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Polycavernoside C and C2, the new analogs of the human lethal toxin polycavernoside A, from the red alga, *Gracilaria edulis*

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Abstract—Two new analogs of the human lethal toxin polycavernoside A, polycavernoside C and C2 (0.1–0.2 mg for each), were isolated from the red alga, *Gracilaria edulis*. The relative stereostructure of polycavernoside C and the absolute structure of polycavernoside C2 were determined by spectroscopic analysis and synthesis of the model of their aglycon.

Polycavernoside A (PA, Fig. 1) and B (PB) were isolated as the causative toxins of the human fatal poisoning (3 death/15 patients) which occurred in Guam in 1991, resulting from ingestion of the red alga, *Gracilaria edulis*.[1,2] PA was also identified from the same alga and *Acanthophora specifera*, which also caused fatal poisoning (8 death/36 patients) in Philippines in 2002–2003.[3] We determined the planar structures of PA[1] and the analogs, PA2, PA3, PB, and PB2,[4] which possess the same macrolide aglycon structure. Structural variation among these analogs are in the conjugated diene or triene side chain at C15, and in O-methylated or O-acetylated L-fucosyl-D-xylose sugar part at C5. The total synthesis of (−)-PA was achieved by three groups,[5–7] and the absolute structure of PA was established by Fujiwara and Murai et al.[5] Further synthetic efforts have been continuously reported.[8] The study of structure–activity relationship[9] and pharmacological details of PA are now progressing.[10] We isolated two other minor analogs, polycavernoside C (PC, **1**) and C2 (PC2, **2**) (0.1–0.2 mg for each), from *G. edulis* in 1992–1994 collected in Guam. These were suggested to have a common aglycon structure, and to be the first analogs which have the different aglycon structure from that of PA. Here we report the relative stereostructure of **1** and the absolute structure of **2** deduced by spectroscopic analysis and synthesis of the model of their aglycon (**3b**).

Compound **1** (0.1–0.2 mg) was isolated together with PA (0.2 mg) and PB (0.1 mg), from *G. edulis* (4 kg) collected on June 25, 1991 in Guam, while **2** (0.1–0.2 mg) was isolated from the same alga (2 kg, June 11, 1992) with PA (0.4 mg), PA2 (0.1 mg), PA3 (0.4 mg), and PB2 (0.1 mg),[4] by successive normal and reversed phase chromatography as reported previously.[1,4] Elution of **1** and **2** from the columns was monitored by diode array UV detector. For final separation of **1** from PB,[1] Capcell pack CN (H_2O–MeCN from 1:1 to 0:10) was used, and **1** was eluted before PB. Compound **2** was eluted after PA2, PB2, and PA from the final column, Develosil ODS-5 (H_2O–MeCN 1:3)[4] and further purified on the same column. Compound **2** has been kept intact until now after storage in CD_3CN at −20 °C for more than 10 years, while **1** was decomposed in pyridine-d_5. Thus, **2** was mainly used for the structural analysis of the common aglycon of **1** and **2**.

Compound **1** was found to have a molecular weight 856 as determined from FABMS $[M-H_2O+H]^+$ m/z 839, $[M+Na]^+$ m/z 879, $[M+K]^+$ m/z 895. **2** was found to have a molecular formula of $C_{45}H_{74}O_{15}$ by HR-FAB MS ($[M+Na]^+$ 877.4930, Δ+0.5 mmu). ^1H NMR spectra and ^1H–^1H COSY of **1** and **2** (CD_3CN), and UV absorption (λ_{max} 220 nm for **1**, 259, 270, 280 nm for **2**, MeCN) suggested the presence of conjugated diene for **1** and conjugated triene for **2**. The $^3J_{HH}$ values (16 Hz for **1**, 15.4 Hz for **2**) indicated *E*, *E* and *E*, *E*, *E* geometry for **1** and **2**, respectively. The same disaccharide

