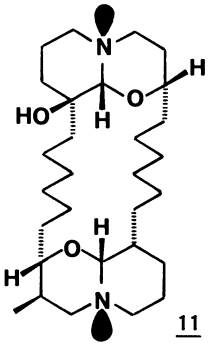

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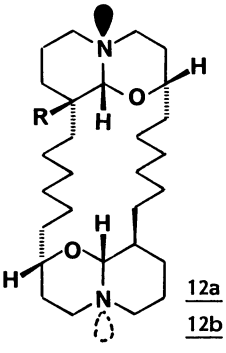
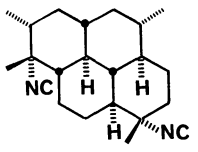


12a : R = H
12b : R = OH

Diisocyanoadociane from *Adocia* sp. Diisocyanoadociane 13 was isolated as a coronary vasodilative constituent of Adocia sp. collected in Rodda Reef, Queensland, Australia; its ED_{50} is 10 μg/heart. The compound has been reported by Baker, et al. and Kazlauskas, et al. (ref. 17) as an antimicrobial constituent of Adocia sp.



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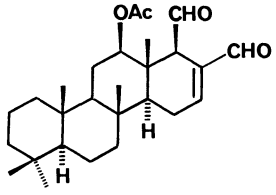

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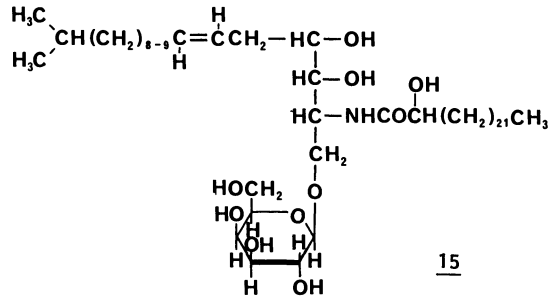
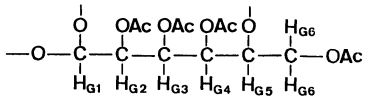


15

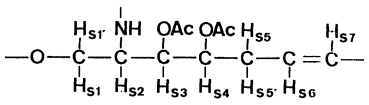
HYPOTENSIVE COMPOUNDS FROM SPONGES

12-Epi-scalaradial from *Hyrtios erecta*. Tetracyclic sesterterpenes from Spongia nitens have been reported by Cimino, et al. (ref. 18). We isolated one of them, 12-epi-scalaradial 14 as a hypotensive constituent of Hyrtios erecta collected in Palau. The compound showed a strong and long-lasting hypotensive effect on anesthetized rats at a dose of 0.05 mg/kg administered intravenously.

Cerebrosides from *Chondropsis* sp. New cerebrosides 15 (as a mixture, ref.19) which inhibited hystidine decarboxylase (IC_{50} = 2.8 × 10^{-4} mg/ml) were isolated from Chondropsis sp. collected in Port Philip, Victoria, Australia. The compounds also showed hypotensive activity in anesthetized rats at a dose of 5 mg/kg. The i.r. (KBr) spectrum showed an amide bond (1656 and 1544 cm^{-1}) and hydroxyl groups (3361, 1076, and 1047 cm^{-1}). The ^1H n.m.r. spectrum of the mixture (in pyridine-d_5) showed a doublet at δ 0.85 (6H, J = 7.3 Hz) due to two secondary methyls and a triplet at δ 0.85 (3H, J = 7.0 Hz) due to a primary methyl. Peaks due to long chain methylene protons at δ 1.25, due to an anomeric proton at δ 4.87 (d, J = 7.5 Hz), due to sugar protons at δ 4.0 - 4.7, and due to olefinic proton at δ 5.71 (dt, J = 15.4, 8.1 Hz), δ 5.94 (dt, J = 15.4, 8.1 Hz) were also observed. The ^{13}C n.m.r. spectrum showed an carbonyl at δ 175.61, and olefinic carbons at δ 133.67 and 126.60; ten low field carbons were also observed between δ 51.39 and 75.82. These data suggested the cerebroside structure which has one primary amide, one double bond, one sugar, and one long chain carbon unit. The proton sequences in part G and part S (Fig. 4) were determined by two dimensional ^1H n.m.r. spectra of the heptaacetate (Fig. 5 and Fig.



