Antifouling Polyhydroxysterols Isolated from a Palauan Octocoral of *Sinularia* sp.

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In our continuing search for effective antifoulants from marine invertebrates which can be used as substitutes for effective but highly toxic agents such as organotin compounds, we found that the lipophilic fraction from the extract of a Palauan octocoral of *Sinularia* sp. inhibited settlement of the blue mussel *Mytilus edulis*. Bioassay-guided isolation afforded five polyhydroxylated sterols with or without a monoacetyl functional group such as 6 β -acetoxy-24-methylenecholestane-3 β ,5 α -diol. The inhibitory activity of one of these compounds against the settlement of the blue mussel was found to be 34% of that of CuSO₄, a standard antifouling agent. The activity of all five compounds was approximately 2 to 5 times stronger than that of cholesterol, β -cholestanol and β -sitosterol, suggesting the importance of the hydroxyl and/or acetoxy groups for antifouling activity.

Key words: *Mytilus edulis*, antifoulants, 6 β -acetoxy-24-methylenecholestane-3 β ,5 α -diol, trihydroxylated sterols, octocoral, *Sinularia* sp.

Marine fouling organisms such as barnacles and mussels cause serious problems to ship hulls, cooling systems of power plants, aquaculture systems, and other man-made submersible structures. Organotin compounds such as TBTO [bis-(*n*-tributyltin) oxide] are currently used as antifoulants and are effective for preventing the settlement of sessile invertebrates. However, their toxic effects on fishes and shellfishes have caused serious concern, therefore non- or less-toxic substitutes for these organometallic chemicals are urgently required. We have been researching chemical signals, such as repellents and attractants, among marine invertebrates, and isolated several antifoulants from marine invertebrates.¹⁻⁵⁾ The bioassay methods used for assessing their settlement inhibitory activities against fouling invertebrates have been applied to the blue mussel *Mytilus edulis*,⁶⁾ and cyprid larvae of the reared barnacle *Balanus amphitrite*.⁷⁾ Among the isolated antifoulants, common sterols such as cholesterol, β -cholestanol and β -sitosterol showed weak inhibitory activity against the settlement of *M. edulis*.¹⁾ In order to clarify the antifouling mechanism of sterols, we started to search for steroidal antifoulants from marine invertebrates. During this study, we were able to isolate several polar sterols containing an acetoxy group from a marine octocoral *Sinularia* sp.

In this report, we describe the isolation, identification and inhibitory activity of these compounds.

Materials and Methods

General Procedure

¹H- and ¹³C-NMR spectra were recorded on a Varian Unity 500 NMR Spectrometer in CDCl₃. Optical rotations

were determined with a JASCO DIP-140 digital polarimeter at 24°C, in CHCl₃. Electron impact mass spectra (EIMS) and high-resolution electron impact mass spectra (HREIMS) were obtained with a JEOL JMS-SX102 mass spectrometer. Thin layer chromatography (TLC) was performed on silica gel 60 pre-coated plates (Merck) and spots were visualized by spraying with a 2% ceric sulfate solution in 5% sulfuric acid and then heating on a hot plate.

The bioassay was carried out according to the method of Ina *et al.*,⁶⁾ using the blue mussel *M. edulis*.

Preparation of Extracts of Marine Invertebrates for the Bioassay

Marine invertebrates, mainly sponges and octocorals, were collected around the palau Islands between October and November in 1991 by scuba diving during the cruise of the R/V Sogen Maru. Approximately 100 specimens were collected, stored in a freezer, and transported to our laboratory. Each frozen specimen (100 g) was soaked in acetone (100 ml); the acetone extract was concentrated under reduced pressure, and partitioned between ethyl acetate and distilled water. Only the organic layer was subjected to the mussel assay.

Isolation of the Active Compounds from Palauan *Sinularia* sp.

Among one hundred invertebrate samples tested, the lipophilic layer from the extract of an octocoral of *Sinularia* sp. inhibited the settlement of *M. edulis*. Colonies of the soft coral were extracted with acetone whose extract gave the residue (650 g). This was partitioned between CH₂Cl₂

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Fig. 1. Structures of antifouling steroids isolated from a Palauan octocoral *Sinularia* sp.

and H_2O . The organic layer (6 g) was chromatographed on a silica gel column (Silica gel 60, 230–400 mesh, Merck) with $\text{CHCl}_3/\text{MeOH}$ systems. Fraction I (500 mg) eluted with CHCl_3 was gelfiltered on a Sephadex LH-20 with CHCl_3 to give a colorless powder, which was then subjected to HPLC on an ODS column (Cosmosil 5C₁₈-AR, 250 × 4 mm, Nacalai tesque) using 90% aqueous methanol to afford compounds **1** (34.3 mg), **2** (3.5 mg), **3** (8.4 mg) and **4** (19.1 mg). Fraction II (73 mg) eluted with $\text{CHCl}_3/\text{MeOH}$ (10:1) was crystallized from methanol to give compound **5** (29 mg).

