New 3β,5α,6β-Trihydroxysteroids from the Octocorals *Bebryce* sp. (Plexauridae) and *Carijoa* sp. (Clavulariidae)

Ping-Jyun SUNG*,[a,b,c] and Chih-Yang LIU[a,b]

[a] *Graduate Institute of Marine Biotechnology, Department of Life Science and Graduate Institute of Biotechnology, National Dong Hwa University; Checheng, Pingtung 944, Taiwan:* [b] *National Museum of Marine Biology and Aquarium; Checheng, Pingtung 944, Taiwan: and* [c] *Department of Marine Biotechnology and Resources, Asia-Pacific Ocean Research Center, National Sun Yat-sen University; Kaohsiung 804, Taiwan.*

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A new 3β,5α,6β-trihydroxysteroid, bebryceoid A (1), has been isolated from an octocoral *Bebryce* sp. In addition, an octocoral *Carijoa* sp. yielded two new 3β,5α,6β-trihydroxysteroids, carijoids A (2) and B (3). The structures of steroids 1—3 were elucidated by spectroscopic methods and by comparison of the spectral data with those of known steroid analogues.

Key words *Bebryce*; bebryceoid; *Carijoa*; carijoid; steroid

Previous studies on the octocorals belonging to the genus *Bebryce* (Cnidaria: Anthozoa: Octocorallia: Alcyonacea: Plexauridae) and *Carijoa* (=*Telesto*) (Cnidaria: Anthozoa: Octocorallia: Alcyonacea: Clavulariidae), have yielded a series of interesting secondary metabolites including guaiane,[1] prostanoid,[2] amide,[3] and steroid derivatives,[3—6] and most of these metabolites were proven to possess various bioactivity. In our studies on the chemical constituents of marine invertebrates collected off Taiwan waters, three new 3β,5α,6β-trihydroxysteroids, bebryceoid A (**1**) and carijoids A (**2**), B (**3**), have been isolated from the octocorals identified as *Bebryce* sp. and *Carijoa* sp., respectively. The structures of steroids **1**—**3** were established by spectroscopic methods and by comparison of spectral data with those of known steroid analogues. In this paper, we describe the isolation, structure determination, and bioactivity of steroids **1**—**3**.

Results and Discussion

Bebryceoid A from *Bebryce* sp. Bebryceoid A (**1**) was isolated as a white powder. The molecular formula of **1** was established as $C_{26}H_{44}O_3$ (five degrees of unsaturation) from a sodiated molecule at *m/z* 427 in the electronspray ionization (ESI)-MS spectrum and supported by high resolution (HR)-ESI-MS (*m/z* 427.3189, Calcd 427.3188, $[C_{26}H_{44}O_3Na]^+$). The IR spectrum contained a broad absorption indicative of hydroxy groups (3390 cm^{-1}). The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra of **1** showed that this compound has 26 carbons (Table 1), including five methyls, eight sp^3 methylenes, eight sp^3 methines, three sp^3 quaternary carbons, and two sp^2 methines. From the ^{13}C- and ^{1}H-NMR spectra (Tables 1, 2), **1** was found to possess a disubstituted olefin (δ_C 134.9, d, CH-23; 133.6, d, CH-22; δ_H 5.26, 1H, dd, *J*=15.2, 6.4 Hz, H-23; 5.16, 1H, dd, *J*=15.2, 7.6 Hz, H-22). From the above data, a degree of unsaturation was accounted for, and the remaining four degrees of unsaturation were assigned to the four rings of a steroid skeleton. The ^{1}H-NMR spectrum of **1** in $CDCl_3$ exhibited various peaks characteristic of a 3β,5α,6β-trihydroxysterol moiety,[3,4,7,8] a commom feature in marine polyhydroxysterols.[9] These mainly include two methyl singlets at δ_H 0.69 (3H, s, H_3-18) and 1.18 (3H, s, H_3-19), three methyl doublets at δ_H 0.99 (3H, d, *J*=6.8 Hz, H_3-21) and 0.94 (2×3H, d, *J*=6.8 Hz, H_3-25, H_3-26), a broad oxygen-bearing methine proton multiplet at δ_H 4.10 (1H, m, H-3), and a broad oxymethine proton singlet at δ_H 3.54 (1H, br s, H-6).