Part G



Part S

Fig. 4. Partial structures of the heptaacetate mixture derived from the cerebroside mixture

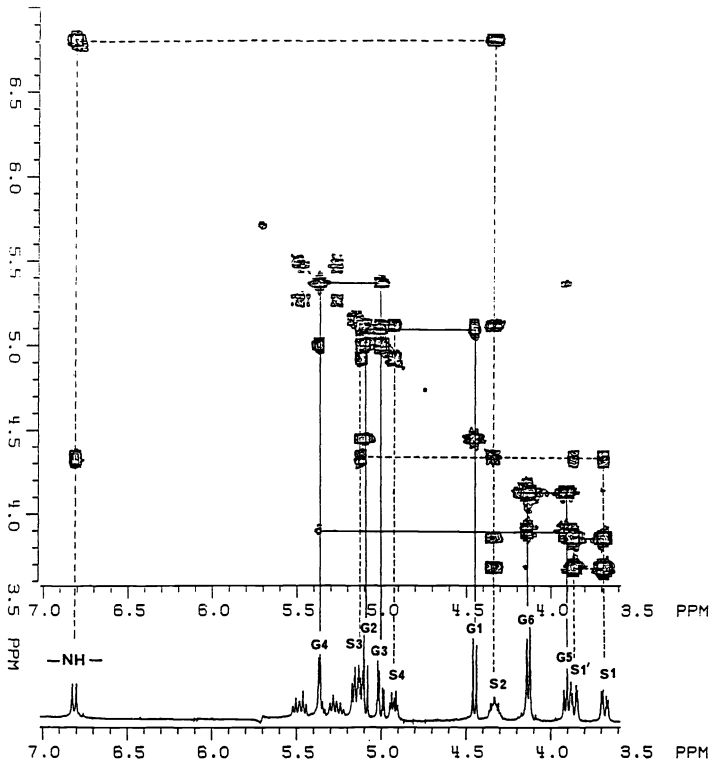


Fig. 5. Partial two dimension ^1H n.m.r. spectrum ($CDCl_3$) of the heptaacetate mixture derived from the cerebroside mixture.

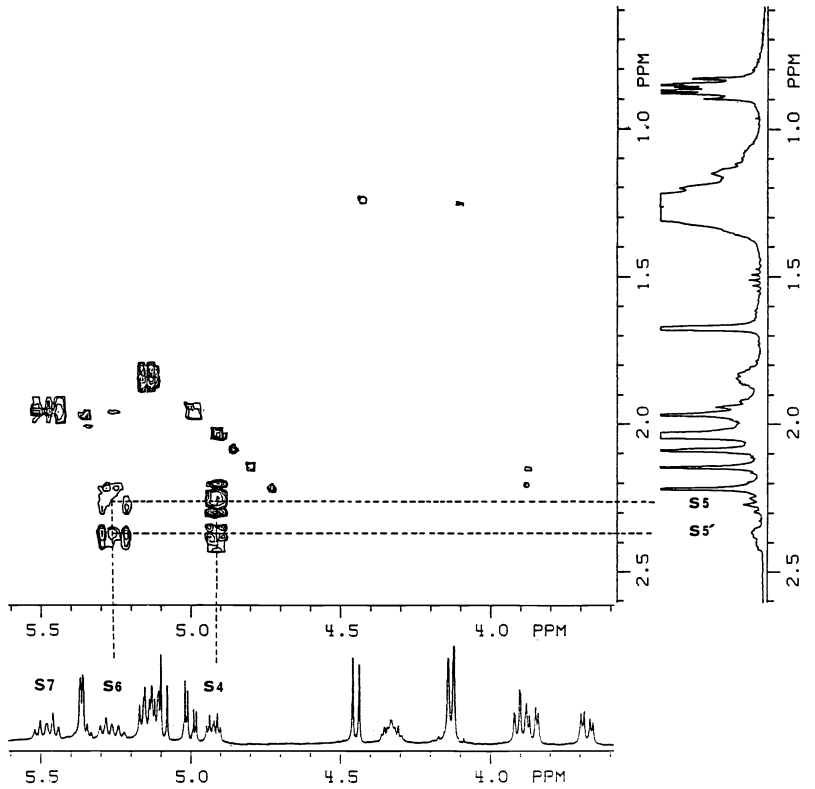


Fig. 6. Partial two dimension ^1H n.m.r. spectrum ($CDCl_3$) of the heptaacetate mixture derived from the cerebroside mixture.

6). The acid hydrolysis of the cerebroside mixture gave three fragments, cerebronic acid, a sugar, and a sphingosin mixture. The sugar was benzoylated and identified as D-galactose pentabenzoate. In order to determine the length(s) of the methylene chain(s) in the sphingosin part(s), the heptaacetate mixture of the cerebroside(s) was ozonized to give an aldehyde mixture, which was oxidized by Jones reagent and subsequently methylated to yield a mixture of methyl esters; GC-MS analysis showed that the main esters are $(CH_3)_2CH(CH_2)_8CO_2CH_3$ and $(CH_3)_2CH(CH_2)_9CO_2CH_3$.

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