Polyhydroxy Steroids and Saponins from China Sea Starfish *Asterina pectinifera* and Their Biological Activities

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A new polyhydroxy sterol ester, (25*S*)-5α-cholestane-3β,6α,7α,8,15α,16β-hexahydroxyl-26-*O*-14′*Z*-eicosenoate (1), together with seven known steroid derivatives (2—8), were isolated from the EtOH extract of the whole body of China Sea starfish *Asterina pectinifera*. The structure of 1 was determined by using extensive spectra analysis (IR, 1D and 2D NMR, and MS), chemical degradation, and comparison with the known compound (25*S*)-5α-cholestane-3β,6α,7α,8,15α,16β,26-heptol (2). All the isolates were evaluated for their antiviral activity against herpes simplex virus type 1 (HSV-1) and their cytotoxicity against human liver carcinoma HepG2 cell line *in vitro*. Compounds 3—6, and 8 exhibited antiviral activity against HSV-1 virus with the minimal inhibitory concentration (MIC) values of 0.2, 0.05, 0.2, 0.22, and 0.07 μM, respectively. While compounds 4 and 5 exhibited cytotoxicity against HepG2 cells with IC_{50} values of 0.2 and 1.6 μM, respectively.

Key words *Asterina pectinifera*; starfish; polyhydroxy sterol ester; antiviral; cytotoxicity

The starfish *Asterina pectinifera* (MÜLLER *et* TROSCHEL) (phylum Echinodermata, class Asteroidea, order Spinulosa), feeding on oyster, abalone, and other echinoderms,[1,2] was widely distributed in North Pacific Ocean.[1] In recent years, it has attracted much attention due to its large scale outbreak in Chinese coastal areas such as Qingdao, Jiaozhou Bay in 2006, *etc.*, which led to severe economy damage to fishery and aquacultural grounds for benthic shellfish. In fisher's opinion, it was an animal without any value, so it was usually thrown away when it appears in aquacultural grounds for benthic shellfish. However, it has a series of pharmaceutical functions in Chinese folk such as treating gostar and relieving cough.[3] Previous studies showed that polyhydroxy steroids and saponins were the predominant secondary metabolites of starfish, which showed various biological activities such as hemolytic, cytotoxic, antibacterial, and anti-inflammatory activities.[4—6] More than twenty polyhydroxy steroids and saponins were isolated from genes *Asterina*.[7—18] In our study of the cytotoxic compounds from starfish *A. pectinifera*, a new polyhydroxy sterol ester, (25*S*)-5α-cholestane-3β,6α,7α,8,15α,16β-hexahydroxyl-26-*O*-14′*Z*-eicosenoate (**1**), along with seven known steroids (**2**—**8**), have been isolated. In this paper, the isolation, structure elucidation and biological activity of the isolated compounds are decribed.

The EtOH extract of starfish *A. pectinifera* was fractioned by petroleum ether, EtOAc, and *n*-BuOH, respectively. The *n*-BuOH extract was chromatgraphed on silica gel column, Sephadex LH-20, and reversed-phase HPLC, to yield compounds **1**—**8** (Fig. 1). Compounds **2**—**8** were identified as (25*S*)-5α-cholestane-3β,6α,7α,8,15α,16β,26-heptol (**2**),[15,19] (25*S*)-5α-cholestane-3β,4β,6α,7α,8,15α,16β,26-octol (**3**),[15,19] (25*S*)-5α-cholestane-3β,4β,6α,7α,8,15β,16β,26-octol (**4**),[15] cholest-7-en-3-sodium sulfate (**5**),[20,21] (24*S*)-5α-cholestane-3β,6α,8,15α,24-pentol (**6**),[15] asterosaponins P_1 (**7**) and P_2 (**8**),[22] respectively, by comparison of their physical and spectroscopic data with those in literatures.

