Aplyronine A, a Potent Antitumor Substance, and the Congeners Aplyronines B and C Isolated from the Sea Hare *Aplysia kurodai*

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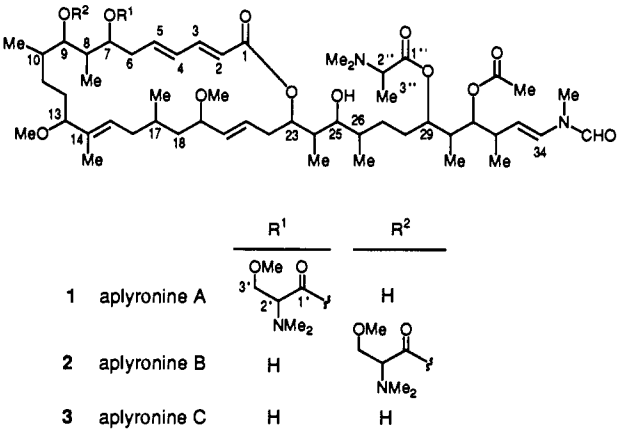
The sea hare *Aplysia kurodai* Baba has been known to contain various unique metabolites.[1] Herein we describe the isolation of a new type of potent antitumor compound termed aplyronine A (**1**) and the congeners aplyronines B (**2**) and C (**3**) from the same animal[2] and report their structures.

Originally, aplyronine A (**1**) was isolated by eight-step chromatographic separation guided by the cytotoxicity against HeLa-S_3 cells in vitro. Subsequently, we developed a more efficient method for the isolation of **1**, which enabled us to isolate the congeners aplyronines B (**2**) and C (**3**). The ethyl acetate-soluble material obtained from the sea hare was separated by solvent partitioning and chromatography[3] to afford aplyronine A (**1**) (2.5×10^{-5}% yield based on wet weight), aplyronine B (**2**) (1.4×10^{-6}% yield), and aplyronine C (**3**) (3×10^{-7}% yield) as amorphous powders, respectively. Aplyronines A (**1**), B (**2**), and C (**3**) showed strong cytotoxicities against HeLa-S_3 cells in vitro with IC_{50}s of 0.039, 4.39, and 159 ng/mL, respectively. Aplyronine A (**1**) exhibited exceedingly potent antitumor activities in vivo against P388 murine leukemia (T/C = 545%, 0.08 mg/kg), Lewis lung carcinoma (T/C = 556%, 0.04 mg/kg), Ehrlich carcinoma (T/C = 398%, 0.04 mg/kg), colon 26 carcinoma (T/C = 255%, 0.08 mg/kg), and B16 melanoma (T/C = 201%, 0.04 mg/kg).[4]

Structure determination was carried out with aplyronine A (**1**) in details $[\alpha]^{28}_D$ +32° (*c* 0.26, MeOH); UV (MeCN) λ_{max} 256 nm (ε 30 000); IR ($CHCl_3$) 3690, 3500, 1730, 1690, and 1655 cm^{-1}. The molecular formula of **1** was established to be $C_{59}H_{101}N_3O_{14}$ by HRFABMS [$(M + H)^+$ *m/z* 1076.7360, Δ −0.2 mmu] and NMR data. The IR spectrum of **1** indicated the presence of hydroxyl groups, which was confirmed by acetylation (Ac_2O, pyridine) to give the diacetate **4**.[5] The ^1H and ^{13}C NMR spectral data showed the presence of an α,β,γ,δ-unsaturated ester (*E*,*E* geometry), an acetate, two additional esters, five olefins (two correspond to the α,β,γ,δ-unsaturated ester), three methoxy groups, and two dimethylamino groups. The UV spectrum confirmed the presence of the α,β,γ,δ-unsaturated ester group. A terminal *N*-methyl-*N*-vinylformamide structure in **1** was deduced when the ^1H NMR data were compared with those for scytophycins,[6] sphinxolide,[7] and macrocyclic trisoxazoles.[8] Owing to the restricted rotation about the *N*-methyl-*N*-vinylformamide terminus (2:1 ratio) and the presence of two scalemic[9] amino acid portions (1.1:1 and 3:1 ratios for *N*,*N*,*O*-trimethylserine and *N*,*N*-dimethylalanine moieties, respectively),[10] doubled NMR signals for some protons and carbons were observed (78 signals were counted in the ^{13}C NMR spectrum), as shown in Table I.