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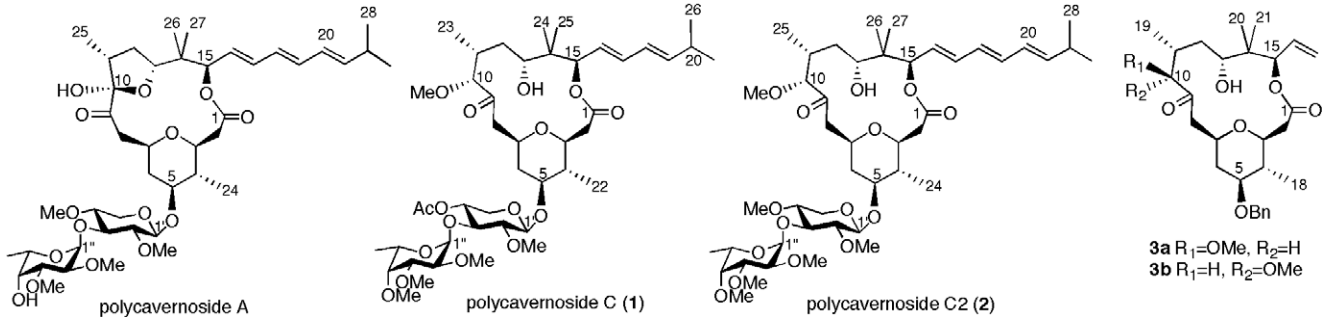


Figure 1. Structures of polycavernoside A, C (**1**), C2 (**2**), and the models (**3a**,**b**) of the aglycon of **1** and **2**.

structure as that in PB[4] (*O*-2,3,4-tri-*O*-methylfucopyranoyl-(1″-3′)-*O*-4-acetyl-*O*-2-methylxylopyranosyl) was suggested in **1**, while the same disaccharide structure as that in PA3[4] (*O*-2,3,4-tri-*O*-methylfucopyranoyl-(1″-3′)-*O*-2,4-dimethylxylopyranosyl) was suggested in **2**, by the agreement of the ^{1}H NMR chemical shifts (the difference was less than 0.03 ppm) and $^{3}J_{HH}$ values of H1′-H5′ and H1″-H6″ signals of **1** and **2** (Table 1) with those of the sugar parts of PB and PA3, respectively. ^{1}H–^{1}H COSY of **1** and **2** suggested that these compounds have a common aglycon structure which was distinctly different from the aglycon of PA and other reported analogs. Partial structures of H2–H8, H10–H13, H15–H23,28, H1′–H5′, and H1″–H6″ of **2** were deduced from analyses of ^{1}H–^{1}H COSY and TOCSY. A ^{1}H signal at 2.96 ppm (d 10.2 Hz) coupling with a methyl substituted methine ^{1}H at 2.01 ppm (H11) showed correlation with the ^{13}C signal at 94 ppm in the HSQC spectrum. This ^{1}H signal was assignable to H10, suggesting that C10 hemiacetal in PA was reduced to oxymethine in **2**. The upfield shifts of the signals corresponding to H11, H12a, H12b, and H13 of **2** (0.75, 0.72, 0.83, and 0.90 ppm, respectively) (Table 1) from those of PA[1] supported that the five membered cyclohemiacetal ring formed by C10–C13 in PA was opened and the hemiacetal was reduced in **2**. A deuterium exchangeable proton signal at 1.71 ppm (d 12.0 Hz) coupling with H13 was assigned to 13-OH. Compared with the signals of PA, the signals corresponding to H3, H7, and H8a of **2** were downfield shifted (0.12, 0.32, 0.12 ppm, respectively), and the signal corresponding to H8b was 0.12 ppm upfield shifted. However, the same partial structure of C2–C8 in **2** as that in PA including a tetrahydropyrane ring was suggested by the similar coupling patterns of H2–H8. Due to the small sample size of **2**, the HSQC spectrum showed only partial ^{1}H–^{13}C correlations. For the same reason, the HMBC spectrum clarified the connectivities only around methyl groups by giving cross peaks due to $^{2,3}J_{CH}$ between C10/10–OMe, C10/Me25, C11/Me25, C3/Me24, C4/Me24, C5/Me24, C14/Me26, C14/Me27, C15/Me26, C15/Me27, C26/Me27, C27/Me26, C4″/Me6″, C5″/Me6″, C2′/2′–OMe, C2″/2″–OMe, C3″/3″–OMe, and C4″/4″–OMe. Among them, the cross peak C10/Me25 supported the connectivity of C10 and C11, and that C10/10–OMe indicated that C10 bears a methoxy group. The presence of carbonyl carbons at C1 and C9 and formation of lactone between C1 and C15 were not evident from HMBC correlations. However, these structural features were suggested by the close ^{1}H NMR chemical shifts of the signals corresponding to 2-CH_2 (2.22/2.62), 8-CH_2 (2.19/2.85), and H15 (4.92) of **2** to those in PA, 2-CH_2 (2.14/2.52), 8-CH_2 (2.07/2.97) and H15 (5.00), respectively. All these data led to the planar structures of **1** and **2** (Fig. 1) and assignments of their NMR data (Table 1).