Results and Discussion

Structural Elucidation of the Active Compounds

The physico-chemical and spectroscopic data for compounds **1** to **5** were as follows:

Compound **1**. Mp 104.5–105.5°C ($\text{CHCl}_3/\text{CH}_3\text{OH}$); $[\alpha]_{\text{D}}^{23.3^\circ}$ (c 0.06, CHCl_3); HRMS: m/z 474.3741 ($\text{C}_{30}\text{H}_{50}\text{O}_4$, calcd. for 474.3709); MS: m/z 474 (M^+ , 27%), 456 ($\text{M}^+ - 18$, 12), 414 ($\text{M}^+ - \text{AcOH}$, 43), 43 (00); $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ : 0.69 (18- CH_3 , s), 0.94 (21- CH_3 , d, $J = 6.8$ Hz), 1.020, 1.022 (26, 27- CH_3 , each d, $J = 7.0$ Hz), 1.15 (19- CH_3 , s), 2.06 (CH_3CO , s), 2.22 (25-H, septet, $J = 7.0$ Hz), 4.08 (3- CH_α , m), 4.66, 4.72 ($=\text{CH}_2$, each 1H, each s),

4.69 (6- CH_α , brs); $^{13}\text{C-NMR}$ (125 MHz, CDCl_3) δ : 12.2 (q, 18-C), 16.5 (q, 19-C), 18.7 (q, 21-C), 21.1 (t, 11-C), 21.4 (q, $\text{C}^*\text{H}_3\text{CO}$), 21.8, 22.0 (each q, 26-C, 27-C), 24.1 (t, 15-C), 28.2 (t, 16-C), 30.7 (t, 2-C), 30.8 (d, 8-C), 31.0 (t, 23-C), 31.4 (t, 7-C), 32.0 (t, 1-C), 33.8 (d, 25-C), 34.7 (t, 22-C), 35.8 (d, 20-C), 38.5 (s, 10-C), 39.9 (t, 12-C), 40.5 (t, 4-C), 42.8 (s, 13-C), 45.4 (d, 9-C), 55.9 (d, 14-C), 56.0 (d, 17-C), 67.3 (d, 3-C), 75.3 (s, 5-C), 76.1 (d, 6-C), 106.0 (t, 28-C), 156.8 (s, 24-C), 170.1 (s, $\text{CH}_3\text{C}^*\text{O}$).

Compound **2**. Mp 101.0–102.0°C ($\text{CHCl}_3/\text{CH}_3\text{OH}$); $[\alpha]_{\text{D}}^{51.3^\circ}$ (c 0.39, CHCl_3); HRMS: m/z 474.3724 ($\text{C}_{30}\text{H}_{50}\text{O}_4$, calcd. for 474.3709); MS: m/z 474 (M^+ , 20%), 456 ($\text{M}^+ - 18$, 17), 414 ($\text{M}^+ - \text{AcOH}$, 21), 69(100), 43 (85); $^1\text{H-NMR}$ (CDCl_3) δ : 0.695 (18- CH_3 , s), 0.82, 0.83 (26, 27- CH_3 , each d, $J = 7.2$ Hz), 0.91 (28- CH_3 , d, $J = 6.7$ Hz), 1.01 (21- CH_3 , d, $J = 6.7$ Hz), 1.15 (19- CH_3 , s), 2.06 (CH_3CO , s), 4.08 (3- CH_α , m), 4.70 (6- CH_α , brs), 5.14–5.24 (22-and 23- CH , 2H, m); $^{13}\text{C-NMR}$ (CDCl_3) δ : 12.4 (q, 18-C), 16.5 (q, 19-C), 17.6 (t, 28-C), 19.7, 20.0 (each q, 26-C, 27-C), 20.9 (4, 21-C), 21.1 (t, 11-C), 21.4 (q, $\text{C}^*\text{H}_3\text{CO}$), 24.1 (t, 15-C), 28.5 (t, 16-C), 30.7 (t, 2-C), 30.8 (d, 8-C), 31.4 (t, 7-C), 32.0 (t, 1-C), 33.1 (d, 25-C), 38.5 (s, 10-C), 39.8 (t, 12-C), 40.1 (d, 20-C), 40.6 (t, 4-C), 42.7 (s, 13-C), 42.8 (d, 24-C), 45.5 (d, 9-C), 56.0 (d, 14-C), 56.1 (d, 17-C), 67.3 (d, 3-C), 75.3 (s, 5-C), 76.1 (d, 6-C), 131.8 (d, 23-C), 135.7 (d, 22-C), 170.2 (s, $\text{CH}_3\text{C}^*\text{O}$).

