Arch Pharm Res Vol 28, No 3, 285-289, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

New Cytotoxic Sulfated Saponins from the Starfish *Certonardoa semiregularis*

Weihong Wang, Hyojin Jang, Jongki Hong[1], Chong-Ok Lee[2], Song-Ja Bae[3], Sook Shin[4], and Jee H. Jung

College of Pharmacy, Pusan National University, Busan 609-735, [1]Basic Science Institute, Seoul, [2]Korea Research Institute of Chemical Technology, Daejon, [3]Silla University, Busan, Korea, [4]and Sahmyook University, Seoul, Korea

(Received November 22, 2004)

Two new sulfated saponins designated as certonardosides P_2 and I_3 (**1** and **2**) were isolated from the brine shrimp active fraction of the MeOH extract of the starfish *Certonardoa semiregularis*. The structures were determined on the basis of spectral analysis. Compounds **1** and **2** were tested for cytotoxicity against five human tumor cell lines (A549, SK-OV-3, SK-MEL-2, XF498, and HCT15), and compound **1** displayed significant cytotoxicity against the SK-MEL-2 skin cancer cell.

Key words: Starfish, *Certonardoa semiregularis*, Cytotoxicity

INTRODUCTION

Saponins composed of a polyhydroxysterol and a monosaccharide or disaccharide unit are widespread in starfish (D' Auria *et al.*, 1993; Minale *et al.*, 1995; Iorizzi *et al.*, 2001), and have been reported to exhibit cytotoxic (Wang *et al.*, 2004a), hemolytic (Ivanchina *et al.*, 2000), antiviral (Wang *et al.*, 2002), antifungal (Chludil *et al.*, 2002), and antimicrobial activities (De Marino *et al.*, 1998).

In our previous study on bioactive metabolites from the starfish *Certonardoa semiregularis* (family Linckiidae), 65 sterols and saponins were isolated (Wang *et al.*, 2002, 2003a, 2003b, 2004a, 2004b). In a continuing study, two new sulfated saponins (**1** and **2**) were isolated from the brine shrimp active fraction of the MeOH extract. The gross structures of the compounds were determined mainly on the basis of COSY, HSQC, and HMBC experiments. The structure elucidation and cytotoxicity of the compounds are described in this paper.

MATERIALS AND METHODS

Instruments

The IR spectra were recorded on a JASCO FT/IR-410 infrared spectrometer. NMR spectra were recorded on Varian Unity Inova AS 500 and Varian Unity Plus 300 instruments. Chemical shifts were reported with reference to the respective solvent peak or residual solvent peak (δ_H 3.30 and δ_C 49.0 for CD_3OD). FABMS data were obtained on a JEOL JMS-SX-102A double-focusing spectrometer. HPLC was performed with C18-10E Shodex packed column (250 × 10 mm, 5 μm, 100 Å) and YMC ODS-H 80 column (250 × 10 mm, 4 μm, 80 Å) using a Shodex RI-71 detector.

Animal material

The starfish were collected in July 2000, at depths of 5-10 m off the coast of Geomun Island, Korea. The collected sample was frozen immediately and kept at -20 °C until processed. The specimen was identified as *Certonardoa semiregularis* by Prof. Sook Shin, Department of Life Science, Sahmyook University, Seoul, Korea. The starfish was moderately sized (10 cm in diameter), five-armed species. Arms were slender, broad at base, cylindrical in cross-section, and tapering evenly to rounded tips. The dorsal surface was a shade of red and the ventral surface was pale. The dorsal surface was covered in plates that are arranged in regular longitudinal and transverse rows up to one-third of its arms. The voucher specimen (J00K-4) of the starfish was deposited at the Marine Natural Product Chemistry Laboratory, Pusan National University, Busan, Korea.

