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Note

Manauealide C and Anhydrodebromoaplysiatoxin, Toxic Constituents of the Hawaiian Red Alga, *Gracilaria coronopifolia*

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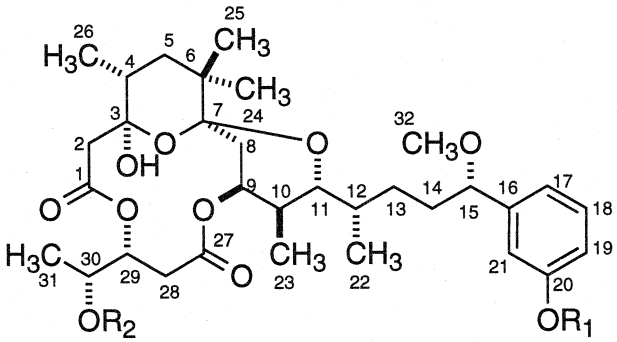
Manauealide C (1) and anhydrodebromoaplysiatoxin (4), toxic constituents of the Hawaiian red alga, *Gracilaria coronopifolia* which has been concerned with food poisoning cases, were studied. The absolute structure of manauealide C was determined as 1 by chemical conversion and spectroscopic methods. The first complete assignment of ^{13}C chemical shifts for anhydrodebromoaplysiatoxin (4) was established. The biological activity of 4 was also investigated.

Key words: *Gracilaria coronopifolia*; manauealide C; anhydrodebromoaplysiatoxin; absolute configuration; marine algal toxin

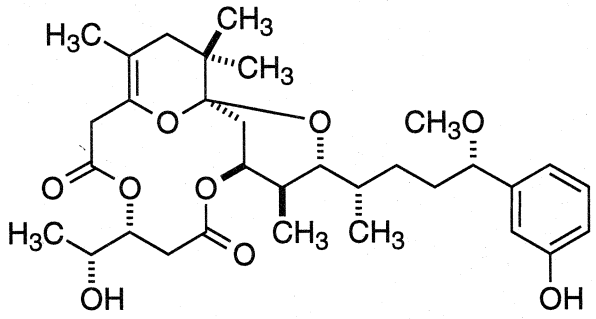
Poisoning from ingesting the red alga, *Gracilaria coronopifolia*, occurred successively in Hawaii in 1994.[1,2] Aplysiatoxin and debromoaplysiatoxin were identified as the main causative agents of the poisoning cases.[2] A further study of the constituents of Hawaiian *G. coronopifolia* led us to find new malyngamide-related compounds[3] and new toxic macrolides, manauealides A-C.[4] However, the partial stereostructure of manauealides A and C remains unknown. We report here an elucidation of the absolute structure of manauealide C (**1**), and an identification of anhydrodebromoaplysiatoxin (**4**)[5] as a toxic constituent of *G. coronopifolia*. We also report here the complete assignment of ^{1}H and ^{13}C chemical shifts for anhydrodebromoaplysiatoxin (**4**) and its biological activity.



1: $R_1 = H$ $R_2 = {}^{33}_{}COCH_3{}^{34}$

2: $R_1 = H$ $R_2 = H$

3: $R_1 = {}^{35}_{}COCH_3{}^{36}$ $R_2 = {}^{33}_{}COCH_3{}^{34}$



4

Manauealide C (**1**) has been obtained as a toxic constituent of *G. coronopifolia*.[4] A previous analysis of the spectroscopic data led us to elucidate the relative stereostructure of manauealide C as **1**, except for the C-12, C-15, and C-30 configurations.[4] The close structural resemblance of manauealide C (**1**) and debromoaplysiatoxin (**2**) is suggested by the similarity of their proton and carbon chemical shifts and proton coupling constants. We converted diacetyldebromoaplysiatoxin (**3**) from authentic debromoaplysiatoxin (**2**)[2,3] and monoacetylmanauealide C (**3**) from manauealide C (**1**),[3] respectively, by acetylation (see Experimental). Each ^{1}H-NMR spectrum of the converted diacetyldebromoaplysiatoxin and monoacetylmanauealide C seemed to be identical. The precise analysis of HSQC, HMBC, ROESY, and ^{1}H- and ^{13}C-NMR spectra of the mixture of diacetyldebromoaplysiatoxin and monoacetylmanauealide C revealed the relative configurations of these compounds to be identical as **3** (Table I). Furthermore, it was biosynthetically deduced that debromoaplysiatoxin (**2**) and manauealide C (**1**) possessed the same absolute stereochemistry based on their co-occurrence (see Experimental). Therefore, the structure of manauealide C was deduced to be **1**, which is that of 30-acetyldebromoaplysiatoxin. During this study, it was found that we previously assigned incorrectly the ^{13}C chemical shift for C-34 of manauealide C (**1**).[4] It should have been δ21.1 ppm.