From the ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum of **1**, several different structural units, including C-1/-2/-3/-4, C-6/-7/-8/-9/-11/-12, C-8/-14/-15/-16/-17/-20/-22/-23/-24/-25(-26), and C-20/-21, were identified (Fig. 1), which were assembled with the assistance of a heteronuclear

Table 1. ^{13}C-NMR Data for Steroids **1**—**3**[a]

Carbons	1	2	3
1	32.4 (t)[b]	32.4 (t)	33.0 (t)
2	30.8 (t)	30.8 (t)	30.8 (t)
3	67.6 (d)	67.6 (d)	67.7 (d)
4	40.7 (t)	40.8 (t)	39.4 (t)
5	76.1 (s)	76.1 (s)	76.0 (s)
6	76.1 (d)	76.1 (d)	73.7 (d)
7	34.5 (t)	34.5 (t)	117.6 (d)
8	30.2 (d)	30.2 (d)	144.0 (s)
9	45.9 (d)	45.9 (d)	43.5 (d)
10	38.3 (s)	38.3 (s)	37.1 (s)
11	21.1 (t)	21.1 (t)	22.0 (t)
12	39.9 (t)	39.8 (t)	39.3 (t)
13	42.6 (s)	42.7 (s)	43.9 (s)
14	56.0 (d)	55.8 (d)	54.7 (d)
15	24.1 (t)	24.1 (t)	22.9 (t)
16	28.4 (t)	28.6 (t)	27.8 (t)
17	56.0 (d)	56.0 (d)	56.1 (d)
18	12.4 (q)	12.3 (q)	12.1 (q)
19	16.9 (q)	16.9 (q)	18.8 (q)
20	39.8 (d)	40.1 (d)	36.0 (d)
21	20.7 (q)	20.7 (q)	18.8 (q)
22	133.6 (d)	139.3 (d)	35.9 (t)
23	134.9 (d)	124.4 (d)	23.3 (t)
		124.5	
24	32.4 (d)	36.3 (t)	33.7 (t)
		36.4	33.9
25	22.8 (q)	32.9 (d)	32.5 (d)
			32.6
26	22.8 (q)	68.9 (t)	69.4 (t)
		69.0	69.6
27		16.6 (q)	16.8 (q)
26-OAc		171.3 (s)	171.3 (s)
		21.0 (q)	21.0 (q)

a) Spectra measured at 100 MHz in $CDCl_3$ at 25 °C. *b*) Attached protons were deduced by DEPT and HMQC spectra.

* To whom correspondence should be addressed. e-mail: pjsung@nmmba.gov.tw

Table 2. Selective Key ^{1}H-NMR Data for Steroids **1**—**3**[a)]

Protons	1	2	3
3	4.10 m	4.10 m	4.08 m
6	3.54 br s	3.54 br s	3.62 br s
7	—	—	5.36 m
18	0.69 s	0.69 s	0.59 s
19	1.18 s	1.18 s	1.08 s
21	0.99 d (6.8)[b)]	1.00 d (6.4)	0.93 d (7.2)
22	5.16 dd (15.2, 7.6)	5.26 br s[c)]	—
23	5.26 dd (15.2, 6.4)	5.25 br s[c)]	—
25	0.94 d (6.8)	—	—
26a	0.94 d (6.8)	3.83 dd (10.8, 2.0)	3.83 dd (10.4, 2.0)
		3.86 dd (10.8, 2.0)	3.85 dd (10.4, 2.0)
26b		3.93 dd (10.8, 6.0)	3.94 dd (10.4, 6.0)
		3.95 dd (10.8, 6.0)	3.97 dd (10.4, 6.0)
27		0.90 d (6.8)	0.92 d (6.4)
		0.90 d (6.8)	0.93 d (6.4)
Acetate methyl		2.06 s	2.06 s

a) Spectra measured at 400 MHz in $CDCl_3$ at 25 °C. *b*) *J* values (in hertz) in parentheses. *c*) Signals overlapped.