The relative configurations of **1** and **2** between the tetrahydropyrane part (C2–C8) and the disaccharides were determined based on the reported data by Fujiwara et al.[11] They synthesized both enantiomers of the C2–C8 part and coupled with the disaccharide of PA. Between these glycosides, the chemical shifts of H5 and H6a were most different (approximately 0.1 ppm), and those of one enantiomer were well agreed with those of PA. Thus, the good agreement of the chemical shifts of H5 [**1** (3.38), **2** (3.38), and PA (3.33)] and those of H6a [**1** (1.32), **2** (1.38) and PA (1.37)] suggested the same relative configurations in **1**, **2**, and PA. However, the combination between the northern part (C10–15) and the southern part (C2–C8) of the aglycon of **1** and **2**, and the stereochemistry of C10 and C13 were kept unknown, since sufficient NOE data of **2** were not obtained.

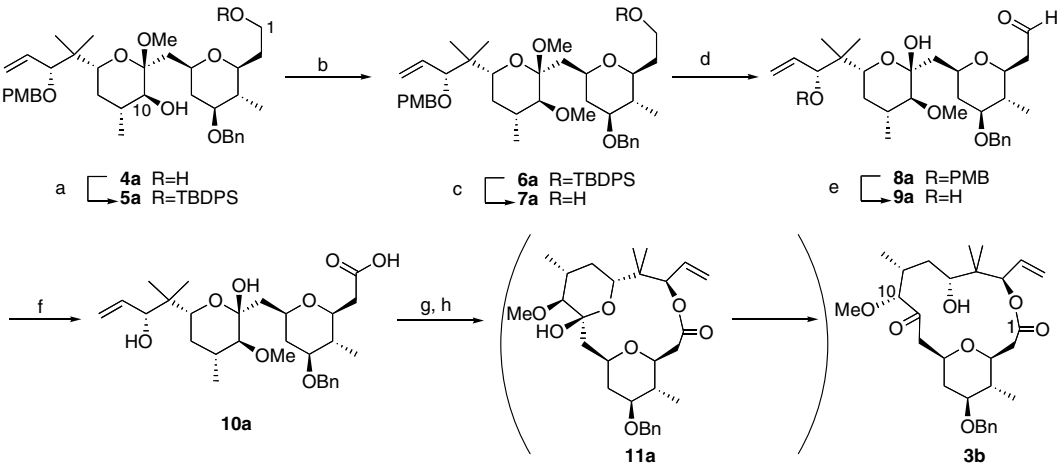
To determine them, keto lactone **3a** (10*S*, 13*R*) and C10-epimeric **3b** (10*R*, 13*R*) (Fig. 1) were planned to be synthesized as the models of aglycon of **1** and **2** from **4a** and **4b** (C10-epimer of **4a**), respectively (Scheme 1). **4a** and **4b** are the synthetic intermediates of PA.[5] The stereochemistry of C13 in **1** and **2** was hypothesized in *R* as same as in PA based on biosynthetic aspect. Selective protection of the primary alcohol of diol **4a** with TBDPS, followed by methylation of secondary alcohol **5a** and deprotection of TBDPS with TBAF afforded alcohol **7a** (Scheme 1). Swern oxidation of **7a** with workup process under the acidic condition gave methyl acetal hydrolyzed aldehyde **8a**. By following the method used for total synthesis of PA,[5] secoic acid **10a** obtained by deprotection of PMB in **8a** with DDQ and by subsequent $NaClO_2$ oxidation was lactonized by modified Yamaguchi's method.[12] NMR spectra of the product in $CDCl_3$ containing trace amount of HCl indicated C10-epimerized keto lactone **3b**, probably formed via acetal lactone **11a**. Detail analysis of stereochemistry of C10 of this keto lactone (**3b**) was described below. Similarly from **4b** (10-epimer of **4a**), corresponding secoic acid was derived by the same way. In this case, methyl acetal was not hydrolyzed by workup process of Swern oxidation. After lactonization of the secoic