Extraction and isolation

The frozen starfish (9 kg) was extracted with MeOH at

Correspondence to: Jee H. Jung, Celloge of Pharmacy, Pusan National University, Busan 609-735, Korea
Tel: 82-51-510-2803, Fax: 82-51-510-2803
E-mail: jhjung@pusan.ac.kr

room temperature. The MeOH extract was partitioned between H_2O and CH_2Cl_2. The CH_2Cl_2 layer was further partitioned between 90% MeOH and *n*-hexane to afford 90% MeOH-soluble (21 g) and *n*-hexane-soluble fractions. The 90% MeOH fraction was subjected to a reversed-phase flash column chromatography (YMC Gel ODS-A, 60 Å, 500/400 mesh), eluting with a step gradient solvent system of 60 to 100% MeOH/H_2O to afford 20 fractions (Fr.1-Fr.20). Fraction Fr.3 (1.08 g) was very active in the brine shrimp assay (ED_{50}, 5 μg/mL) and was further separated by reversed-phase MPLC (YMC Gel ODS-A, 60 Å, 230 mesh), eluting with a solvent system of 50 to 100% MeOH/H_2O, to afford 10 fractions (Fr.3-1–Fr.3-10). Compound **1** was obtained by separation of Fr.3-6 on a MPLC (silica gel 60, 400/230 mesh), eluting with a solvent system of 30 to 100% MeOH/$CHCl_3$, followed by purification on a reversed-phase HPLC (Shodex C18-10E) with the solvent system of 70% MeOH/H_2O. The fraction Fr 3-7 was separated by a MPLC (silica gel 60, 400/230 mesh) and then purified by a reversed-phase HPLC (YMC ODS-H80) with a solvent system of 72% MeOH/H_2O to afford compound **2**.

Solvolysis of compound 1

A solution of compound **1** (2 mg) in a mixture of pyridine (0.5 mL) and dioxane (0.5 mL) was heated at 120 °C in a stoppered reaction vial. After 2 h, TLC analysis [ODS plate with MeOH-H_2O (3:1)] showed that the starting compound had disappeared. The residue was evaporated to dryness and purified by HPLC (C18-10E Shodex packed column, 250 × 10 mm, 5 μm, 100 Å) with MeOH-H_2O (78:22) as eluant to give compound **1a** as a major product.

Certonardoside P_2 (1)

Light yellow needles; IR (KBr pellet) λ_{max} 3430, 2938, 1646, 1457, 1420, 1375, 1252, 1223, 1166, 1055, 895, 588 cm^{-1}; ^1H- and ^{13}C-NMR data, see Tables I and II; FABMS (+ve) *m/z* 899 $[M + Na]^+$, FABMS (–ve) *m/z* 853 $[M - Na]^-$.

Compound 1a

Light yellow needles; The NMR data of the aglycon were almost identical to those of compound **1** (see Table I), ^1H-NMR (500 MHz, CD_3OD), δ (sugar) 4.72 (1H, d, *J* = 7.0 Hz, H- 1"), 4.36 (1H, d, *J* = 7.0 Hz, H-1'), 3.88 (1H, dd, *J* = 11.5, 5.0 Hz, H_{eq}-5"), 3.86 (1H, dd, *J* = 11.5, 5.0 Hz, H_{eq}-5'), 3.60 (3H, s, 2"-OCH_3), 3.50 (1H, m, H-3'), 3.48 (2H, m, H-4',4"), 3.40 (1H, m, H-2'), 3.37 (1H, m, H-3"), 3.15 (2H, m, H_{ax}-5',H_{ax}-5"), 2.92 (1H, dd, *J* = 8.5, 7.0 Hz, H-2"); ^{13}C-NMR (75 MHz, CD_3OD), δ (sugar) 103.5 (C-1"), 102.5 (C-1'), 84.2 (C-2"), 81.5 (C-2'), 77.0 (C-3'), 76.0 (C-3"), 71.3 (C-4'), 71.1 (C-4"), 65.6 (C-5"), 65.5 (C-5'), 60.0 (2"-OCH_3); FABMS (+ve) *m/z* 797 $[M + Na]^+$, FABMS (–ve) *m/z* 773 $[M - H]^-$.