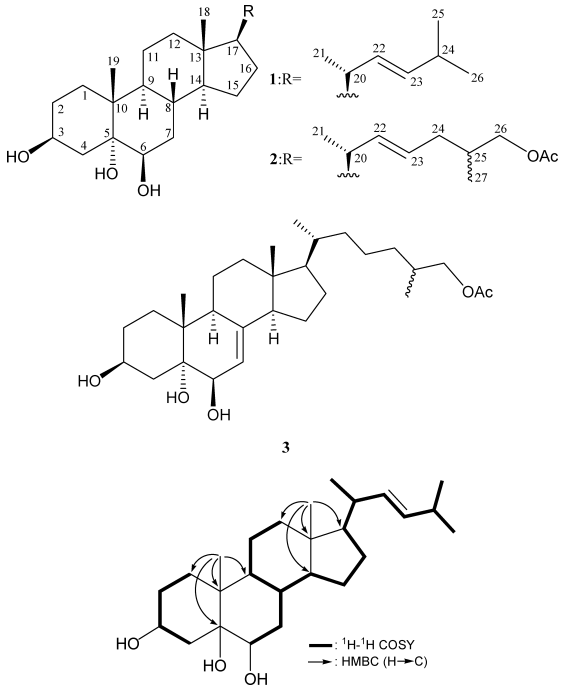

Fig. 1. ^{1}H–^{1}H COSY and Selective HMBC Correlations of **1**

multiple bond coherence (HMBC) experiment permitted elucidation of the carbon skeleton of **1**. The ring junctions C-18 and C-19 methyl groups were positioned at C-13 and C-10 from the HMBC correlations between H_3-18/C-12, -13, -14, -17 and H_3-19/C-1, -5, -9, -10, respectively (Fig. 1). The oxymethine units at δ_C 67.6 and 76.1 correlated to the methine protons at δ_H 4.10 and 3.54 in the heteronuclear multiple quantum coherence (HMQC) spectrum, proving the attachments of hydroxy groups at C-3 and C-6, respectively. The remaining hydroxy group at C-5, as indicated by analysis of HMBC correlations and characteristic NMR signals analysis. It was found that the structure of **1** was very similar with those of a known steroid, (22*E*)-5α-cholest-22-ene-3β,5,6β-triol,[7)] excepting that less one sp^3 methylene unit was found in **1** (nine sp^3 methylene units were found in (22*E*)-5α-cholest-22-ene-3β,5,6β-triol) and this observation

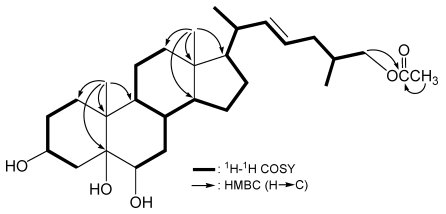

Fig. 2. ^{1}H–^{1}H COSY and Selective HMBC Correlations of **2**

was supported by comparing the spectral data of **1** with those of another known steroid, halistanol sulfate E,[10)] which possesses the same side chain (from C-20 to C-26) as that of **1**. Based on the above findings, the structure of **1** was established.

In the biological activity testing, bebryceoid A (**1**) exhibited weak cytotoxicity toward the DLD-1 (human colon adenocarcinoma, ED_{50}=7.2 μg/ml) and P388D1 (murine macrophage cells, ED_{50}=18.5 μg/ml) tumor cells, and this compound was not active toward the CCRF-CEM (human T-cell acute lymphoblastic leukemia) and HL-60 (human premyelocytic leukemia) tumor cells (ED_{50}>40 μg/ml).

Carijoids from *Carijoa* sp. Carijoid A (**2**) was obtained as a white powder. The HR-ESI-MS of **2** revealed a sodiated molecular ion peak at *m*/*z* 499.3396 $(M+Na)^+$ consistent with the molecular formula $C_{29}H_{48}O_5$ (Calcd for $C_{29}H_{48}O_5Na$, 499.3399) and six degrees of unsaturation. The IR spectrum of **2** showed bands at 3408 and 1742 cm^{-1}, consistent with the presence of hydroxy and ester groups. From the ^{13}C-NMR data of **2** (Table 1), the presence of a disubstituted olefin was deduced from the signals of two carbons at δ_C 139.3 (d, CH-22) and 124.4 (d, CH-23) and two olefin proton signals at δ_H 5.26 (1H, br s, H-22) and 5.25 (1H, br s, H-23) in the ^{1}H-NMR spectrum of **2** (Table 2). In the ^{1}H- and ^{13}C-NMR spectra, an acetoxy group was observed (δ_H 2.06, 3H, s; δ_C 171.3, s, 21.0, q). Thus, from the above NMR data, two degrees of unsaturation were accounted for, and **2** was identified as a tetracyclic compound.