During the course of the study, we newly isolated a toxic compound from a *G. coronopifolia* sample. An analysis of the one-dimensional and two-dimensional NMR spectra showed that the planar structure of the compound was identical to that of an-

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Table I. ^{1}H- and ^{13}C-NMR Data for Monoacetylmanauealide C and Diacetyldebromoaplysiatoxin (**3**)[a]

Position	^{1}H-NMR	^{13}C-NMR
1	—	168.9
2	2.47 (β, d, 12.5 Hz)	46.9
	2.81 (α, d, 12.1 Hz)	
3	—	98.7
4	1.85 (m)	35.7
5	1.62 (ax, t, 13.3 and 12.8 Hz)	41.1
	1.07 (eq, dd, 13.4 and 3.6 Hz)	
6	—	38.9
7	—	100.8
8	1.71 (ax, dd, 3.5 and 14.6 Hz)	33.6
	2.68 (eq, dd, 3.0 and 14.7 Hz)	
9	5.24 (q, 3.1, 3.1 and 3.1 Hz)	73.6
10	1.71 (m)	35.4
11	3.93 (dd, 2.3 and 10.9 Hz)	69.6
12	1.54 (m)	34.1
13	1.34 (m)	31.2
	1.38 (m)	
14	1.63 (m)	36.0
	1.99 (m)	
15	4.09 (t, 6.4 and 6.5 Hz)	85.4
16	—	145.9
17	7.24 (br. d, 7.5 Hz)	125.3
18	7.35 (t, 7.8 and 7.9 Hz)	129.7
19	7.01 (ddd, 0.9, 2.3 and 8.0 Hz)	121.4
20	—	136.2
21	7.15 (t, 1.8 and 2.0 Hz)	120.8
22	0.80 (3H, d, 6.2 Hz)	13.6
23	0.72 (3H, d, 6.9 Hz)	13.0
24	0.84 (3H, s)	26.7
25	0.79 (3H, s)	23.6
26	0.89 (3H, d, 6.7 Hz)	16.4
27	—	169.6
28	2.99 (α, dd, 11.8 and 18.1 Hz)	36.0
	2.81 (β, dd, 2.2 and 18.0 Hz)	
29	5.38 (ddd, 2.1, 3.9 and 11.7 Hz)	71.6
30	5.13 (dq, 3.9 and 6.6 Hz)	70.4
31	1.21 (3H, d, 6.6 Hz)	15.8
32 (CH_3O-)	3.19 (3H, s)	56.7
33 (CH_3CO-)	—	170.3
34 (CH_3CO-)	2.04 (3H, s)	21.1
35 (CH_3CO-)	—	169.6
36 (CH_3CO-)	2.26 (3H, s)	21.1

[a] Spectra determined in acetone-d_6; data reported in ppm

Table II. ^{1}H- and ^{13}C-NMR Data for Anhydrodebromoaplysiatoxin (**4**)[a]