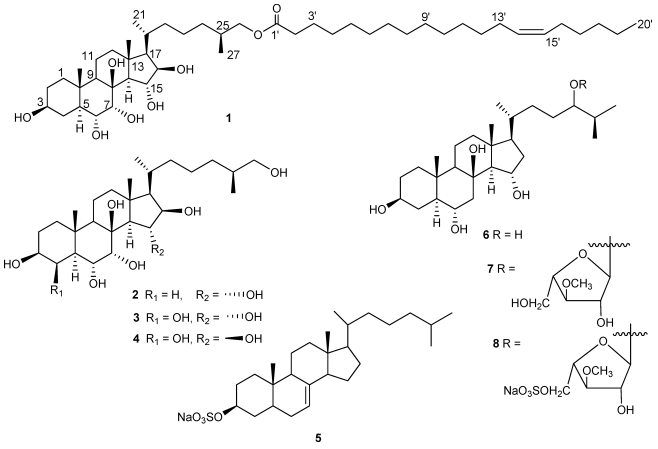

Fig. 1. Structures of Compounds **1**—**8** from *Asterina pectinifera*

Compound **1** was obtained as white amorphous powder, with a molecular formula of $C_{47}H_{84}O_8$ being determined by high-resolution (HR)-electrospray ionization (ESI)-MS (*m*/*z* 776.6160, Calcd 776.6166) and ^{1}H- and ^{13}C-NMR spectra. The IR spectrum of **1** suggested the presence of hydroxyl (3425 cm^{-1}) groups, which also indicated by the intense peaks at *m*/*z* 759, 741, 723, 705, and 687 for stepwise water loss in the atmospheric pressure chemical ionization (APCI)-MS spectrum. The ^{1}H-NMR (Table 1) spectrum showed signals for four methyl protons at δ 1.04 (3H, s, H-18), 0.88 (3H, s, H-19), 0.86 (3H, d, J=6.5 Hz, H-21), and 0.83 (3H, d, J=6.5 Hz, H-27), five oxymethine groups at δ 3.26 (1H, m, H-3), 3.54 (1H, m, H-6), 3.62 (1H, m, H-7), 4.04 (1H, dd, J=11.5, 2.5 Hz, H-15), and 3.81 (1H, dd, J=8.5, 2.5 Hz, H-16), and one oxymethylene group at 3.80 (1H, m, H-26a) and 3.89 (1H, m, H-26b). The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra displayed 47 carbons, including five methyl carbons, 25 methylene car-

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Table 1. ^{1}H- and ^{13}C-NMR Spectral Data of **1** (in DMSO-d_6)

Position	**1**		Position	**1**	
	δ_H (J in Hz)	δ_C		δ_H (J in Hz)	δ_C
1	0.83 m, 1.55 m	38.0	19	0.88 s	13.8
2	1.23 m, 1.56 m	30.6	20	1.77 m	28.7
3	3.26 m	69.7	21	0.86 d (6.5)	17.4
4	0.97 m, 1.94 m	31.5	22	0.95 m, 1.47 m	35.0
5	1.36 m	42.8	23	1.14 m, 1.36 m	22.8
6	3.54 m	66.3	24	1.06 m, 1.34 m	33.5
7	3.62 m	74.4	25	1.71 m	32.0
8		75.8	26	3.80 m, 3.89 m	68.2
9	1.00 m	49.3	27	0.83 d (6.5)	16.8
10		36.0	1′		172.7
11	1.36 m, 1.61 m	17.7	2′	2.27 t (7.0)	33.3
12	1.08 m, 1.80 m	41.4	3′	1.52 m	24.3
13		43.7	4′—12′, 17′	1.23—1.38 m	27.9—28.9
14	1.38 m	58.3	13′, 16′	1.97 m	26.3—26.4
15	4.04 dd (11.5, 2.5)	77.8	14′, 15′	5.32 t-like (5.5)	129.5—129.6
16	3.81 dd (8.5, 2.5)	80.2	18′	1.23 m	31.2
17	1.08 m	59.3	19′	1.23 m	22.0
18	1.04 s	16.2	20′	0.85 t (5.0)	13.2