Table I. ^1H- and ^{13}C-NMR data of the aglycons of compounds **1** and **2**[a]

Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1	1.70 (m), 1.16 (m)	39.3	1.74 (m), 0.95 (m)	38.0
2	1.74 (m), 1.47 (m)	31.3	1.84 (m), 1.58 (m)	25.2
3	3.46 (m)	72.1	3.38 (m),	75.5
4	2.18 (dd, 10.0, 2.0), 1.16 (m)	62.9	4.48 (m)	70.0
5	1.04 (m)	53.7	1.23 (m)	54.8
6	3.71 (m)	67.6	4.92 (dd, 10.8, 4.2)	73.5
7	2.39 (dd, 12.0, 3.5), 1.32 (t, 11.5)	49.7	2.70 (m), 1.16 (m)	46.5
8		77.2		77.2
9	0.88 (dd, 12.3, 3.5)	57.3	0.84 (dd, 12.3, 2.6)	56.5
10		37.9		38.0
11	1.70 (m), 1.47 (m)	19.5	1.74 (m), 1.42 (m)	18.5
12	1.92 (m), 1.16 (m)	43.4	1.93 (m), 1.23 (m)	43.6
13		44.5		44.6
14	1.04 (m)	62.6	1.03 (m)	59.5
15	4.38 (dd, 7.0, 5.5)	71.2	4.38 (dd, 7.0, 5.5)	70.2
16	4.24 (t, 7.0)	72.6	4.20 (t, 7.0)	71.5
17	1.04 (t, 7.5)	60.9	0.95 (m)	61.5
18	1.23 (s)	14.0	1.27 (m)	17.2
19	0.98 (s)	17.9	1.27 (s)	16.5
20	1.47 (m)	31.4	1.93 (m)	30.4
21	0.95 (d, 6.5)	18.5	0.95 (d, 6.5)	17.0
22	1.70 (m), 1.16 (m)	34.6	1.66 (m), 1.23 (m)	33.0
23	1.32 (m), 1.16 (m)	28.2	1.42 (m), 1.03 (m)	27.5
24	1.32 (m)	42.7	1.24 (m)	43.0
25	1.92 (m)	36.1	1.74 (m)	29.5
26	3.71 (m), 3.24 (m)	73.5	0.87 (m)	19.5
27	0.86 (d, 6.5)	13.8	0.84 (d, 6.5)	18.0
24^1	1.42 (m), 1.16 (m)	23.3	1.66 (m), 1.42 (m)	27.5
24^2	0.90 (t, 7.0)	12.6	3.85 (d, 7.5), 3.50 (m)	68.5

[a] Spectra were recorded in CD_3OD at 500 MHz for ^1H-NMR, and 75 MHz for ^{13}C-NMR.

Certonardoside I_3 (2)

Light yellow needles; ^1H- and ^{13}C-NMR data, see Tables I and II; FABMS (+ve) *m/z* 929 $[M + Na]^+$, FABMS (-ve) *m/z* 883 $[M-Na]^-$.

Cytotoxicity evaluation

Cytotoxicity assay was performed at the Korea Research Institute of Chemical Technology. SRB (sulforhodamine B)

Table II. ^{1}H- and ^{13}C-NMR data of the sugar residues of compounds **1** and **2**[a]

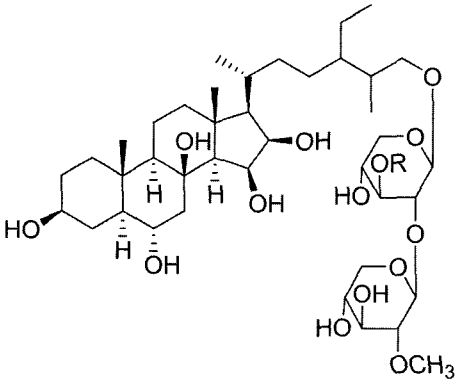
Position	1		2	
	δ_H	δ_C	δ_H	δ_C
1'	4.54 (d, 5.5)	102.7	4.36 (d, 7.5)	102.5
2'	3.70 (dd, 6.5, 5.5)	76.8	3.40 (m)	80.5
3'	4.39 (t, 6.5)	81.5	3.47 (m)	73.0
4'	3.78 (m)	69.8	2.96 (m)	84.7
5'	3.98 (dd, 12.0, 4.0)	64.5	4.00 (brd, 11.0)	62.5
	3.35 (m)		3.20 (m)	
4'- OMe			3.46 (s)	59.5
1''	4.80 (d, 6.5)	103.1	4.70 (d, 7.0)	103.5
2''	3.00 (t, 6.5)	83.6	2.94 (t, 7.0)	83.5
3''	3.45 (m)	75.4	3.38 (m)	75.5
4''	3.49 (m)	71.0	3.47 (m)	71.5
5''	3.98 (dd, 12.0, 4.0)	65.8	3.85 (m)	65.5
	3.20 (dd, 12.0, 8.0)		3.15 (dd, 11.5, 7.5)	
2''- OMe	3.58 (s)	60.6	3.58 (s)	60.7

