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Sokodosides, Steroid Glycosides with an Isopropyl Side Chain, from the Marine Sponge *Erylus placenta*

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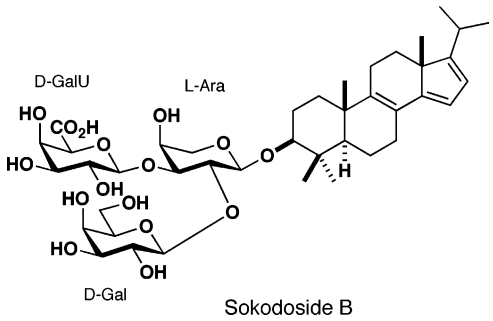

Sokodoside B

Two novel steroid glycosides, sokodosides A and B (**1** and **2**, respectively), were isolated from the marine sponge *Erylus placenta* as growth-inhibitory principles against several strains of yeast and a cancer cell line. Sokodosides possess the novel carbon skeleton as characterized by the presence of a combination of isopropyl side chain and the 4,4-dimethyl steroid nucleus. Sokodoside B has another unique characteristic in the presence of $\Delta^{8,14,16}$ unsaturation. The structures of sokodosides were determined by analysis of spectral data and chemical degradation. The absolute stereochemistry of sokodoside A (**1**) was determined by the application of the modified Mosher analysis to the aglycon obtained by acid hydrolysis, whereas the absolute stereochemistry of the monosaccharide units in **1** and **2** was determined by chiral GC analyses of the acid hydrolysates.

Introduction

Since the discovery of the first sponge-derived triterpene glycosides from *Asteropus sarasinosum*,[1,2] a considerable number of this class of metabolites have been reported from sponges of significant taxonomical diversity, viz. Ancorinidae,[3,4] Mycalidae,[5] Pachastrellidae,[6] Niphatidae,[7] and Raspalidae.[8] Some of them were reported to possess antimicrobial and/or cytotoxic activities. In the course of our screening of growth inhibitory activity against genetically engineered yeasts, the marine sponge *Erylus placenta* collected off Hachijo Island exhibited a broad spectrum of activity against several strains of yeasts and a fungus. This paper reports the isolation and structure elucidation of two novel hexanortriterpene glycosides from the sponge.

Results and Discussion

The aqueous *n*-PrOH extract of the sponge *E. placenta* was subjected to standard solvent partitioning scheme to afford the *n*-BuOH fraction, which was separated by ODS flash chromatography followed by several rounds of reversed-phase HPLC

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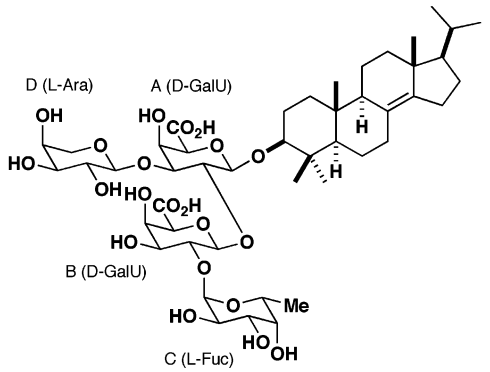
to afford sokodosides A (**1**) and B (**2**) as a colorless and yellowish powder, respectively.



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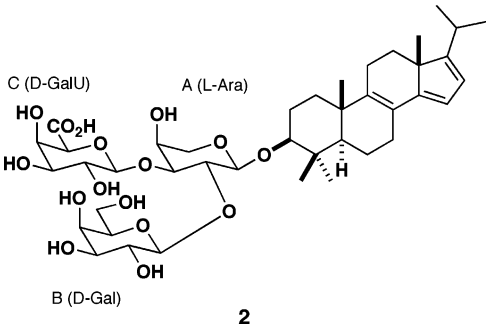

