New Pregnane Steroids from Formosan Red Alga *Ceratodictyon spongiosum* and Symbiotic Sponge *Sigmadocia symbiotica*

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Four new pregnane steroids, ceratosteroid A (**1**), B (**2**), C (**3**), and D (**4**), were isolated from the Formosan red alga *Ceratodictyon spongiosum* and symbiotic sponge *Sigmadocia symbiotica*. The structures were elucidated by 1D and 2D NMR spectral analyses. Compounds **2** and **4** showed cytotoxicity against P-388 cell line.

Red alga *Ceratodictyon spongiosum* and symbiotic sponge *Sigmadocia symbiotica* were reported to contain ceramides and cyclic heptapeptides.[1,2] As part of our search for bioactive substances from marine organisms, the Formosan red alga *Ceratodictyon spongiosum* and symbiotic sponge *Sigmadocia symbiotica* were studied because their EtOAc extracts showed significant cytotoxicity (IC_{50} 9.2 μg mL^{-1}) against P-388 (mouse lymphocytic leukemia) cell cultures as determined by standard procedures.[3,4] Bioassay-guided fractionations resulted in the isolation of four new pregnane steroids, ceratosteroid A (**1**), B (**2**), C (**3**), and D (**4**) (Figure 1). Compounds **2** and **4** showed cytotoxicity against P-388 cell lines with IC_{50} of 2.99 and 3.52 μg mL^{-1}, respectively.

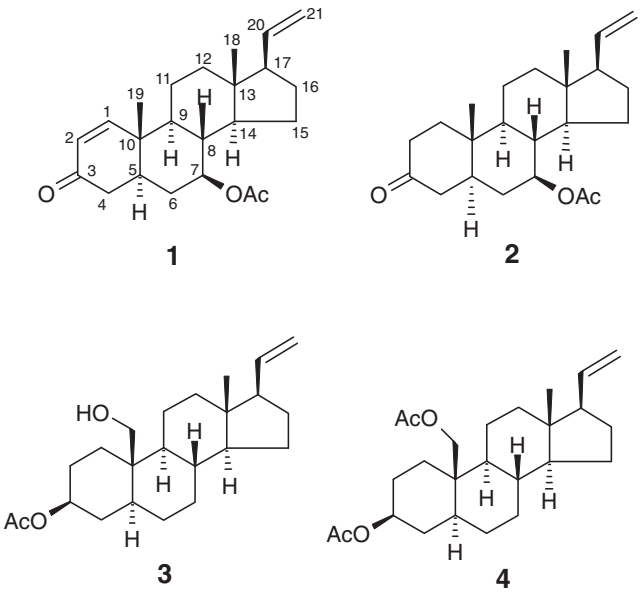

Figure 1. Structures of compounds **1**–**4**.

Compound **1** had a molecular formula of $C_{23}H_{32}O_3$ as established by HR-ESI-MS. The NMR spectrum revealed the presence of two tertiary methyls (δ_H 0.66 (s), 1.05 (s); δ_C 13.2 (CH_3), 13.2 (CH_3)), a terminal vinyl group (δ_H 5.74 (ddd, $J = 17.4$, 10.4, 7.5 Hz), 4.98 (d, $J = 17.4$ Hz), 4.99 (d, $J = 10.4$ Hz); δ_C 139.3 (CH), 115.2 (CH_2)), a secondary acetoxyl (δ_H 4.61 (dt, $J = 5.1$, 10.2 Hz), 2.02 (s); δ_C 75.6 (CH), 21.7 (CH_3), 170.7 (qC)). The presence of an α,β-unsaturated carbonyl group (partial structure **a** in Figure 2) was straightforward from NMR signals (Tables 1 and 2) at δ_H 5.89/δ_C 128.0, 7.14/157.2, and 199.2 (qC), as well as from an IR absorption at 1684 cm^{-1}. The 1D NMR data could account for 4 of the 8 degrees of unsaturation, suggesting the tetracyclic nature of **1**. Interpretation of the ^{1}H–^{1}H COSY spectrum led to partial structure **b** (Figure 2). Rings A and B were elucidated on the basis of HMBC cross-peaks between Me-19/C-1, C-5, C-9, C-10 and H-4/C-3, whereas rings C and D were completed on the basis of HMBC correlations between Me-18/C-12, C-13, C-14, C-17. Comparison of ^{13}C NMR