[a] Spectra were recorded in CD_3OD at 500 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR.

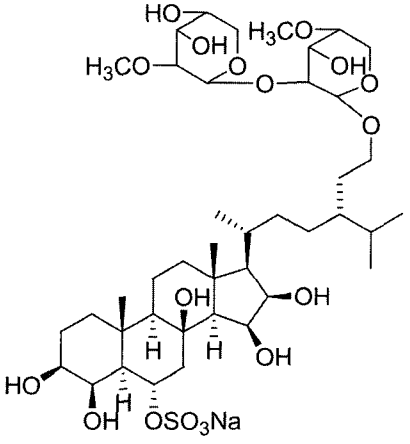
assay, developed for measuring the cellular protein content of the cultures, was applied for the measurement of the cytotoxicity of the compounds against tumor cells. The rapidly growing cells were harvested, counted, and inoculated at the appropriate concentrations ($1\text{-}2 \times 10^4$ cells/well) into 96-well microtiter plates. After incubation for 24 h, the compounds dissolved in culture medium were applied to the culture wells in triplicate, followed by incubation for 48 h at 37 °C under a 5% CO_2 atmosphere. The cultures fixed with cold TCA (trichloroacetate) were stained by 0.4% SRB dissolved in 1% acetic acid. After solubilizing the bound dye with 10 mM unbuffered tris base by a gyrotory shaker, the absorbance at 520 nm was measured with a microplate reader (Dynatech Model MR 700). Fifty percent inhibitory concentration (ED_{50}) was defined as the concentration, which reduced absorbance by 50% compared to the control level in the untreated wells.

RESULTS AND DISCUSSION

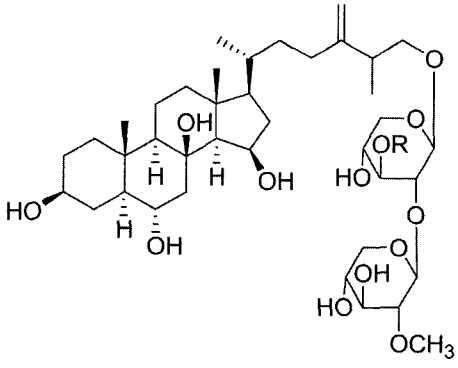
Certonardoside P_2 (**1**) was isolated as light yellow needles. The positive and negative FABMS gave molecular ion peaks at *m/z* 899 $[M + Na]^+$ and 853 $[M - Na]^-$, respectively. The presence of the sulfate group was supported by the absorption bands at 1252 and 1223 cm^{-1} in the IR spectrum. The ^{1}H- and ^{13}C-NMR data of compound **1**