Compound **3**. Mp 100.5–101.0°C ($\text{CHCl}_3/\text{CH}_3\text{OH}$); $[\alpha]_{\text{D}}^{32.3^\circ}$ (c 0.13, CHCl_3); HRMS: m/z 402.3494 [$\text{C}_{27}\text{H}_{46}\text{O}_2$ for ($\text{M}^+ - \text{AcOH}$), calcd. for 402.3498]; MS: m/z 462 (M^+ , 2%), 444 ($\text{M}^+ - 18$, 8), 402 ($\text{M}^+ - \text{AcOH}$, 100), 43(95); $^1\text{H-NMR}$ (CDCl_3) δ : 0.68 (18- CH_3 , s), 0.860, 0.862 (26, 27- CH_3 , each d, $J = 7.2$ Hz), 0.90 (21- CH_3 , d, $J = 6.7$ Hz), 1.14 (19- CH_3 , s), 2.06 (CH_3CO , s), 4.08 (3- CH_α , m), 4.70 (6- CH_α , brs); $^{13}\text{C-NMR}$ (CDCl_3) δ : 12.2 (q, 18-C), 16.5 (q, 19-C), 18.7 (q, 21-C), 21.1 (t, 11-C), 21.4 (q, $\text{C}^*\text{H}_3\text{CO}$), 22.6, 22.8 (each q, 26-C, 27-C), 23.9 (t, 23-C), 24.1 (t, 15-C), 28.0 (d, 25-C), 28.2 (t, 16-C), 30.6 (t, 2-C), 30.7 (d, 8-C), 31.4 (t, 7-C), 32.0 (t, 1-C), 35.8 (d, 20-C), 36.2 (t, 22-C), 38.4 (s, 10-C), 39.5 (t, 24-C), 39.9 (t, 12-C), 40.5 (t, 4-C), 42.7 (s, 13-C), 45.4 (d, 9-C), 55.9 (d, 17-C), 56.2 (d, 14-C), 67.3 (d, 3-C), 75.3 (s, 5-C), 76.1 (d, 6-C), 170.3 (s, $\text{CH}_3\text{C}^*\text{O}$).

Compound **4**. Mp 102.0–104.0°C ($\text{CHCl}_3/\text{CH}_3\text{OH}$); $[\alpha]_{\text{D}}^{26.3^\circ}$ (c 0.18, CHCl_3); HRMS: m/z 458.3778 [$\text{C}_{30}\text{H}_{50}\text{O}_3$ for ($\text{M}^+ - \text{H}_2\text{O}$), calcd. for 458.3760]; MS: m/z 476 (M^+ , 2%), 458 ($\text{M}^+ - 18$, 8), 416 ($\text{M}^+ - \text{AcOH}$, 100), 43(95); $^1\text{H-NMR}$ (CDCl_3) δ : 0.68 (18- CH_3 , s), 0.780, 0.780 (26, 27- CH_3 , each d, $J = 7.0$ Hz), 0.85 (28- CH_3 , d, $J = 6.7$ Hz), 0.91 (21- CH_3 , d, $J = 6.7$ Hz), 1.14 (19- CH_3 , s), 2.06 (CH_3CO , s), 4.08 (3- CH_α , m), 4.70 (6- CH_α , brs); $^{13}\text{C-NMR}$ (CDCl_3) δ : 12.2 (q, 18-C), 15.5 (q, 28-C), 16.5 (q, 19-C), 17.6, 20.5 (each q, 26-C, 27-C), 18.8 (q, 21-C), 21.1 (t, 11-C), 21.4 (q, $\text{C}^*\text{H}_3\text{CO}$), 24.1 (t, 15-C), 28.1 (t, 16-C), 30.6 (t, 2-C), 30.6 (t, 23-C), 30.7 (d, 8-C), 31.4 (t, 7-C), 31.5 (d, 25-C), 32.0 (t, 1-C), 33.7 (t, 22-C), 36.2 (d, 20-C), 38.5 (s, 10-C), 39.1 (d, 24-C), 39.9 (t, 12-C), 40.5 (t, 4-C), 42.7 (s, 13-C), 45.4 (d, 9-C), 55.9 (d, 17-C), 56.1 (d, 14-C), 67.3 (d, 3-C), 75.3 (s, 5-C), 76.0 (d, 6-C), 170.3 (s, $\text{CH}_3\text{C}^*\text{O}$).