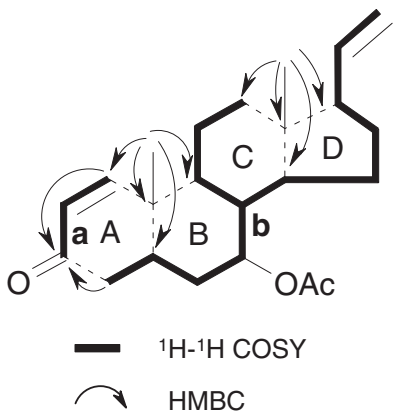

Figure 2. Key COSY and HMBC correlations of **1**.

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Table 1. ^{1}H NMR Spectral Data (δ) of **1**–**4**[a)]

H	**1**	**2**	**3**	**4**
1	7.14 d (10.2)[b)]	1.35 m, 2.04 m	0.91 m, 2.32 m	0.96 m, 2.23 dt (13.6, 3.4)
2	5.89 d (10.2)	2.35 m	1.55 m, 1.92 m	1.42 m, 1.82 m
3			4.73 m	4.73 m
4	2.28 m, 2.37 m	2.12 m, 2.23 m	1.48 m, 1.73 m	1.48 m, 1.74 m
5	2.07 m	1.66 m	1.34 m	1.38 m
6	1.45 m, 1.65 m	1.35 m, 1.73 m	1.30 m, 1.82 m	1.32 m, 1.88 m
7	4.61 dt (5.1, 10.2)	4.58 td (10.2, 5.1)	1.54 m, 1.75 m	0.90 m, 1.75 m
8	1.80 m	1.72 m	1.55 m	1.40 m
9	1.14 m	0.92 m	0.75 m	0.77 m
11	1.51 m, 1.87 m	1.43 m, 1.66 m	1.52 m, 1.73 m	1.35 m, 1.66 m
12	1.78 m	1.19 m, 1.72 m	1.03 m, 1.72 m	0.98 m, 1.68 m
14	1.23 m	1.17 m	1.06 m	1.03 m
15	1.44 m	1.53 m	1.20 m, 1.73 m	1.20 m, 1.51 m
16	1.45 m, 1.80 m	1.28 m, 1.76 m	1.56 m, 1.82 m	1.45 m, 1.76 m
17	1.94 m	1.92 m	1.98 m	1.97 m
18	0.66 s	0.64 s	0.63 s	0.57 s
19	1.05 s	1.06 s	3.82 d (11.4), 3.96 d (11.4)	4.24 d (12.0), 4.34 d (12.0)
20	5.74 ddd (17.4, 10.4, 7.5)	5.74 ddd (18.3, 9.3, 7.2)	5.76 ddd (16.5, 11.1, 7.5)	5.74 ddd (16.2, 10.8, 7.8)
21	4.98 d (17.4), 4.99 d (10.4)	4.98 d (18.3), 4.99 d (9.3)	4.96 d (16.5), 4.97 d (11.1)	4.95 d (16.2), 4.96 d (10.8)
OAc	2.02 s	2.02 s	2.00 s	2.02 s, 2.06 s

a) Recorded in $CDCl_3$ at 300 MHz. b) J values (in Hz) in parentheses.

Table 2. ^{13}C NMR Spectral Data (δ) of **1**–**4**[a)]

