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CYTOTOXIC CHOLIC ACID TYPE STERONES FROM A MARINE SOFT CORAL *Paraminabea* sp.

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*Chemical investigation of an Okinawan marine soft coral resulted in the isolation of one new compound (**1**) and five known cholic acid type keto-steroids **2–6** possessing enone or dienone A-rings and desmosterol (**7**). The structures of all compounds were elucidated from spectral data, mainly by 1D and 2D NMR techniques, mass spectra, comparison of NMR data with those of reported compounds, and by chemical transformations. Compounds **1–7** showed potent cytotoxic activity.*

Keywords: cholic acid, 2D NMR, soft coral, cytotoxicity, NBT-T2 cell.

Marine organisms produce steroidal components with a variety of structures and biological activities. Steroidal carboxylic acids and derivatives have been detected in marine organisms more frequently than terrestrial organisms. The occurrence of bioactive steroidal carboxylic acid derivatives, which were sometimes oxygenated at various sides, has been shown in nudibranch, starfish, sponge, octocoral, scleractinian coral and, specially, soft coral [1–8]. Some cytotoxic sterols have also been reported from marine alga [9]. In the course of our studies on bioactive metabolites of marine organisms, we isolated one new methyl ester **1** and six known steroidal carboxylic acids **2–7**, including desmosterol (**7**), from a marine soft coral collected in Okinawa. We describe herein the isolation and structure elucidation of new compound with 1D and complete 2D NMR characterization.

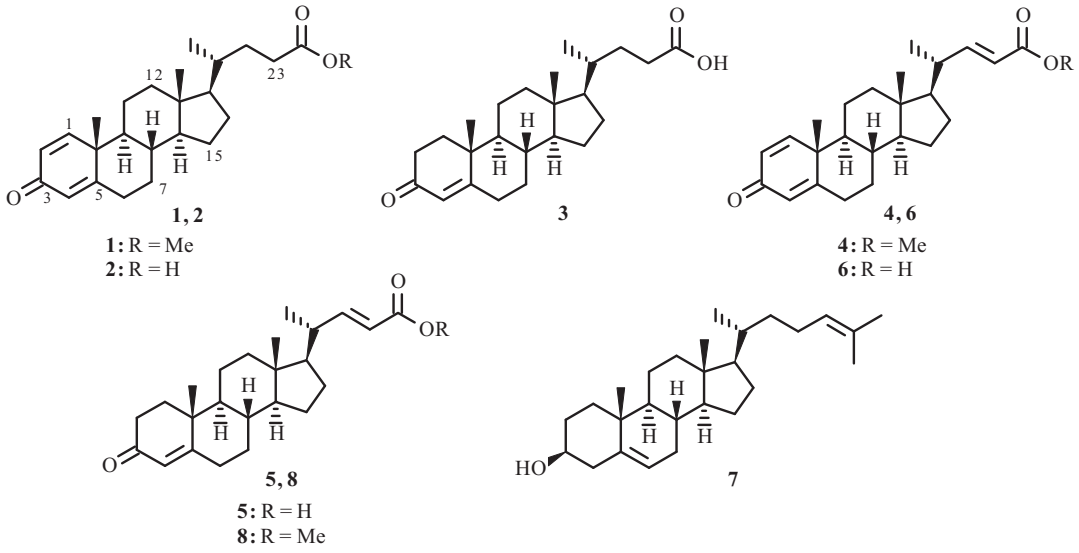

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TABLE 1. ^{1}H and ^{13}C NMR Data of Compound **1** in $CDCl_3$

C atom	δ_C	δ_H, J/Hz	C atom	δ_C	δ_H, J/Hz
1	156.0 d	7.05 (d, J = 10.0)	14	55.4 d	1.05 m
2	127.4 d	6.23 (dd, J = 10.0, 2.0)	15	24.3 t	1.15 m, 1.62 m
3	186.5 s		16	28.0 t	1.28 m, 1.70 m
4	123.8 d	6.07 br.s	17	55.7 d	1.26 m
5	169.4 s		18	12.0 q	0.74 s
6	32.9 t	2.30 m, 2.47 m	19	18.7 q	1.23 s
7	33.6 t	1.02 m, 1.81 m	20	35.3 d	2.34 m
8	35.5 d	2.03 m	21	19.2 q	0.92 (d, J = 6.6)
9	52.3 d	1.01 m	22	31.0 t	1.03 m, 1.30 m
10	43.6 s		23	30. 9 t	1.16 m, 1.33 m
11	22.8 t	1.43 m, 1.55 m	24	171.1 s	
12	39.4 t	1.22 m, 2.02 m	OMe	51.5 q	3.66 s
13	42.8 s				