Compound **5**. Mp 223–224.5°C ($\text{CHCl}_3/\text{CH}_3\text{OH}$); $[\alpha]_{\text{D}}^{1.7^\circ}$ (c 0.26, CHCl_3); HRMS: m/z 432.3601 ($\text{C}_{28}\text{H}_{48}\text{O}_3$, calcd. for 432.3604); $^1\text{H-NMR}$ (CDCl_3) δ : 0.68 (18- CH_3 , s),

0.94 (21-CH₃, *d*, *J*=6.8 Hz), 1.020, 1.022 (26, 27-CH₃, each *d*, *J*=7.0 Hz), 1.184 (19-CH₃, *s*), 2.22 (25-H, *septet*, *J*=7.0 Hz), 3.54 (6-CH_α, *brs*), 4.10 (3-CH_α, *m*), 4.66, 4.72 (=CH₂, each 1H, each *s*); ¹³C-NMR (CDCl₃) δ : 12.2 (*q*, 18-C), 16.9 (*q*, 19-C), 18.7 (*q*, 21-C), 21.2 (*t*, 11-C), 21.8, 22.0 (each *q*, 26-C, 27-C), 24.2 (*t*, 15-C), 28.2 (*t*, 16-C), 30.2 (*d*, 8-C), 30.9 (*t*, 2-C), 31.1 (*t*, 23-C), 32.4 (*t*, 1-C), 33.8 (*d*, 25-C), 34.6 (*t*, 7-C), 34.7 (*t*, 22-C), 35.8 (*d*, 20-C), 38.3 (*s*, 10-C), 40.0 (*t*, 12-C), 40.8 (*t*, 4-C), 42.8 (*s*, 13-C), 45.9 (*d*, 9-C), 56.0 (*d*, 14-C), 56.1 (*d*, 17-C), 67.7 (*d*, 3-C), 76.1 (*s*, 5-C), 76.1 (*d*, 6-C), 106.0 (*t*, 28-C), 156.8 (*s*, 24-C).

The data for compound **5** coincide well with those for 24-methylenecholestane-3 β ,5 α ,6 β -triol.⁸⁾

On comparison of the NMR data of compound **1** with those of compound **5**, they were closely related to each other, except for a signal due to an acetyl group at δ 2.06 (3H, *s*), and a low-field shift of the 6-CH_α signal δ 4.69 (1H, *brs*) in compound **1**, while the latter appeared at δ 3.54 (1H, *brs*) in compound **5**. These data indicate that compound **1** was the 6-*O*-acetyl derivative of compound **5**, *i.e.* 6 β -acetoxy-24-methylenecholestane-3 β , 5 α -diol, which is found as a novel sterol with an acetyl group.

The ¹H-NMR spectra for compounds **1**–**4** showed essentially the same chemical shifts for the 3-CH, 6-CH, 18-CH and 19-CH, indicating that they possessed the common steroidal nucleus with different side chain structures. The structures of the side chains were elucidated by comparing of the ¹H- and ¹³C-NMR data with those of known steroids.^{9–11)} The configuration of C-24 for compounds **2** and **4** was determined according to the method reported by Wright *et al.*¹¹⁾ Thus, the structures of compounds **2**, **3** and **4** were established as 6 β -acetoxy-24(*R*)-methylcholest-22-ene-3 β ,5 α -diol, 6 β -acetoxy-cholestane-3 β ,5 α -diol and 6 β -acetoxy-24(*S*)-methylcholestane-3 β ,5 α -diol, respectively. Compounds **1** and **4** were first isolated from the octocoral *Sinularia dissecta* by Bortolotto *et al.*,¹²⁾ although both compounds were shown to be a mixture under chromatographic conditions. Compound **4** was also chemically synthesized by Pruna *et al.*⁸⁾ Compounds **1** to **4** are monoacetates of trihydroxy sterols, which are rare in marine organisms.

Settlement inhibitory activities of compounds **1** to **5**

Compound **1** showed “++” activity⁶⁾ at a dose of 0.7 mg/cm² (34 Units;⁶⁾ 34% of the activity of CuSO₄, which is a standard antifoulant). Compounds **2**, **3** and **4** exhibited “+” activity⁶⁾ at a dose of 0.35 mg/cm², while **5** had “+” activity at a dose of 1.0 mg/cm². Although further examination was not possible owing to the limited samples, the acetyl group seems to play an important role in antifouling activity.

The activities of compounds **1** to **5** were approximately 2 to 5 times stronger than those of such conventional sterols

as cholesterol, β -cholestanol and β -sitosterol, which were isolated by our group as antifoulants with weak activity,¹⁾ again suggesting the importance of multiple hydroxyl and/or acetoxy groups for the antifouling activity. These steroids might act as defense substances for preventing other organisms from settling.

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