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TABLE 1. ^{1}H and ^{13}C NMR Data for Sokodoside A (1)

position	^{13}C	^{1}H	position	^{13}C	^{1}H
1	36.8	1.57 m	1′	103.7	4.24 d (7.7)
		1.05 m	2′	72.4	3.94 dd (7.7, 9.2)
2	25.9	1.53 m	3′	82.8	3.59 m
		1.92 m	4′	69.9	4.10 d (3.1)
3	89.4	3.02 dd (3.9, 11.9)	5′	73.8	4.12 s
4	38.8	-	6′	169.8	-
5	54.2	0.93 m	1″	98.8	5.17 d (7.7)
6	21.5	1.56 m	2″	75.8	3.47 dd (7.7, 9.4)
		1.20 dd (4.2, 12.7)	3″	73.9	3.63 m
7	29.8	1.66 br t (13)	4″	70.1	3.90 d (3.5)
		2.38 br t (13)	5″	73.6	4.03 d (1.2)
8	126.0		6″	169.8	
9	50.8	1.56 m	1‴	99.3	5.05 d (4.2)
10	37.8		2‴	69.0	3.52 m
11	19.0	1.39 m	3‴	70.2	3.69 m
		1.48 m	4‴	71.7	3.51 m
12	36.8	1.05 m	5‴	66.0	4.12 br q (6.5)
		1.87 ddd (3.2, 4.0, 12.7)	6‴	16.8	1.07 (3H, d, 6.5)
13	42.4		1⁗	104.7	4.43 d (6.5)
14	141.4		2⁗	70.8	3.45 m
15	25.2	2.13 br dt (16, 9, 7.3)	3⁗	72.2	3.40 m
		2.18 br dd (11.6, 16.9)	4⁗	67.5	3.65 br s
16	26.8	1.34 m	5⁗	65.4	3.73 dd (3.9, 12.3)
		1.74 m			3.38 dd br d (12.3)
17	58.7	0.93 m			
18	18.2	0.80 (3H, s)			
19	14.5	0.70 (3H, s)			
20	29.2	1.53 m			
21	22.9	0.92 (3H, d, 6.5)			
22	23.0	0.86 (3H, d, 6.5)			
23	16.5	0.82 (3H, s)			
24	28.2	1.05 (3H, s)			

Sokodoside A (**1**) had a molecular formula of $C_{47}H_{74}O_{21}$ which was determined by HRFABMS in conjunction with the NMR data. The ^{1}H NMR spectrum in DMSO-d_6 displayed signals for seven methyls (four singlets at δ 0.70, 0.80, 0.81, and 1.05 and three doublets at δ 0.86, 0.92, and 1.07), eight pairs of methylenes, four methines between δ 1.05 and 2.18, and numerous oxygenated methylenes and methines, which were reminiscent of steroid glycosides (Table 1). The ^{13}C NMR spectrum exhibited four anomeric carbons, one oxygenated methylene, 19 oxygenated methines, two olefinic carbons (δ142.0 and 141.4), three quaternary carbons (δ 37.0, 38.8, and 42.4), and two carbonyl carbons (δ 169.8, 2C).

Interpretation of the COSY, TOCSY, and HMQC data revealed four partial structures which comprised the aglycon: C-1 to C-3 (unit a); C-5 to C-7 (unit b); C-9, C-11, and C-12 (unit c); C-15 to C-17 and C-17 to C-21/C-22 (unit d) (Figure 1a). These partial structures were connected to each other through nonprotonated carbons on the basis of the following HMBC correlations: H-1/C-5, C-10, and C-19; H-3/C-4, C-5, C-23, and C-24; H-11/C-8 and C-13; H_3-18/C-12, C-13, C-14, and C-17; $H_3$19/C-1, C-5, C-9, and C-10; H_3-23/C-3, C-4 and C-5; H_3-24/ C-3, C-4, and C-5. HMBC cross-peaks from H-6, H-7, H-9, and H-11 to C-8, and H-15, H-16, H-17, and H_3-18 to C-14 required the $\Delta^{8\ (14)}$ unsaturation in the 4,4-dimethyl steroid skeleton. NOESY correlations, H-3/H-5; H_3-19/H_3-24; H_3-19/H-11β (δ 1.39); H-11β/H_3-18, H_3-18/H-20, and H-12α (δ 1.05)/H17 showed that H_3-18, H_3-19, H-20, and H_3-24 were on the same face of the molecule, while H-3, H-5, H-9, and H-17 were on the other (Figure 1b).