	1	**2**	**3**	**4**
1	157.2 (CH)[b)]	38.0 (CH_2)	32.1 (CH_2)	32.0 (CH_2)
2	128.0 (CH)	38.0 (CH_2)	28.0 (CH_2)	27.6 (CH_2)
3	199.2 (qC)	211.0 (qC)	73.4 (CH)	73.1 (CH)
4	40.2 (CH_2)	43.9 (CH_2)	34.5 (CH_2)	34.3 (CH_2)
5	40.9 (CH)	43.5 (CH)	44.9 (CH)	44.9 (CH)
6	33.2 (CH_2)	34.3 (CH_2)	28.2 (CH_2)	28.2 (CH_2)
7	75.6 (CH)	76.2 (CH)	31.2 (CH_2)	31.7 (CH_2)
8	40.1 (CH)	39.8 (CH)	36.2 (CH)	36.0 (CH)
9	49.0 (CH)	52.1 (CH)	56.0 (CH)	54.6 (CH)
10	38.3 (qC)	35.1 (qC)	39.3 (qC)	38.1 (qC)
11	21.2 (CH_2)	21.3 (CH_2)	22.6 (CH_2)	21.9 (CH_2)
12	37.3 (CH_2)	37.3 (CH_2)	38.1 (CH_2)	37.9 (CH_2)
13	44.4 (qC)	44.3 (qC)	43.9 (qC)	43.7 (qC)
14	54.3 (CH)	54.1 (CH)	54.9 (CH)	55.9 (CH)
15	26.2 (CH_2)	26.4 (CH_2)	24.8 (CH_2)	24.8 (CH_2)
16	27.5 (CH_2)	27.5 (CH_2)	27.2 (CH_2)	27.2 (CH_2)
17	54.6 (CH)	54.7 (CH)	55.4 (CH)	55.4 (CH)
18	13.2 (CH_3)	13.0 (CH_3)	13.2 (CH_3)	13.1 (CH_3)
19	13.2 (CH_3)	11.6 (CH_3)	60.9 (CH_2)	62.8 (CH_2)
20	139.3 (CH)	139.5 (CH)	139.9 (CH)	139.8 (CH)
21	115.2 (CH_2)	115.1 (CH_2)	114.6 (CH_2)	114.6 (CH_2)
OAc				170.7 (qC)
	170.7 (qC)	170.7 (qC)	171.1 (qC)	171.2 (CH_3)
	21.7 (CH_3)	22.2 (CH_3)	21.5 (CH_3)	21.2 (CH_3)
				21.5 (CH_3)

a) Recorded in $CDCl_3$ at 75 MHz. b) Assigned by DEPT and HSQC.

chemical shift values of **1** with those of five pregn-1-en-3-ones reported from the octocoral *Alcyonium gracillimum*[5] inferred normal stereochemistry of the ring junctures of **1**. The NOESY correlations (Figure 3) observed between H-7 and H-5, H-9 and H-5, H-14 and H-9, H-11β and H-8, H-11β and H_3-18, H-11β and H_3-19, H_3-18 and H-8, H_3-19 and H-8, H_3-18 and H-20, and H-9 and H-12α in **1** confirmed the relative configurations for each ring junction and chiral center.

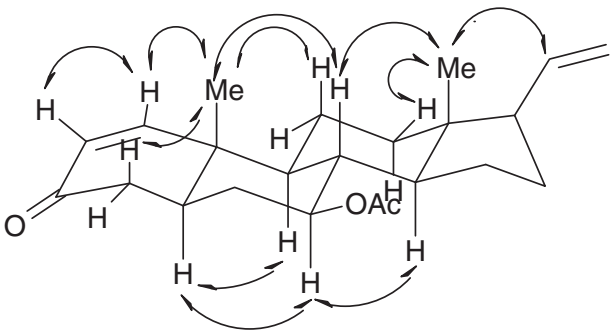

Figure 3. Selective NOESY correlations of **1**.

Compound **2** had a molecular formula of $C_{23}H_{34}O_3$ as determined by HR-ESI-MS. The ^{1}H and ^{13}C NMR spectral data (Tables 1 and 2) were analogous to those of **1**. However, the ^{13}C NMR spectrum of **2** showed that the olefinic methines at δ_C 128.0 and 157.2 were replaced by two sp^3 methylenes at δ_C 38.0 while the carbonyl carbon at δ_C 199.2 was shifted downfield to δ 211.0. Corresponding differences were found in the ^{1}H NMR spectrum in which the olefinic proton signals at δ 5.89 and 7.14 were replaced by upfield signals. In addition, the carbonyl absorption band at 1684 cm^{-1} in the IR spectrum of **1** was shifted to 1716 cm^{-1} in **2**. Therefore, **2** must be 7β-acetoxypregn-20-en-3-one.