bons (one bearing oxygen), 11 methine carbons (five bearing oxygen), three quaternary carbons (one bearing oxygen), one acyl carbon, and two olefinic carbons. These data, together with analysis of the ^{1}H–^{1}H correlation spectroscopy (COSY) and the key heteronuclear multiple bond coherence (HMBC) spectra of **1**, revealed that **1** possessed the skeleton of 5α-cholestane-3β,6α,7α,8,15α,16β,26-heptahydroxyl steroid, isolated for the first time from the starfish *Protoreaster nodosu*.[19] In addition, a twenty-carbon enoyl, –COCH$_2$(CH$_2$)$_x$CH=CHCH$_2$(CH$_2$)$_y$CH$_3$ (x+y=14), was deduced from the ^{1}H-NMR signals at δ 2.27 (2H, t, J=7.0 Hz, H-2′), 1.52 (2H, m, H-3′), 1.23—1.38 (20H, m, H-4′—12′, 17′), 0.85 (3H, t, J=5.0 Hz, H-20′), and 5.32 (2H, t-like, J=5.5 Hz, H-14′, 15′), ^{13}C-NMR signals at δ 172.7 (C-1′), 129.5—129.6 (C-14′, 15′), 27.9—28.9 (C-4′—12′, 17′), 33.3 (C-2′), and 24.3 (C-3′), and the molecular ion peak of **1** at m/z 777 [M+H]$^+$ in the APCI-MS spectrum. All evidence mentioned above suggested **1** should be eicosenoate acid ester of the 5α-cholestane-3β,6α,7α,8,15α,16β,26-heptahydroxy steroid.

This suggestion was proved by the hydrolysis of compound **1** in alkaline EtOH–H$_2$O solution and co-TLC with authentic sample (**2**). One of the derivatives (**1a**) obtained by alkali hydrolysis of **1** has the same *Rf* value as authentic sample **2** by co-TLC indicated that **1** was the derivative of 5α-cholestane-3β,6α,7α,8,15α,16β,26-heptahydroxy steroid (**2**). The linkage of 5α-cholestane-3β,6α,7α,8,15α,16β,26-heptahydroxyl steroid and eicosenoate acyl was established by the key HMBC correlations between δ 3.80 (1H, m, H-26a), 3.89 (1H, m, H-26b), and δ 172.7 (C-1′) (Fig. 2).

In addition, the double bond in the eicosenoic acyl was located at C-14′ by the characteristic fragment-ion peaks at m/z 705 [M+H−C$_5$H$_{11}$]$^+$ and 679 [M+H−C$_7$H$_{13}$]$^+$ in the APCI-MS of **1**, which indicated that the bond cleavage between carbon 15′ and 16′, and carbon 13′ and 14′ (Fig. 3). The geometry of the double bond is determined as *Z* type by the δ value (26.4 ppm) of the allylic carbon because the allylic carbon in a long-chain alkene signals were observed at *ca.* 27 ppm in *Z* type and at *ca.* 32 ppm in *E* type,[23] which was also determined by olefinic protons (14′-H and 15′-H) signals appearing at δ 5.32 ppm as a triplet-like because olefinic proton signals were observed as a triplet-like in *Z* type and a multiplet in *E* type.[24] The stereochemistry at C-25 was determined as *S* configuration by comparison of the chemical shifts of NMR spectra and optical rotation value of **1a** with those of (25*S*)-5α-cholestane-3β,6α,7α,8,15α,16β,26-heptol (**2**) reported in literature.[15,19]

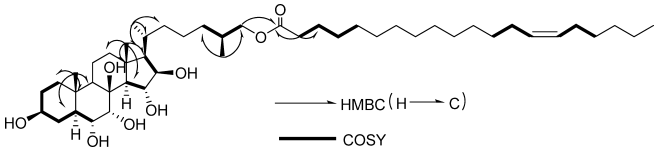

Fig. 2. Key COSY and HMBC Correlations of **1**

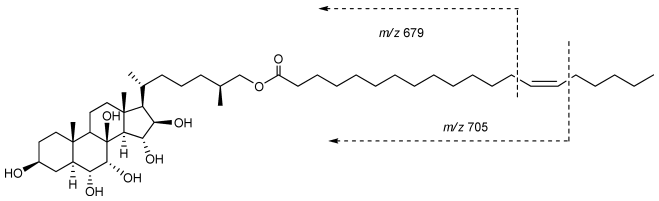

Fig. 3. Diagnostic Ion Masses of **1**

Table 2. Antiviral and Antitumor Activities of Compounds **1**—**8**

Compounds	Minimum inhibitory concentration (μM)/HSV-1	IC_{50} (μM)/HepG2
1	—	—
2	—	—
3	0.2	—
4	0.05	0.2
5	0.2	1.6
6	0.22	—
7	—	—
8	0.07	—

—: no activity.