Detailed analysis of ^1H–^1H COSY and phase-sensitive ^{13}C–^1H COSY (J_{CH} = 135 Hz) spectra of **1** allowed construction of six partial structures C2–C9, C14–C17, C18–C25, C27–C34, C2′–C3′, and C2″–C3″. The remaining partial structure C10–C13 and the connectivity of C25–C26 were established by ^1H–^1H COSY experiment using aplyronine A diacetate (**4**). The locations of two hydroxyl groups in aplyronine A (**1**) were disclosed by the ^1H–^1H COSY correlations (H9/9-OH and H25/25-OH) as well as by the acetylation shifts observed for H9 and H25 in the ^1H NMR of **4**.[5] To connect the partial structures described above and further to determine the positions of four kinds of esters (C1, C1′, C1″, and an acetate), three methoxy groups, and two dimethylamino groups, HMBC experiments on **1** were carried out, and the results are shown in Table I. The connectivity of C9–C10 was established by observing a cross peak of 10-Me/C9 in the HMBC spectra of **4**. Although no cross peak was observed between C1′ and H7 in the HMBC spectra of **1** and **4**, the trimethylserine portion could be bonded to C7, because all other connectivities have already been established. The *E* geometry of the C14–C15 double bond was clarified by NOEs between H13 and H15 (8%) and between 14-Me and H16 (6% and 3%). These

(1) (a) Yamamura, S.; Hirata, Y. *Tetrahedron* **1963**, *19*, 1485–1496. (b) Ojika, M.; Kigoshi, H.; Yoshikawa, K.; Nakayama, Y.; Yamada, K. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 2300–2302 and references cited therein.

(2) The animal was collected on the Pacific coast of Mie Prefecture, Japan.

(3) The EtOAc-soluble material was partitioned between 7:3 MeOH/H_2O and 1:1 CH_2Cl_2/CCl_4, and the latter was further partitioned between 8:2 MeOH/H_2O and CCl_4. The material obtained from the 80% MeOH portion was successively separated by column chromatography [(i) Silica gel, EtOAc/MeOH. (ii) Al_2O_3, EtOAc/MeOH. (iii) Silica gel, hexane/EtOAc/MeOH], by TLC (silica gel, $CHCl_3$/acetone/MeOH), and by reversed-phase HPLC (ODS, 65% MeCN–0.02 M NH_4OAc).

(4) The drug was administered intraperitoneally once daily for the first 5 days of the test. Tumor cells were inoculated intraperitoneally on day zero.

(5) **4**: amorphous powder; $[\alpha]^{27}_D$ +18° (*c* 0.22, MeOH); UV (MeCN) λ_{max} 253 nm (ε 35 400); IR ($CHCl_3$) 1730, 1695, and 1655 cm^{-1}; FABMS $(M + H)^+$ *m/z* 1160; ^1H NMR (500 MHz, acetone-d_6) δ 4.88 (1 H, br d, *J* = 9.4 Hz, H9), 4.70 (1 H, dd, *J* = 7.6, 4.2 Hz, H25), 2.04 (6 H, s, 2 × Ac), 1.95 (3 H, s, Ac). See supplementary material for detailed NMR data.

(6) (a) Moore, R. E.; Patterson, G. M. L.; Mynderse, J. S.; Barchi, Jr., J.; Norton, T. R.; Furusawa, E.; Furusawa, S. *Pure Appl. Chem.* **1986**, *58*, 263–271. (b) Ishibashi, M.; Moore, R. E.; Patterson, G. M. L.; Xu, C.; Clardy, J. *J. Org. Chem.* **1986**, *51*, 5300–5306. (c) Carmeli, S.; Moore, R. E.; Patterson, G. M. L. *J. Nat. Prod.* **1990**, *53*, 1533–1542. (d) Jung, J. H.; Moore, R. E.; Patterson, G. M. L. *Phytochemistry* **1991**, *30*, 3615–3616.