Compound **3** had a molecular formula of $C_{23}H_{36}O_3$ as established by HR-ESI-MS, indicating 6 degrees of unsaturation. ^{13}C NMR and DEPT spectra of **3** exhibited the presence of two methyl, ten sp^3 methylenes, six sp^3 methines, one sp^2 methine, two sp^3 quaternary, one sp^2 methylene, and one carbonyl, indicating **1** was tetracyclic.

The ^{1}H and ^{13}C NMR (including DEPT and HSQC) NMR spectra implied the presence of a tertiary methyl (δ_H 0.63 (s); δ_C 13.2 (CH_3)), a terminal vinyl group (δ_H 5.76 (ddd, $J = 16.5$, 11.1, 7.5 Hz), 4.96 (d, $J = 16.5$ Hz), 4.97 (d, $J = 11.1$ Hz); δ_C 139.9 (CH), 114.6 (CH_2)), a secondary acetoxy (δ_H 4.73 (m), 2.00 (s); δ_C 73.4 (CH), 21.5 (CH_3), 171.1 (qC)), and an oxygenated methylene (δ_H 3.82 (d, $J = 11.4$ Hz), 3.96 (d, $J = 11.4$ Hz); δ_C 60.9 (CH_2)). The foregoing spectral data and literature survey provided evidence that **1** has a 3-*O*-acetoxypregnane skeleton, with an oxygenated methylene group. This methylene group was assigned to C-19, based on the absence of a methyl singlet (δ 0.80) assignable to the C-19 angular methyl and the presence of an AB doublet at δ 3.82 ($J = 11.4$ Hz) and 3.96 ($J = 11.4$ Hz). HMBC correlations between H_2-19 and C-10, C-9, C-1, and C-5 confirmed this assignment. The relative stereochemistry of **3** was established by NOESY experiment. The NOESY correlations observed from H-20 to H_3-18, from H-14 to H-17/H-9, from H_2-19 to H-8/H-2β, and from H-5 to H-3/H-9/H-1α indicated the relative configurations for each ring junction and chiral center. Based on these findings the structure of **3** was established as 3β-acetoxypregn-20-en-19-ol.[6,7]

Compound **4** was analyzed for $C_{25}H_{38}O_4$ by mass spectrometry in combination with interpretation of ^{13}C NMR data. The ^{1}H and ^{13}C NMR spectral data (Tables 1 and 2) were similar to those of **3**. However, the ^{1}H NMR spectrum of **4** showed that the hydroxymethylene proton signals at δ_H 3.82 (d, $J =$ 11.4 Hz) and 3.96 (d, $J = 11.4$ Hz) in **3** were replaced by an acetoxymethylene proton signals at δ_H 4.24 (d, $J = 12.0$ Hz) and 4.34 (d, $J = 12.0$ Hz). Corresponding differences were found in the ^{13}C NMR spectrum in which the the hydroxymethylene signal at δ_C 60.9 were replaced by acetoxymethylene signal at δ_C 62.8. HMBC correlations between H_2-19 and C-10, C-9, C-1, and C-5 confirmed this assignment. The relative stereochemistry of **4** was established by a NOESY experiment. Based on these findings, 3β,19-diacetatoxypregn-20-ene was presumed for **4**.

Experimental

General Experimental Procedures. Optical rotations were determined on a JASCO DIP-181 polarimeter. UV spectra were obtained on a Shimadzu UV-160A spectrophotometer, and IR spectra were recorded on a Hitachi 26-30 spectrophotometer. The NMR spectra were recorded on a Bruker AVANCE 300 FT-NMR at 300 MHz for ^{1}H and 75 MHz for ^{13}C, in $CDCl_3$, unless otherwise stated. Low-resolution mass spectral data were obtained by EI or ESI with a VG QUATTRO GC/MS spectrometer. HRMS were recorded by ESI FT-MS on a BRUKER APEX II mass spectrometer. Silica gel 60 (Merck, 230–400 mesh) was used for column chromatography; precoated Silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) were used for TLC analysis.