Therefore, the structure of compound **1** was elucidated as (25*S*)-5α-cholestane-3β,6α,7α,8,15α,16β-hexahydroxyl-26-*O*-14′*Z*-eicosenoate, which is a first example from natural resources to our best knowledge.

Compounds **1**—**8** were evaluated for their antiviral activity against herpes simplex virus type 1 (HSV-1) virus and cytotoxicity against human liver carcinoma HepG2 cells *in vitro*, due to some sulfated sterols from marine invertebrates being reported to be active against human immunodeficiency virus (HIV).[25,26] Compounds **3**—**6**, and **8** showed antiviral activity against HSV-1, with minimal inhibitory concentration (MIC) values ranging from 0.05 to 0.22 μM, meanwhile, compounds **4** and **5** showed marginal cytotoxicity against HepG2 with IC_{50} of 0.2 μM and 1.6 μM, respectively (Table 2). These results suggest that hydroxyl group at C-4, and C-24, sulfate group at C-3 may be essential for anti-HSV-1 activity, and β configuration of C-15 and sulfate at C-3 may be also necessary for anti-HepG2 activity. Interestingly, sugar unit attached at the C-24 position affects anti-HSV-1 activity greatly. Furthermore, when the configuration of C-15 was transformed from α form to β form, the anti-HSV-1 activity

was stronger.

Experimental

General Procedures Optical rotation was recorded on a Perkin Elmer model 341 polarimeter. IR spectra were recorded with a Bruker vector 33 infrared spectrometer with KBr pellets. NMR spectra were recorded on a Brucker Avance 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C), with TMS as internal standard. APCI mass spectrum was obtained using an Esquire HCT PLUS LC-Mass spectrometer. HR-ESI-MS data were obtained from MAT 95XP (Thermo) mass spectrometer. Silica gel (Yantai Jiangyou Silica gel Development Co., Ltd., 100—200 and 200—300 mesh) and Sephadex LH-20 (Pharmacia Biotech, Sweden) were used for column chromatography. Reversed-phase HPLC separations were performed on HitachiL-2400 system, detected by a ELSD detector and equipped with a YMC semipreparative C18 column (5 μm, 10×250 mm) running with flow rate of 2 ml/min. Spots on TLC plates (Merck silica gel 60 GF254) were detected with a UV lamp or by spraying with 98% H_2SO_4 followed by heating. All solvents used were either spectral grade or analytical reagents.

Animal Material The starfish *A. pectinifera* were first collected in April 2008 around Dalian waters, Liaoning province, China, then dried in the sun. The specimens were identified by Professor Qingchao Chen, South China Sea Institute of Oceanology, Chinese Academy of Sciences, China. A small amount of the sample (No. 200804) is kept in the key Laboratory of Marine Bio-resources Sustainable Utilization, South China Sea Institute of Oceanology, Chinese Academy of Sciences, China.