(7) Guella, G.; Mancini, I.; Chiasera, G.; Pietra, F. *Helv. Chim. Acta* **1989**, *72*, 237–246.

(8) (a) Roesener, J. A.; Scheuer, P. J. *J. Am. Chem. Soc.* **1986**, *108*, 846–847. (b) Matsunaga, S.; Fusetani, N.; Hashimoto, K.; Koseki, K.; Noma, M. *J. Am. Chem. Soc.* **1986**, *108*, 847–849. (c) Fusetani, N.; Yasumuro, K.; Matsunaga, S.; Hashimoto, K. *Tetrahedron Lett.* **1989**, *30*, 2809–2812 and references cited therein.

(9) The term scalemic is used to describe an unequal mixture of enantiomers: Heathcock, C. H.; Finkelstein, B. L.; Jarvi, E. T.; Radel, P. A.; Hadley, C. R. *J. Org. Chem.* **1988**, *53*, 1922–1942.

(10) By four-step reactions [(i) Acidic hydrolysis of the *N*-methyl-*N*-vinylformamide part in **1**. (ii) Acetalization of the resulting aldehyde. (iii) $LiAlH_4$ reduction. (iv) *p*-Bromobenzoylation of the amino alcohols], two amino acid portions in **1** were converted into *p*-bromobenzoates of 2-(dimethylamino)-3-methoxypropanol and 2-(dimethylamino)propanol, respectively, and their HPLC analysis using chiral columns showed that the ratios *R*/*S* for the trimethylserine ester and for the dimethylalanine ester were 48:52 and 28:72, respectively.

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Table I. NMR Data for Aplyronine A (**1**) in Acetone-d_6

no.	$^1H^a$	$^{13}C^b$	HMBC (H no.)^c	no.	$^1H^a$	$^{13}C^b$	HMBC (H no.)^c
1		167.5 (s)	2, 3, 23	24	1.74	41.9 (d)	23, 24-Me
2	5.98	121.7 (d)^d	3, 4	24-Me	0.90	10.7 (q)	23, 24
3	7.23	145.0 (d)	4, 5	25	3.06	77.0 (d)	23, 24, 24-Me
4	6.43^d	131.7 (d)^d	2, 3, 5	25-OH	3.57		
5	6.29	141.1 (d)	2, 3, 4, 6	26	1.63	34.8 (d)	
6	2.16, 2.46	32.7 (t)^d	4	26-Me	0.98	17.9 (q)	25
7	4.75^d	76.6 (d)		27	1.16, 1.38	25.3 (t)	25, 29
8	2.01	39.0 (d)^d		28	1.51, 1.61	30.7 (t)	29
8-Me	1.01^d	11.7 (q)^d		29	5.03	72.7 (d)	
9	3.30	77.9 (d)		30	1.97	38.0 (d)	
9-OH	3.50			30-Me	1.00	10.1 (q)^e	29, 30
10	1.65	33.3 (d)		31	4.80^e	77.4 (d)	29, 30
10-Me	1.02	16.3 (q)		32	2.66	37.6 (d)^e	34
11	1.30	22.6 (t)		32-Me	1.00	19.9 (q)	32
12	1.65	29.2 (t)	13	33	5.05^e	110.0 (d)^e	31, 32, 34
13	3.52	86.8 (d)^d	13-OMe, 15	34	6.84^e	131.0 (d)^e	CHO, 34-NMe
13-OMe	3.13	55.5 (q)	13	34-NMe	2.97^e	27.3 (q)^e	34, CHO
14		135.5 (s)^d	14-Me	CHO	8.36^e	162.9 (d)^e	34, 34-NMe
14-Me	1.51^d	10.3 (q)	13, 15	1′		170.4 (s)	2′, 3′
15	5.18	130.7 (d)^d	13, 14-Me	2′	3.37^d	68.0 (d)^d	2′-NMe_2, 3′
16	1.57, 1.92	38.1 (t)^d	17-Me	2′-NMe_2	2.37^d	42.6 (q)	2′
17	1.19	30.5 (d)	17-Me	3′	3.60	72.4 (d)^d	3′-OMe
17-Me	0.76^d	20.3 (q)	18		3.69^d		
18	1.14, 1.56	41.1 (t)	17-Me	3′-OMe	3.34^d	59.0 (q)^d	3′
19	3.47	82.4 (d)	18, 19-OMe	1″		172.8 (s)	29, 2″, 3″
19-OMe	3.11	55.4 (q)	19	2″	3.20	63.5 (d)^f	2″-NMe_2, 3″
20	4.95	133.4 (d)		2″-NMe_2	2.34^f	41.6 (q)	2″
21	5.61	132.8 (d)	22, 23	3″	1.26^f	15.9 (q)^f	2″
22	2.27, 2.42	38.2 (t)		Ac		170.7 (s)	31, Ac
23	5.47	72.7 (d)			2.03^f	21.0 (q)	