Algal Material. The red alga *Ceratodictyon spongiosum* and symbiotic sponge *Sigmadocia symbiotica* was collected at Ken-ting, Taiwan, in September 2003. A voucher specimen, KT-100, was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

Extraction and Isolation. The bodies of the red alga *C. spongiosum* and symbiotic sponge *S. symbiotica* (2.6 kg) were extracted with EtOH (3.0 L × 3). After removal of solvent in vacuo, the residue was partitioned between EtOAc and water. The EtOAc extract (16.6 g) was chromatographed over silica gel 60 using *n*-hexane and *n*-hexane–EtOAc mixtures of increasing polarity. Elution by *n*-hexane–EtOAc (1:1) afforded fractions containing compounds **1** and **2**. Elution by *n*-hexane–EtOAc (2:3) afforded fractions containing compound **3**. Elution by EtOAc afforded fractions containing compound **4**. Compounds **1** (4 mg, t_R 98 min) and **2** (2 mg, t_R 56 min) were further purified by HPLC (LiChrosorb RP-18, 7 μ, 25 i.d. × 250 mm, 4 mL min^{-1}), eluting with MeOH/H_2O (8:2). Compound **3** (3 mg, t_R 31 min) was further purified by HPLC (LiChrosorb RP-18, 7 μ, 25 i.d. × 250 mm, 4 mL min^{-1}), eluting with MeOH/H_2O (9:1). Compound **4** (8 mg, t_R 98 min) was further purified by HPLC (LiChrosorb Si 60, 7 μ, 25 i.d. × 250 mm, 4 mL min^{-1}), eluting with *n*-hexane/EtOAc (15:1).

Ceratosteroid A (**1**): amorphous solid; $[\alpha]_D^{25}$ +36° (*c* 0.05, $CHCl_3$); UV (MeOH): λ_{max}/nm (log ε) 229 (4.16); IR (KBr): ν_{max} 2930, 2852, 1684, 1630, 1438, 1390, 1271, 1012, 920 cm^{-1}; ^{1}H and ^{13}C NMR see Table 1; HR-ESI-MS *m/z* 379.2247 (calcd for $C_{23}H_{32}O_3Na$, 379.2249).

Ceratosteroid B (**2**): amorphous solid; $[\alpha]_D^{25}$ +112° (*c* 0.05, $CHCl_3$); IR (KBr): ν_{max} 2940, 2862, 1716, 1620, 1436, 1396, 1273, 1022, 890 cm^{-1}; ^{1}H and ^{13}C NMR see Table 1; HR-ESI-MS *m/z* 381.2405 (calcd for $C_{23}H_{34}O_3Na$, 381.2406).

Ceratosteroid C (**3**): amorphous solid; $[\alpha]_D^{25}$ +28° (*c* 0.2, $CHCl_3$); IR (KBr): ν_{max} 3580, 2930, 2852, 1730, 1628, 1438, 1386, 1283, 1032, 910 cm^{-1}; ^{1}H and ^{13}C NMR see Table 1; HR-ESI-MS *m/z* 383.2560 (calcd for $C_{23}H_{36}O_3Na$, 383.2562).

Ceratosteroid D (**4**): amorphous solid; $[\alpha]_D^{25}$ +5° (*c* 1.7, $CHCl_3$); IR (KBr): ν_{max} 2938, 2856, 1732, 1627, 1432, 1385, 1288, 1022, 918 cm^{-1}; ^{1}H and ^{13}C NMR see Table 2; HR-ESI-MS *m/z* 425.2667 (calcd for $C_{25}H_{38}O_4Na$, 425.2668).

Cytotoxicity Assay. To measure the cytotoxicity of natural product against P-388 (mouse lymphocytic leukemia), HT-29 (human colon adenocarcinoma), and A-549 (human lung epithelial carcinoma), each cell line was initiated at 1500, 750, and 750 cells/well, respectively, in 96-well microplates. Three to eight concentrations encompassing an 8- to 128-fold range were on each cell line. P-388, A-549, and HT-29 cells were enumerated using MTT after the exposure to test samples for 3, 6, and 6 days, respectively. Fifty μL of 1 mg mL^{-1} MTT were added to each well, and plates were incubated at 37 °C for 5 h. Supernatant was aspirated. Formazan crystals were redissolved in DMSO for 10 min with shaking, and the plate was read immediately on a microplate reader at a wavelength of 540 nm.[8]

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Supporting Information

HSQC spectra of compounds **1**–**4**. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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