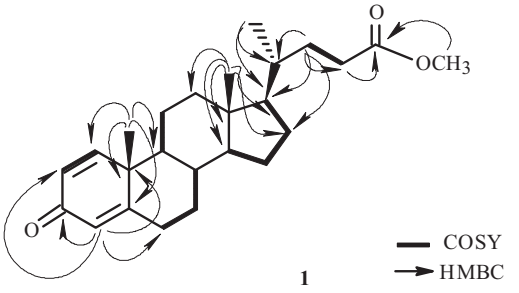

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Fig. 1. Key COSY and HMBC correlations observed for **1**.

As part of our investigation on biologically active metabolites from marine invertebrates, we performed a cytotoxic screening assay on 24 crude extracts (both EtOAc and methanolic extracts) of Okinawan marine specimens. The crude EtOAc extract of a soft coral, *Paraminabea* sp., displayed significant cytotoxic activity (1 μg/mL). On the basis of the biological activity, subsequent chemical investigation was carried out on this extract. Repetitive normal phase HPLC separation of the active fractions afforded one new bioactive methyl ester and six bioactive known compounds. The structures of all known compounds were assigned on the basis of their spectroscopic data and comparison with those of the reported compounds [2–9]. This is the first instance of isolating all the compounds from the same species.

Compound **1** had the molecular formula $C_{25}H_{36}O_3$ as established by mass and NMR spectral data. The ^{1}H NMR spectrum of **1** revealed two tertiary methyl signals (δ 0.74 s, 1.23 s) and one secondary methyl signal (δ 0.92 d, J = 6.6 Hz) ascribable to methyl groups of a steroidal compound. The presence of one tertiary methyl (δ 3.66 s), indicative of the ester functionality, and three olefinic protons (δ 6.07 br.s, 6.23 dd, J = 10.0, 2.0 Hz, and 7.05 d, J = 10.0 Hz) were also revealed from the ^{1}H NMR spectrum. The 1D NMR data could account for four of the eight degrees of unsaturation, suggesting the tetracyclic nature of **1**. The presence of an α,β-unsaturated carbonyl group was evident from ^{1}H and ^{13}C NMR signals (Table 1) at δ 7.05 d, 6.23 dd; δ 156.0, 127.4, 186.5 as well as from the IR absorption at 1664 cm^{-1}. The structure of **1** was elucidated on the basis of 2D NMR (COSY, HMQC, and HMBC) techniques. Rings A and B were elucidated on the basis of HMBC cross peaks between H-19 (1.23 s)/C-1, C-5, C-9, C-10 and H-4 (6.07 br.s)/C-3, C-2, C-6, C-10, whereas rings C and D were completed on the basis of HMBC correlations between H-18 (0.74 s)/C-12, C-13, C-14, C-17 (Fig. 1). The methyl ester was linked to C-23 by the HMBC correlations from H-23 to C-24 of the ester carbonyl at δ 171.1 and from methyl signal at δ 3.66 s to the ester carbonyl at δ 171.1. Comparison of ^{1}H and ^{13}C chemical shifts of **1** with those of reported compounds [2–5] inferred the normal relative stereochemistry of the ring junctures and stereo centers of **1**. Thus, compound **1** was elucidated as methyl-3-oxochola-1,4-dien-24-oate. Compound **1** was also prepared by treatment of **2** with trimethylsilyldiazomethane ($TMSCH=N_2$) which showed identical NMR data and chromatographic behavior as an isolated one. This transformation further confirmed the structure and stereochemistry of **1**.

To confirm the structure of compound **5** (due to lack of detailed NMR data) [2], it was also treated with trimethylsilyldiazomethane (TMSCH=N_2) to give **8**, which showed identical NMR data with those reported in [3], confirming the structure and stereochemistry of **5**. Similarly, compound **6** gave a product **4** which showed identical NMR data with those reported, confirming the structures and stereochemistries of **4** and **6** [5].

All compounds were evaluated for cytotoxicity using NBT-T2 rat bladder epithelial cells. The IC_{50} values for **1–7** are 6.7, 3.1, 17, 5.6, 3.2, 2.1, and 12 μg/mL, respectively.