Position	^{1}H-NMR	^{13}C-NMR
1	—	171.0
2	3.05 (br. d, 13.4 Hz)	36.6
	3.32 (br. d, 12.1 Hz)	
3	—	137.2
4	—	104.7
5	2.19 (ax, br. d, 15.6 Hz)	40.9
	1.35 (eq, br. d, 15.6 Hz)	
6	—	36.4
7	—	100.5
8	1.73 (ax, dd, 3.6 and 14.8 Hz)	31.0
	2.22 (eq, dd, 2.7 and 14.8 Hz)	
9	4.84 (q, 2.9, 2.9 and 3.0 Hz)	74.1
10	1.72 (m)	34.5
11	3.77 (dd, 1.8 and 10.7 Hz)	72.3
12	1.52 (m)	34.5
13	1.46 (2H, m)	31.3
14	1.73 (2H, m)	37.3
15	3.99 (t, 6.4 and 6.7 Hz)	85.6
16	—	146.0
17	6.82 (br. d, 7.6 Hz)	118.9
18	7.15 (t, 7.8 and 7.8 Hz)	130.0
19	6.72 (ddd, 1.0, 2.5 and 8.0 Hz)	115.0
20	—	158.4
21	6.86 (t, 1.7 and 2.3 Hz)	114.5
22	0.83 (3H, d, 6.0 Hz)	13.6
23	0.82 (3H, d, 6.0 Hz)	12.6
24	0.95 (3H, s)	24.6
25	0.82 (3H, s)	23.3
26	1.59 (3H, s)	17.5
27	—	170.5
28	2.76 (dd, 3.8 and 17.9 Hz)	35.7
	2.72 (dd, 10.6 and 17.7 Hz)	
29	5.30 (dt, 3.8, 3.8 and 10.4 Hz)	73.6
30	3.84 (m)	67.8
31	1.11 (3H, d, 6.5 Hz)	18.4
32 (CH_3O-)	3.17 (3H, s)	56.8

[a] Spectra determined in acetone-d_6; data reported in ppm

hydrodebromoaplysiatoxin (**4**).[5] Thus, we converted anhydrodebromoaplysiatoxin (**4**) from authentic debromoaplysiatoxin (**2**)[2] by dehydration under acidic conditions (see Experimental). It was confirmed that this toxic compound was identical to anhydrodebromoaplysiatoxin (**4**) by the spectroscopic data analysis. Anhydrodebromoaplysiatoxin (**4**) and anhydroaplysiatoxin were first obtained as artifacts during the purification of debromoaplysiatoxin (**2**) and aplysiatoxin from the sea hare *Stylocheilus longicauda*.[6] However, Moore *et al.* found **4** as a natural product from a mixture of two blue-green algae, *Schizothrix calcicola* and *Oscillatoria nigroviridis*.[5] During this study, aplysiatoxin and debromoaplysiatoxin (**2**) were obtained from the sample together with anhydrodebromoaplysiatoxin (**4**) (see Experimental). However, anhydroaplysiatoxin could not be detected from the same sample, we therefore believe that anhydrodebromoaplysiatoxin (**4**) existed as a natural product in the *G. coronopifolia* sample. The first complete assignment of ^{13}C chemical shifts for anhydrodebromoaplysiatoxin (**4**) was established (Table II). The previously reported ^{1}H-NMR data for **4**[6] agree well with our data, except for δ0.23 ppm of H-10[6] which is thought to be an error.

Anhydrodebromoaplysiatoxin (**4**) did not show any antimicrobial activity against the two fungi, *Aspergillus niger* and *Penicilium funiculosum*, and against a Gram-negative bacterium *Escherichia coli* at the dose of 50 μg/disc with the paper disc method. The cytotoxicity to mouse neuro-blastoma cells was not observed at the concentration of 38 μg/ml of **4**. However, one, ten, and a hundred-μg injections of **4** caused diarrhea in mice for 1–2 h, 4–5 h, and 7–12 h periods, respectively. This strong diarrhea goes against the normal level; and reveals the potential hazardous nature of anhydrodebromoaplysiatoxin (**4**), together with manauealide C (**1**),[4] when it contaminates the edible algae such as *G. coronopifolia*. These toxic compounds are likely to have originated from epiphytic blue-green algae, which grow

on *G. coronopifolia* as reported elsewhere.[2,4] Aplysiatoxin and its derivatives, including **4**, have been reported as potent tumor promoters.[7] These results alert us the potential danger of noxious marine blue-green algae in addition to toxic freshwater ones[8] from a public health point of view.