1 R = SO_3Na
1a R = H



2



3 R = SO_3Na

were reminiscent of those of certonardoside C (**3**) (Wang *et al.*, 2002). However, certain differences in the ^{1}H- and ^{13}C-NMR data were noticed. The olefinic methylene was replaced by an ethyl group [H-24^1: δ 1.42 (m), δ 1.16 (m); H-24^2: δ 0.90, t, J = 7.0 Hz]. In the upfield region of the ^{13}C-NMR spectrum, methyl carbon signals at δ 12.6 (C-24^2), δ 13.8 (C-27), and δ 18.5 (C-21) were observed. In addition, two oxymethine signals at δ_H 4.38/δ_C 71.2 (C-15) and δ_H 4.24/δ_C 72.6 (C-16) suggested the 15β,16β-dihydroxy configuration because the chemical shifts were close to those of 15β,16β-dihydroxy steroids but dissimilar to those of 15α,16β-dihydroxy steroids (ca. 80-83 ppm) (Iorizzi *et al.*, 1986). The 15α,16α-dihydroxy configuration could be eliminated by the downfield shifts of the H_3-18 methyl proton signal (δ 1.23) and the C-18 carbon signal (δ 14.0) (Iorizzi *et al.*, 1986). The upfield chemical shifts of C-15 and C-16 agree with the strong steric interactions between the vicinal *cis*-hydroxy groups (Van Antwerp *et al.*, 1977). The ^{13}C-NMR shifts of A ring, especially the downfield shift of C-3 (δ 72.1), indicated the presence of a 3β-hydroxy substituent (Eggert *et al.*, 1976). The presence of a 6α-hydroxy group was established also by the ^{13}C-NMR data (δ 67.6). The C-6 signal was shifted downfield to δ 74.8 in 3β,6β,8,15β- tetrahydroxy steroidal nucleus such as halityloside F (Iorizzi *et al.*, 1986). The COSY, HSQC, and HMBC data corroborated the assignment of the 3β,6α,8,15β,16β-pentahydroxy steroidal nucleus and the 26-hydroxy-24-ethylcholestane side chain. The common 20*R* configuration was assumed on the basis of the chemical shift of H-21 (ca. δ 0.95 in sterols with saturated side chain) (Iorizzi *et al.*, 1986). The stereochemistry at C-24 and C-25 remains unassigned.

The ^{1}H- and ^{13}C-NMR data suggested that compound **1** had the same 2-*O*-methyl-β-D-xylopyranosyl-(1→2)-3-*O*-sulfonato-β-D-xylopyranosyl sugar unit as that of certonardoside C (**3**) (Wang *et al.*, 2002). The interglycosidic linkages were defined on the basis of the long-range correlations between C-1' and H-26, and between C-2' and H-1". The common D-configuration for xylose was presumed.

Solvolysis of **1** in dioxane-pyridine afforded the desulfonated derivative (**1a**), whose negative ion FABMS exhibited a pseudomolecular ion peak at *m/z* 773 [M – H]. Solvolysis of **1** to the desulfonated derivative (**1a**) was further confirmed by upfield shift of H-1' (δ 4.54→4.36), H-2' (δ 3.70→3.40), H-3' (δ 4.39→3.50), H-4' (δ 3.78→3.48), and H_{eq}-5' (δ 3.98→3.86) of the internal xylopyranosyl unit. The signals of C-2', 3', and 4' were shifted by +4.7, -4.5, and +1.5 ppm, respectively. Thus, it was deduced that sulfate group was located at C-3'. Therefore, the structure of compound **1a** was established as 26-*O*-[2-*O*-methyl-β-D-xylopyranosyl-(1→2)-β-D-xylopyranosyl]-24-ethyl-5α,24ζ,25ζ-cholestane-3β,6α,8,15β,16β,26-hexol.

Accordingly, the structure of compound **1** is defined as a sodium salt of 26-*O*-[2-*O*-methyl-β-D-xylopyranosyl-(1→2)-3-*O*-sulfonato-β-D-xylopyranosyl]-24-ethyl-5α,24ζ,25ζ-cholestane-3β,6α,8,15β,16β,26-hexol. Compound **1** is the second example of naturally occurring saponin with the 26-hydroxy-24-ethylcholestane side chain. Attenuatoside S-III isolated from the starfish *Hacelia attenuata* was the first saponin of this type (Minale *et al.*, 1984).