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured on a Jasco P-1010 polarimeter. The ^1H and ^{13}C and all 2D NMR spectra were recorded on a Jeol A500 NMR spectrometer using TMS as internal standard. Carbon multiplicities were determined using DEPT-135 and DEPT-90 sequences. Atom connectivities were determined using HMQC, HMBC, and COSY data. The chemical shifts are given in δ (ppm) and coupling constants in Hz. MS and IR spectra were obtained on a LTQ Orbitrap mass spectrometer and on a Varian FTIR FTS3000 spectrometer. HPLC was performed on a Hitachi L-6000 pump equipped with a Shodex RI-101 monitor and a Hitachi L-4000 UV detector using a Mightysil Si 60 column or a Develosil Si 60 column. Merck silica gel was used for vacuum flash chromatography and normal phase column chromatography. All solvents used were reagent grade. A Tecan sunrise microplate reader was used for cytotoxicity testing.

Animal Material. A specimen of the soft coral *Paraminabea* sp. was collected with Scuba diving at a depth of 40–45 m at the island of Okinawa. They were frozen immediately after collection and were kept frozen until extraction.

Extraction and Isolation. The soft coral (22 g, wet) was cut into pieces and extracted by steeping in acetone (300 mL) for one day, and the extraction process was repeated three times. The combined extracts were concentrated under reduced pressure, and the residue was partitioned between EtOAc and water. The organic extract (133 mg) was separated by vacuum flash chromatography on silica gel to give four fractions. The third fraction (101 mg) was further separated on HPLC (silica, *n*-hexane–EtOAc, 2:3) to give ten subfractions. The ninth subfraction (5.0 mg) was subjected to repetitive separation on HPLC (silica, *n*-hexane–EtOAc, 2:1) to give new compound **1** (1.2 mg). Similarly, the seventh subfraction (8.0 mg) yielded the known compounds **2** (1.7 mg), **3** (1.6 mg), and **4** (0.9 mg). The eighth subfraction (16.8 mg) gave the known compound **5** (6.5 mg), the tenth subfraction (7.4 mg) gave the known compound **6** (5.4 mg), and the fifth subfraction (20.3 mg) gave the known compound desmosterol (**7**, 3.5 mg).

Preparation of 1 from 2. Compound **2** (0.8 mg) was dissolved in 100 μL of MeOH, and a few drops of trimethylsilyldiazomethane (TMSCH=N_2) solution was added. After standing half an hour, the solvent was removed by nitrogen flush. The product showed identical ^1H NMR data and chromatographic behavior as natural **1**.

Synthesis of 8 from 5. Compound **5** (1.3 mg) was dissolved in 200 μL of MeOH, and a few drops of trimethylsilyldiazomethane (TMSCH=N_2) solution was added. After standing half an hour, the solvent was removed by nitrogen flush. The product **8** showed identical ^1H NMR data with those of the reported compound [3].

Methylation of 6. Compound **6** (1.2 mg) was dissolved in 200 μL of MeOH, and a few drops of trimethylsilyldiazomethane (TMSCH=N_2) solution was added. After standing half an hour, the solvent was removed by nitrogen flush. The product showed identical ^1H NMR data and chromatographic behavior as **4** [5].

Compound 1 (methyl-3-oxochola-1,4-dien-24-oate). Colorless solid, $[\alpha]_D^{26}$ +52° (*c* 0.09, CH_2Cl_2); IR (neat) 2939, 1736, 1664, 1242 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HR-MS *m/z* 385.3715 $[M + H]^+$ (calcd for $C_{25}H_{37}O_3$ 385.3712).

Cytotoxicity Assay. NBT-T2 cells (BRC-1370) were purchased from Riken Bioresource Center and cultured under a standard protocol using Dulbecco′s modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and antimicrobials. Cultured cells were inoculated into each well (96-well plate) with 200 μL of the medium. After preincubation (24 h, 37°C, 5% CO_2), aliquots of the test compounds in MeOH were added to culture wells in duplicate. After incubating the sample wells for 2 days (24 h, 37°C, 5% CO_2), the toxic effect of the compounds was observed under a microscope. The IC_{50} values were measured by the MTT colorimetric method. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (15 μL, 5 mg/mL in PBS) was added to each well after removal of the medium by aspiration and incubated for 3 h. The residual formazan was dissolved in 100 μL of dimethyl sulfoxide (DMSO). The absorbance was measured at 560 nm with a Tecan sunrise microplate reader. The IC_{50} values were estimated by plotting the absorbance values against concentrations [10].

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