Experimental

Instruments. UV spectra were recorded with a Shimadzu UV-250 spectrophotometer, and ^{1}H- and ^{13}C-NMR spectra were measured on a Bruker DMX500 spectrometer, using acetone-d_6 as a solvent. FAB mass spectra were obtained with a JEOL JMS-HX/HX110 spectrometer. Optical rotation was determined with a JASCO DIP-1000 instrument, and CD spectra were recorded on a JASCO J-600 spectropolarimeter.

Algal material. G. coronopifolia (4.8 kg, wet weight) was collected one week after the *G. coronopifolia* food poisonings[1] (September, 1994) at the same site at Waiehu, in Maui where the toxic specimen had been collected. The sample was transferred to the University of Hawaii cooled with ice and then kept at −15°C until extraction could be carried out.

Extraction and isolation. The sample (4.8 kg) was thawed and lightly washed in distilled water. The alga was soaked overnight in 6 liters of acetone at room temperature, and the acetone extract was filtered. The sample was further extracted twice with acetone and then twice with MeOH. The extracts were combined and evaporated. The dried residue was partitioned between H_2O (600 ml) and $CHCl_3$ (400 ml). The aqueous phase was evaporated to eliminate $CHCl_3$ and extracted three times with EtOAc (500 ml). The EtOAc fraction was evaporated to dryness. The EtOAc fraction was dissolved in a small amount of MeOH, and the solution was purified by HPLC in a TSK-GEL ODS 120-T column (7.8×300 mm; Tosoh, Japan) with 80 or 90% CH_3CN in H_2O. Manauealide C (**1**, 1.25 mg) and anhydrodebromoaplysiatoxin (**4**, 6.8 mg) were isolated, together with aplysiatoxin, debromoaplysiatoxin, and malyngamide-related compounds from the same algal sample.[3]

Acetylation of manauealide C (**1**) *and debromoaplysiatoxin* (**2**). Manauealide C (600 μg) was dissolved in 100 μl of pyridine while cooling with ice, acetic anhydride (50 μl) was added, and the mixture left for 17 hours at room temperature. The mixture was then poured into distilled water (1.5 ml), cooled with ice and extracted with ethyl acetate. The ethyl acetate fraction was successively washed with distilled water, a saturated $NaHCO_3$ solution, a 5% $CuSO_4$ solution, and finally distilled water. The washed ethyl acetate fraction was evaporated to dryness. After HPLC purification, 140 μg of pure monoacetylmanauealide C was obtained. Debromoaplysiatoxin (**2**, 500 μg) was also treated by a similar procedure, and diacetyldebromoaplysiatoxin (**3**, 120 μg) was obtained.

Dehydration of debromoaplysiatoxin (**2**). Debromoaplysiatoxin (**2**, 800 μg) was dissolved in a mixture of H_2O (500 μl) and acetic acid (150 μl). After 6 days of reaction at room temperature, the solution was completely dried by evaporation. The reacted mixture was subjected to ODS HPLC (Shiseido, Capcell Pak 10 mm×25 cm, 80%CH_3CN), and pure anhydrodebromoaplysiatoxin (**4**, 220 μg) was obtained.

Mouse toxicity test. The sample was dissolved in 0.8 ml of a 1% Tween 60 solution and intraperitoneally injected into a *ddY* male mouse (18–20 g).

Mixture of diacetyldebromoaplysiatoxin (**3**) *and monoacetylmanauealide C.* HRFABMS (positive ion) $[M+Na]^+$ m/z 699.3376, $C_{36}H_{52}O_{12}Na$ requires 699.3356; FABMS (positive ion) $[M+Na]^+$ 699 and $[M+H-H_2O]^+$ 659; UV (MeOH) λ_{max} nm (ε) 221 (7037), 263 (650); ^{1}H- and ^{13}C-NMR (acetone-d_6), see Table I.

Anhydrodebromoaplysiatoxin (**4**). HRFABMS (positive ion) $[M+H]^+$ m/z 575.3234, $C_{32}H_{47}O_9$ requires 575.3230; UV (MeOH) λ_{max} nm (ε) 208 (6800), 274 (1790); $[\alpha]_D=+25.6°$ (c 0.02, MeOH); CD (MeOH) $[\theta]_{276}$ +2583°; ^{1}H- and ^{13}C-NMR (acetone-d_6), see Table II.

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