Extraction and Isolation The sun-dried starfish (10 kg) were chopped and extracted with 75% EtOH for 7 d at room temperature for three times to give the crude extract 300.0 g. The crude extract was suspended in H_2O, and then partitioned with petroleum ether, EtOAC, and *n*-BuOH for three times, respectively. The petroleum ether, EtOAc, and *n*-BuOH layers were concentrated *in vacco* to afford 20.0 g, 18.0 g, and 75.5 g, respectively. The *n*-BuOH fraction was chromatographed on a silica gel column using $CHCl_3$–MeOH (from 40 : 1 to 0 : 1) as eluent. By combining the same fractions with TLC (GF_{254}) monitoring, twenty fractions were obtained. Fraction 11 was chromatographed over Sephadex LH-20 eluted with $CHCl_3$–MeOH (1 : 1), and then repeatedly subjected to column chromatography on silica gel, eluted with EtOAc–MeOH (from 20 : 1 to 10 : 1) to yield **1** (5 mg). Fraction 15 was subjected to column chromatography using $CHCl_3$–MeOH (10 : 1) as eluent, and further purified by Sephadex LH-20 and reversed-phase HPLC to give **2** (10 mg), **3** (10 mg), and **4** (6 mg). Fraction 17 was subjected to column chromagraphy on silica gel using $CHCl_3$–MeOH (from 9 : 1 to 8 : 1), and repeatedly purified by Sephadex LH-20 eluted with $CHCl_3$–MeOH (from 1 : 1 to 0 : 1) to yield **5** (4 mg), **6** (6 mg), **7** (5 mg), and **8** (4 mg).

(25*S*)-5α-cholestane-3β,6α,7α,8,15α,16β-hexahydroxyl-26-*O*-14′*Z*-eicosenoate (**1**): white amorphous power, $[\alpha]_D^{20}$ +12.0° (c=0.5, MeOH). IR (KBr) cm^{-1}: 3425, 2926, 1743, 1477, 1381, 961. ^{1}H- and ^{13}C-NMR (500 and 125 MHz, respectively, DMSO-d_6) data, see Table 1. APCI-MS (positive-ion mode) m/z: 777 ([M+H]$^+$). HR-ESI-MS m/z: 776.6160 (Calcd for $C_{47}H_{84}O_8$: 776.6166).

Alkali Hydrolysis Compound **1** (2 mg) was dissolved in 2 ml of 5% KOH ethanolic (aqueous, 95%) solution and refluxed for 3 h. The reaction mixture was cooled to room temperature. Ten milliliters of water was added to the reaction solution and then extracted with chloroform. Organic layer was concentrated *in vacco* to give the residue, monitored by co-TLC with authentic sample (**2**), the residue was purified by HPTLC ($CHCl_3$/MeOH, 10 : 1), to get the hydrolysis product (**1a**, 0.95 mg). The optical rotation of **1a** ($[\alpha]_D^{20}$ +31.5° (c=0.01, MeOH)) was comparable to that of compound **2** ($[\alpha]_D^{20}$ +38.4° (c=0.5, MeOH)).

Anti-HSV-1 Assay Anti-HSV-1 activities of compounds **1**—**8** were evaluated by the cytopathic effect (CPE) inhibitory assay.[27,28] In general, Vero cells were seeded into a 96-well plate and allowed to form monolayer. After removing the growth medium, 50 μl of 100TCID$_{50}$ viral suspensions and the same volume of different concentrations of test compounds were added to each well. Noninfected and infected cells without isolated compounds acted as cell and virus control, respectively. The 96-well plates were incubated at 37 °C in humidified 5% CO_2 atmosphere. When virus showed the maximum CPE, the virucidal effects were determined by the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay,[29] and the MIC values of test compounds was calculated.

Cytotoxicity HepG2 cells in the logarithmic growth phase were trypsinized and diluted with medium to 2×10^5 cells/ml, and then 100 μl of this cell suspension solution was added to each well of a 96-well plate. Thereafter, the plate was incubated for 24 h and the supernate was removed. Then the test compounds were diluted with test medium to different concentrations (200—3.125 μg/ml), and 100 μl of this diluted solution was placed in the 96-well plate. After the plate was incubated for 48 h, the toxicity concentration of test compounds were investigated and recorded. The inhibition effects of test compounds on proliferation of HepG2 cell was determined by MTT method as described previously.[29]