From the ^{1}H–^{1}H COSY spectrum of **2** (Fig. 2), it was possible to establish the separate spin systems that map out the proton sequences from H_2-1/H_2-2/H-3/H_2-4, H-6/H_2-7/H-8/H-9/H_2-11/H_2-12, H-8/H-14/H_2-15/H_2-16/H-17/H-20/H-22/H-23/H_2-24/H-25/H_2-26(H_3-27), and H-20/H_3-21. Based on these data and HMBC correlations, the carbon skeleton of **2** could be established. The C-18 and C-19 methyl groups positioned at C-13 and C-10 from the HMBC correlations between H_3-18/C-12, -13, -14, -17 and H_3-19/C-1, -5, -9, -10 (Fig. 2). It was found that the structure of **2** was very similar with that of a known metabolite, 25ξ-cholestane-3β,5α,6β-26-tetrol-26-acetate,[3,4)] excepting that the signals corresponding to a disubstituted olefin between C-22/23 was found in **2**. However, the doubling of the 26-acetoxymethylene and H_3-27 signals indicate that **2** consists of a stereoisomeric mixture (25*R*/25*S*).

The trihydroxysteroid **3** (carijoid B) had the same molecular formula $C_{29}H_{48}O_5$ as that of **2** on the basis of HR-ESI-MS (see Experimental). By detailed analysis, it was found that the spectral data of **3**, including 1D- and 2D-NMR data, were similar to those of **2** and the known steroid 25ξ-cholestane-3β,5α,6β-26-tetrol-26-acetate,[3,4)] excepting that the signals corresponding to a trisubstituted olefin between C-7/8 was

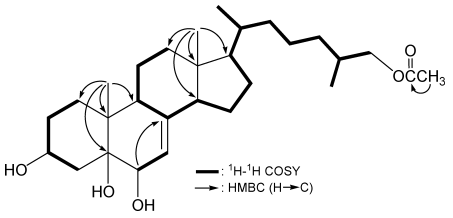

Fig. 3. ^{1}H–^{1}H COSY and Selective HMBC Correlations of **3**

found in **3**. Like as those of **2**, the doubling of the 26-acetoxymethylene and H_3-27 signals indicate that **3** consists of a stereoisomeric mixture (25*R*/25*S*).

Carijoids A (**2**) and B (**3**) were not active toward the DLD-1, P388D1, CCRF-CEM, and HL-60 tumor cells (ED_{50}>40 μg/ml). Due to the screening platforms are limited; and lots of material were consumed in physical and spectral experiments. The other possible biological activities for these interesting substances will not be assayed at this stage. The extensive assay platforms for the natural products will be set up by the National Science and Technology Program for Biotechnology and Pharmaceuticals (NSTPBP), Taiwan. The possible bioactivity for these compounds will be studied if we can get enough material in the future.

Experimental

General Melting points were measured on a FARGO apparatus and were uncorrected. Optical rotation values were measured with a JASCO P-1010 digital polarimeter. IR spectra were obtained on a VARIAN DIGLAB FTS 1000 FT-IR spectrophotometer. NMR spectra were recorded on a VARIAN MERCURY PLUS 400 FT-NMR at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR, respectively, in $CDCl_3$. Proton chemical shifts were referenced to the residual $CHCl_3$ signal (δ_H 7.26 ppm). ^{13}C-NMR spectra were referenced to the center peak of $CDCl_3$ at δ_C 77.1 ppm. ESI-MS and HR-ESI-MS data were recorded on a BRUKER APEX II mass spectrometer. Gravity column chromatography was performed on silica gel (230—400 mesh, Merck, Darmstadt, Germany). TLC was carried out on precoated Kieselgel 60 F_{254} (0.2 mm, Merck) and spots were visualized by spraying with 10% H_2SO_4 solution followed by heating. HPLC was performed using a system comprised of a HITACHI L-7100 pump, a HITACHI L-7455 photodiode array detector, and a RHEODYNE 7725 injection port. A semi-preparative reverse phase column (Hibar 250—10 mm, Purospher Star RP-18e, 5 μm) and a semi-preparative normal phase column (Hibar 250—25 mm, LiChrospher Si 60, 5 μm) were used for HPLC.