Certonardoside I$_3$ (**2**) was isolated as light yellow needles. The FABMS gave a molecular ion peak at *m/z* 929 [M + Na]$^+$. Comparison of the ^{1}H- and ^{13}C-NMR spectral data with those of certonardoside I suggested the presence of a 3β,4β,6α,8,15β,16β-hexahydroxylation pattern (Wang *et al.*, 2002). The location of the sulfate group at C-6 was implied by the downfield shift of the signals of H-6 and C-6 to δ_H 4.92 and δ_C 73.5, respectively. The H-6 and C-6 signals were observed at δ_H 3.71 and δ_C 67.6, respectively, in compound **1** with free hydroxyl group at C-6. In particular, the HMBC correlations from C-4 (δ 62.9), C-5 (δ 53.7), C-7 (δ 49.7), C-8 (δ 77.2), and C-10 (δ 37.9) to H-6 (δ 4.92) were observed. In the ^{1}H-NMR spectrum, two coupled oxymethine signals were observed at δ 4.37 and 4.20, which could be assigned to H-15α and H-16α, respectively. Comparison of the ^{1}H-NMR data with those of compound **1a** revealed the presence of a methoxyl group on C-4' of the internal β-xylose residue. The upfield shift of the H-4' to δ 2.96 (δ 3.48 in compound **1a**) along with the downfield shift of H_{eq}-5' to δ 4.00 (δ 3.86 in compound **1a**) corroborated the location of the methoxyl group at C-4' of the internal xylopyranose. The cross-peaks in the HMBC spectrum between the methoxyl group and C-4' or H-4' were observed. The chemical shift of the anomeric carbons (δ 102.5, 103.5) and the coupling constant of the anomeric protons (δ 4.36, J = 7.5 Hz; δ 4.70, J = 7.0 Hz) suggested β anomeric configurations. The inter-glycosidic linkages were defined on the basis of the long-range correlations between C-1' and H-24^2, and between C-2' and H-1".

The 24*R* configuration was assigned on the basis of comparison of the ^{1}H- and ^{13}C-NMR spectral data with those of 24^2-hydroxylated model compounds (Anastasia *et al.*, 1986). The $\Delta\delta_H$ of the isopropyl methyl proton signals of (24*R*)-24^2-hydroxy steroids ranged from 0.03 to 0.06 ppm, while that of the 24*S* isomer was always under 0.03 ppm. Likewise, the $\Delta\delta_C$ of the isopropyl methyl carbon signals of (24*R*)-24^2-hydroxy steroids ranged from 1.1 to 1.4 ppm, while that of the 24*S* isomer ranged between 0.1 and 0.4 ppm. The Δδ of the isopropyl methyl proton and carbon signals of **2** were 0.03 and 1.1 ppm, respectively, which were close to those of the 24*R* isomer (Anastasia *et al.*, 1986). Thus, the structure of compound **2** was defined as a sodium salt of (24*R*)-24^2-*O*-[(2-*O*-methyl-β-D-xylopyranosyl-(1→2)-4-*O*-methyl-β-D-xylopyranosyl)]-

Table III. Cytotoxicity data of compounds **1** and **2** against five human solid tumor cells[a]

Compound	A549	SK-OV-3	SK-MEL-2	XF498	HCT15
1	>30.0	19.5	2.67	26.5	18.2
2	>30.0	>30.0	>30.0	>30.0	>30.0
Doxorubicin	0.10	0.09	0.08	0.13	0.22

[a]Data as expressed in ED_{50} values (mg/mL). A549: human lung cancer cell; SK-OV-3: human ovarian cancer; SK-MEL-2: human skin cancer; XF498; human CNS cancer; HCT 15: human colon cancer.

6-*O*-sulfonato-24-ethyl-5α-cholestane-3β,4β,6α,8,15β,16β, 24^2-heptol.

Compounds **1** and **2** were evaluated for cytotoxicity against a small panel of human solid tumor cell lines. Compound **1** showed somewhat selective cytotoxicity against the SK-MEL-2 skin cancer cell line with an ED_{50} value of 2.67 μg/mL. Compound **2** showed no activity (ED_{50} > 30 μg/mL) (Table III).

ACKNOWLEDGEMENT

This study was supported by a grant from the Ministry of Maritime Affairs and Fisheries (Marine Bio 21).

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