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References and Notes

1) Huang Z. G., "China Marine Species and Distribution," Ocean Press, Beijing, 1994, p. 635.
2) Goggin C. L., Bouland C., *Int. J. Parasitol.*, **27**, 1415—1418 (1997).
3) Editorial Committee of Chinese Materia Medica, State Administration of Traditional Chinese Medicine, "Chinese Herbs," Shanghai Science and Technology Press, Shanghai, 1999, p. 246.
4) D'Auria M. V., Minale L., Riccio R., *Chem. Rev.*, **93**, 1839—1895 (1993).
5) Stonik V. A., *Russ. Chem. Rev.*, **70**, 673—715 (2001).
6) Minale L., Pizza C., Riccio R., Zollo F., *Pure Appl. Chem.*, **54**, 1935—1950 (1982).
7) Kicha A. A., Ivanchina N. V., Gorshkoya I. A., Ponomarenko L. P., Likhatskava G. N., Stonik V. A., *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.*, **128**, 43—52 (2001).
8) Kicha A. A., Ivanchina N. V., Stonik V. A., *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.*, **136**, 897—903 (2003).
9) Kicha A. A., Ivanchina N. V., Stonik V. A., *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.*, **139**, 581—585 (2004).
10) Iorizzi M., Minale L., Riccio R., *Gazz. Chim. Ital.*, **120**, 147—153 (1990).
11) Kicha A. A., Ivanchina N. V., Kalinovsky A. I., Dmitrenok P. S., Stonik V. A., *Russ. Chem. Bull.*, **49**, 1794—1795 (2000).
12) Noguchi Y., Higuchi R., Marubayashi N., Komori T., *Liebigs Ann. Chem.*, **1987**, 341—348 (1987).
13) Dubois M. A., Noguchi Y., Higuchi R., Komori T., *Liebigs Ann. Chem.*, **1988**, 495—500 (1988).
14) Dubois M. A., Higuchi R., Komori T., Sasaki T., *Liebigs Ann. Chem.*, **1988**, 845—850 (1988).
15) Higuchi R., Noguchi Y., Komori T., Sasaki T., *Liebigs Ann. Chem.*, **1988**, 1185—1189 (1988).
16) Cho Y., Higuchi R., Marubayashi N., Ueda I., Komori T., *Liebigs Ann. Chem.*, **1992**, 79—81 (1992).
17) Guo C., Zhang H., Liu L., *Zhongguo Haiyang Yaowu*, **19**, 12—14 (2000).
18) Yasumoto T., Tanaka M., Hashimoto Y., *Nippon Suisan Gakkaishi*, **32**, 673—676 (1966).
19) Riccio R., Minale L., Pagonis S., Pizza C., Zollo F., Pusset J., *Tetrahedron*, **38**, 3615—3622 (1982).
20) Barton D. H. R., Rosenfelder W. J., *Helv. Chim. Acta*, **32**, 975—978 (1949).
21) D'Auria M. V., Riccio R., Minale L., La Barre S., Pusset J., *J. Org. Chem.*, **52**, 3948—3952 (1987).
22) Kicha A. A., Kalinovskii A. I., Levina E. V., Stonik V. A., Elyakov G. B., *Tetrahedron Lett.*, **24**, 3893—3896 (1983).
23) Fusetani N., Yasumuro K., Matsunaga S., Hirota H., *Tetrahedron Lett.*, **30**, 6891—6894 (1989).
24) Rossi R., Carpita A., *Tetrahedron*, **33**, 2447—2450 (1977).
25) McKee T. C., Cardellina J. H., Riccio R., D'Auria M. V., Iorizzi M., Minale L., Moran R. A., Gulakowski R. J., Mcmahon J., *J. Med. Chem.*, **37**, 793—797 (1994).
26) Roccatagliata A. J., Maiere M. S., Seldes A. M., Pujol C. A., Damonte E. B., *J. Nat. Prod.*, **59**, 887—889 (1996).
27) Makarov V. A., Riabova O. B., Granik V. G., Wutzler P., Schmidtke M., *J. Antimicrob. Chemother.*, **55**, 483—488 (2005).
28) Schmidtke M., Schnittler U., Jahn B., Dahse H. M., Stezner A., *J. Virol. Methods*, **95**, 133—143 (2001).
29) Mosmann T., *J. Immunol. Methods*, **65**, 55—63 (1983).