^{a} 500 MHz. ^{b} 67.8 MHz. ^{c} 400 MHz, optimized for J_{CH} = 8.3 Hz. ^{d,e,f} The minor counterparts of doubled signals in the ratios of 1.1:1 (superscript *d*), 2:1 (superscript *e*), and 3:1 (superscript *f*), respectively, are omitted. See supplementary material for the detailed NMR data.

findings allowed us to establish the gross structure of aplyronine A as **1**. Thus, the structure of aplyronine A (**1**) has been determined to be a 24-membered macrolide containing two amino acid ester groups and an *N*-methyl-*N*-vinylformamide grouping.

Structures of the minor congeners, aplyronines B (**2**)[11] and C (**3**),[11] were elucidated by comparison of the FABMS and ^1H NMR data with those of aplyronine A (**1**). Aplyronine B (**2**) had the same molecular formula as **1**. The ^1H NMR data (acetone-d_6), assigned from the ^1H–^1H COSY experiment, showed that the trimethylserine ester group in **2** was located at C9 instead of at C7 in **1**. Except for the chemical shifts of H7 (δ 3.67) and H9 (δ 4.94), the ^1H NMR data were virtually identical with those for **1**. On the other hand, the ^1H NMR spectrum of aplyronine C (**3**) was almost superimposable on that of **1**, except for the absence of the trimethylserine signals and the chemical shift of H7 (δ 3.68). Interpretation of the ^1H NMR and FABMS data coupled with the molecular formula indicated that **3** was the de-*O*-trimethylseryl derivative of **1**. Comparison of the ^1H NMR spectra of **2** and **3** with the spectrum of **1** revealed that the two amino acid esters in **2** and the amino acid ester in **3** were also scalemic,[9] as in the case of **1**.

The structure–bioactivity relationship among the aplyronines is of interest. Comparison of the cytotoxicities of aplyronines A (**1**) and B (**2**) showed that they depend largely on the location of the trimethylserine moiety in the aplyronines. Further, aplyronine A (**1**) was found to be approximately 4000-fold more cytotoxic than aplyronine C (**3**) that lacks the trimethylserine group in **1**. It is noteworthy that the slight changes of the chemical structures of the aplyronines dramatically alter their cytotoxicities. Studies on the stereochemistry of aplyronine A (**1**) are currently in progress.

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Supplementary Material Available: Tables of ^1H and ^{13}C NMR assignments of **1**–**4** and 1D and 2D NMR spectra of **1**–**4** (21 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(11) **2**: $[\alpha]^{27}_D$ +3.7° (*c* 0.19, MeOH); UV (CH_3CN) λ_{max} 258 nm (ϵ 30 200); IR ($CHCl_3$) 3680, 3490, 1730, 1690, and 1655 cm^{-1}; HRFABMS obsd *m/z* 1076.7370, $C_{59}H_{102}N_3O_{14}$ $(M + H)^+$ requires *m/z* 1076.7362. **3**: $[\alpha]^{27}_D$ +18° (*c* 0.017, MeOH); UV (CH_3CN) λ_{max} 260 nm (ϵ 30 000); IR ($CHCl_3$) 3680, 3490, 1735, 1725, 1690, and 1655 cm^{-1}; HRFABMS obsd *m/z* 947.6553, $C_{53}H_{91}N_2O_{12}$ $(M + H)^+$ requires *m/z* 947.6572. See supplementary material for the ^1H NMR data (500 MHz, acetone-d_6) of **2** and **3**.