Table 1. NMR spectral data of **1**, **2** and **3b** in CD_3CN

Pos	**1**			**2**				**3b**			
	^{1}H (ppm)	Mult.	*J* (Hz)	^{1}H (ppm)	Mult.	*J* (Hz)	^{13}C (ppm)	^{1}H (ppm)	Mult.	*J* (Hz)	^{13}C (ppm)
1				.			—				170
2	2.18	t	12	2.19	m		—	2.20	m		40
	2.62	dd	12, 3	2.62	dd	13.8, 3.0		2.62	dd	13.8, 2.9	
3	3.40	m		3.41	m		81	3.41	m		80
4	1.32	m		1.28	m		43	1.24	m		42
5	3.38	m		3.38	m		82	3.23	dt	14.7, 4.1	80
6	1.32	q	11	1.36	m		—	1.22	m		36
	1.95	m		1.98	m			2.08	m		
7	3.97	t	11	3.96	t	11.3	72	3.97	t	11.0	71
8	2.14	d	14	2.19	m		—	2.20	m		42
	2.85	dd	14, 9	2.85	dd	13.6, 8.7		2.89	dd	17.2, 10.9	
9							—				210
10	2.97	d	9.4	2.96	d	10.2	94	2.97	d	10.6	94
11	2.00	m		2.01	m		32	1.99	m		32
12	0.70	m		0.84	t	9.0	—	0.85	t	8.8	33
	1.17	dd	9.3	1.15	q	12.0		1.15	t	10.3	
13	3.10	t	11	3.15	m		79	3.11	t	10.6	78
14							41				40
15	4.90	d	7	4.92	d	7.8	81	4.90	d	6.5	80
16	5.45	dd	16, 8	5.55	dd	15.4, 9.8	—	5.70	ddd	17.3, 10.8	140
										6.5	
17	6.10	dd	16, 10	6.18	dd	15.4, 9.8	—	5.09	d	17.3	118
								5.17	d	10.5	
18	5.98	dd	16, 10	6.12	dd	15.4, 9.8	—	0.87	d	6.5	12
19	5.69	dd	16, 7	6.22	dd	15.4, 9.8	—	0.98	d	6.5	15
20	2.25	m		6.05	ddd	15.4, 9.8, 1.2					
21	0.95	d	7	5.73	dd	15.4, 6.4	—				
22	0.92	d	7	2.29	m		—				
23	0.98	d	7	0.95	d	6.7	22				
24	0.40	s		0.91	d	6.7	13				
25	1.03	s		0.97	d	6.7	16				
26	0.95	d	7	0.70	s		25	0.74	s		23
27				1.02	s		20	1.01	s		20
28				0.95	d	6.7	22				
1′	4.38	d	8	4.31	d	7.2	107				
2′	2.97	t	10	2.84	t	7.8	86				
3′	3.68	t	10	3.43	m		80				
4′	4.60	m		3.11	m		79				
5′	3.13	t	11	3.05	t	10.8	64				
	3.83	dd	12, 5	3.91	dd	11.3, 5.4					
1″	5.24	d	2	5.18	d	3.6	98				
2″	3.37	br s		3.38	m		79				
3″	3.50	d	12	3.43	m		—				
4″	3.48	br s		3.47	br s		80				
5″	3.88	q	6	4.1	q	6.6	67				
6″	1.09	d	7	1.05	d	6.7	16				
10-OMe	3.19	s		3.19	s		59	3.20	s		58
OMe	3.36	s		3.30	s		59				
OMe	3.38	s		3.38	s		58				
OMe	3.43	s		3.38	s		58				
OMe	3.48	s		3.44	s		62				
OMe				3.47	s		61				
13-OH	1.72	d	11	1.71	d	12.0		1.72	d	12.0	
4-OAc	1.97	s									
Bn–CH_2								4.36	d	11.5	70
								4.58	d	11.5	
Bn–arom								7.31	s		120, 134

^{1}H NMR spectra: **1** (400 MHz), **2** (600 MHz).—Denotes not determined.
^{1}H internal reference (CHD_2CN) was set at 1.9 ppm.
^{13}C internal reference ($CD_3{}^{13}CN$) was set at 118 ppm.
^{13}C chemical shifts of **2** and **3b** were roughly determined based on HSQC and HMBC.

acid derived from **4b**, crude NMR spectra measured in $CDCl_3$ suggested the formation of methyl acetal lactone (9-*O*-methyl C10-epimer of **11a**). This methyl acetal lactone in $CDCl_3$ was shaken with aqueous 0.05 M acetic



Scheme 1. Synthesis of **3b** from **4a**. Reagents and conditions: (a) TBDPSCl, Et_3N, DMAP, CH_2Cl_2, 20 °C, 96%. (b) MeI, NaH, CH_2Cl_2, 20 °C, 86%. (c) TBAF, THF, 0 °C, 93%. (d) $(COCl)_2$, DMSO, CH_2Cl_2, Et_3N, −75 to 0 °C, 83%. (e) DDQ, CH_2Cl_2, H_2O, 20 °C, 87%. (f) $NaClO_2$, NaH_2PO_4, 2-methyl-2-butene, *i*-PrOH–H_2O (3.5:1), 0 °C, 89%. (g) 2,4,6-trichlorobenzoyl chloride, DIPEA, THF, 20 °C, then DMAP, toluene, 60 °C and (h) $CDCl_3$ (+HCl), 20 °C, 50% in two steps.