Animal Material Specimen of the octocorals *Bebryce* sp. and *Carijoa* sp. were collected off the coast of Pingtung, southern Taiwan, in December, 2008 and August 2008, respectively, and these two organisms were identified by comparison with previous descriptions.[11] The voucher specimens were deposited in the National Museum of Marine Biology and Aquarium, Taiwan.

Extraction and Isolation 1) *Bebryce* sp. The freeze-dried and minced material of *Bebryce* sp. (weight 666 g, dry weight 240 g) was extracted with a mixture of MeOH and CH_2Cl_2 (1 : 1) at room temperature. The extract was partitioned between EtOAc and H_2O. The EtOAc layer was separated by silica gel and eluted using hexane/EtOAc (stepwise, 20 : 1–pure EtOAc) to yield 30 fractions. Fraction 17 was separated by silica gel and eluted using hexane/acetone (stepwise, 10 : 1—1 : 1) to afford 15 fractions, 17A—17O. Fraction 17N was repurified with reverse phase HPLC, using a mixture of H_2O and CH_3CN to afford **1** (1.1 mg, 10/90).

Bebryceoid A (**1**): White powder; mp 210—212 °C; $[\alpha]_D^{23}$ −15° (c=0.06, $CHCl_3$); IR (neat) ν_{max} 3390, 2927, 2854, 1457, 1375 cm^{-1}; ^{13}C-NMR ($CDCl_3$, 100 MHz) and ^{1}H-NMR ($CDCl_3$, 400 MHz) data see Tables 1 and 2; ESI-MS m/z 427 $(M+Na)^+$; HR-ESI-MS m/z 427.3189 (Calcd for $C_{26}H_{44}O_3Na$, 427.3188).

2) *Carijoa* sp. The freeze-dried and minced material of *Carijoa* sp. (wet weight 1588 g, dry weight 422 g) was extracted with a mixture of MeOH and CH_2Cl_2 (1 : 1). The extract was partitioned between EtOAc and H_2O. The EtOAc layer was separated by silica gel and eluted using hexane/EtOAc (stepwise, 20 : 1–pure EtOAc) to yield 35 fractions. Fractions 19 and 20 were combined and separated by silica gel and eluted using hexane/acetone (stepwise, 15 : 1—1 : 1) to afford 15 fractions, 19A—19O. Fraction 19N was repurified with normal phase HPLC, using hexane/acetone (stepwise, 10 : 1—1 : 1) to afford **3** (0.6 mg, 2 : 1) and the other 31 fractions. A fraction from 19N (19N21) was further separated by reverse phase HPLC, using a mixture of MeOH and H_2O to afford **2** (1.1 mg, 85 : 15).

Carijoid A (**2**): White powder; mp 105—106 °C; $[\alpha]_D^{24}$ −18° (c=0.06, $CHCl_3$); IR (neat) ν_{max} 3408, 2935, 2866, 1742, 1458, 1375, 1242, 1039 cm^{-1}; ^{13}C-NMR ($CDCl_3$, 100 MHz) and ^{1}H-NMR ($CDCl_3$, 400 MHz) data see Tables 1 and 2; ESI-MS m/z 499 $(M+Na)^+$; HR-ESI-MS m/z 499.3396 (Calcd for $C_{29}H_{48}O_5Na$, 499.3399).

Carijoid B (**3**): White powder; mp 151—153 °C (decomp.); $[\alpha]_D^{22}$ +1° (c=0.03, $CHCl_3$); IR (neat) ν_{max} 3416, 2930, 2868, 1738, 1659, 1458, 1378, 1240, 1033 cm^{-1}; ^{13}C-NMR ($CDCl_3$, 100 MHz) and ^{1}H-NMR ($CDCl_3$, 400 MHz) data see Tables 1 and 2; ESI-MS m/z 499 $(M+Na)^+$; HR-ESI-MS m/z 499.3401 (Calcd for $C_{29}H_{48}O_5Na$, 499.3399).

Cytotoxicity Testing The cytotoxicity of tested compounds was assayed with a modification of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method. Cytotoxicity assays were carried out according to the procedures described previously.[12,13]

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