acid and allowed to stand for one day at room temperature. Finally, keto lactone **3b** was obtained in 30% yield from the corresponding secoic acid in two steps. The yields of the other steps were almost same as those in the case from **4a** to **3b**. To determine the stereochemistry of C10 of keto lactone (**3b**) derived from both of **4a** and **4b**, NOE experiments were performed on this keto lactone. Positive NOEs, H6a/H8b, H8b/H11, H10/H12b, H10/Me19, H12a/10-OMe, H12b/Me19, and H13/Me19 were observed by NOESY1D measured in CD_3CN at 20 °C, whereas NOEs, Me19/10-OMe and H10/H11 were not observed (Fig. 2). By these NOEs together with $^3J_{H10/H11}$ (10.6 Hz), Me19, and 10-OMe, and H10 and H11 were suggested to be in anti-conformation for each, as shown in Figure 2. Based on these data, the stereochemistry of C10 of the obtained keto lactone was determined to be in *R*, therefore, C10 in **3a** was suggested to be epimerized, probably via formation of 9,10-enolate under acidic condition. Steric repulsion between 10-OMe and Me19 in **3a** was presumed to induce this epimerization. The NMR data of **3b** were compared with those of **2** (Table 1). The difference of the ^1H NMR chemical shifts of H2–H15, H24–27, 10-OMe, 13-OH of **2** from those of H2–H15, H18–21, 10-OMe, 13-OH of **3b**, respectively, was less than 0.1 ppm, except H5 (0.15 ppm), H6a (0.14 ppm), and H6b (0.10 ppm) which were probably due to difference of the substituents at C5. The differences of the ^{13}C NMR chemical shifts of C2–C15, C24–C27, 10-OMe of **2** from C2–C15, C18–C21, 10-OMe of **3b,** respectively, were not more than 1 ppm except at C5 (2 ppm). In addition, $^3J_{H10/H11}$ value of **2** (10.2 Hz) was close to that of **3b** (10.6 Hz). According to these data, the planar structure of the aglycon of **1** and **2** was confirmed, and the relative stereostructures of **1** and **2** ($10R^*$,$13R^*$) were determined as shown in Figure 1. To determine the absolute configuration of **2**, CD spectra of **2** and **3b** were measured.[13] Both of these CD spectra showed a similar characteristic single Cotton effect of negative sign in the region of the transition of ketone (n-π^*) around 300 nm, which was also shown in the CD spectrum of PA.[5] Based on these data, the absolute configuration of **2** was determined as same as those of **3b** and PA.

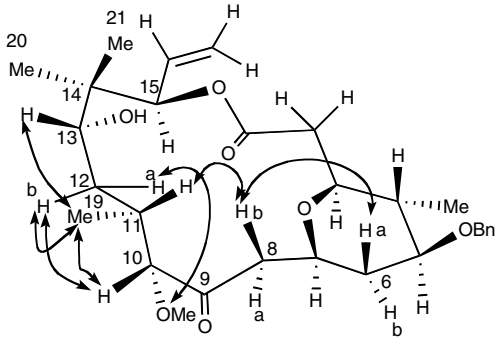


Figure 2. Key NOEs observed in keto-lactone (**3b**) derived from **4a** and **4b**.

The mouse (ddY, male, 12 g) administrated **2** (2.4 μg, i.p.) did not show any symptoms and survived, suggesting the value of LD_{99} for **2** was more than 0.2 mg/kg.[15] Compound **3b** did not show cytotoxicity at 20 μM in two cell lines, mouse neuroblastoma cells (Neuro-2a) and human acute monocytic leukemia cells (THP-1), by WST-8 assay (Dojindo, Japan).[14] Cyanobacterium was proposed for the origin of polycavernosides.[3,16] The structures of **1** and **2** provide information for the biosynthetic pathway or metabolism of polycavernosides, which are necessary for monitoring the occurrence of these human lethal toxins.

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13. CD spectral data of **2** and **3b** (MeCN, 22 °C). Compound **2** (0.12 mM): λ_{ext} 210.0 ($\Delta\varepsilon$ −1.22), 221.6 (−1.14), 251.0 (−0.47), 272.6 (−0.41), 301.6 (−0.53). 320.0 (−0.33), **3b** (0.4 mM): 226.6 (−0.23), 292.2 (−0.95), 301.0 (−1.13), 309.0 (−1.04), 321.2 (−0.57).
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