The remaining signals consisted of four monosaccharide units, which were assigned by interpretation of 2D NMR data in conjunction with the analysis of ^{1}H−^{1}H coupling constant values. Analysis of the COSY spectrum starting from the anomeric proton at δ 4.24 revealed the presence of five contiguous methines in sugar A. H5′ was correlated to a carbon at δ 169.8 in the HMBC spectrum, demonstrating that C6′ was oxidized to carboxylic acid. Large coupling constants between H-1′ and H-2′ (7.7 Hz) and between H-2′ and H-3′ (9.2 Hz) as well as small coupling constants between H-3′ and H-4′ (3.1 Hz) and H-4′ and H-5′ (<1 Hz) suggested that this unit was β-galactopyranosyluronic acid residue, which was in agreement with the NOESY data. The signals in sugar unit B, which comprised five contiguous methines, were almost identical with those of unit A. There was an HMBC cross-peak between H-5″ and a carbon at δ 169.8, indicating that unit B was also β-galactopyranosyluronic acid residue. Sugar unit C also contained five contiguous methines, the terminus of which was coupled with a methyl as analyzed by the COSY data. Although it was not possible to determine coupling constant values except for the one between H-1‴ and H-2‴ (4.2 Hz), a large coupling between H-2‴ and H-3‴ as implied by an intense COSY cross-peak together with NOESY cross-peaks, H-1‴/H-2‴ and H-3‴/H-5‴, suggested this unit to be α-fucopyranose. Starting from an anomeric proton at δ 4.43, analysis of the COSY and TOCSY spectra established that sugar D was a pentopyranose. The NOE correlations H-1⁗/H-3⁗, H-1⁗/H-5⁗, H-4⁗/H-3⁗, H-4⁗/H-5a⁗ together with a large coupling constant between H-1⁗ and H-2⁗ revealed this unit to be β-arabinopyranose (Ara).

HMBC correlations H-1⁗/C-3′, H1″/C-2′, and H-1‴/C-2‴ demonstrated the sequence of the tetrasaccharide unit to be α-fucopyranosyl-(1→2)-β-galactopyranosyluronic acid-(1→2)-[β-arabinopyranosyl-(1→3)]-β-galactopyranosyluronic acid. This unit was shown to be attached to the aglycon at C-3 on the basis of the HMBC cross-peaks H-1′/C-3 and H-3/C-1′.

The chirality of the sugar units was determined by chiral GC analysis[9] of the acid hydrolysate: **1** was subjected to methanolysis followed by trifluoroacetylation and analyzed by GC equipped with a Chirasil-L-Val column as a stationary phase.

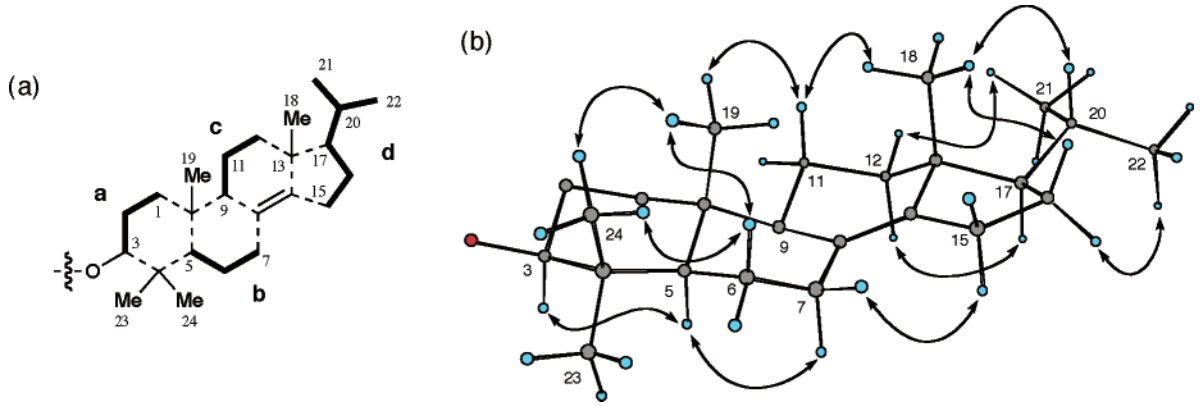

FIGURE 1. (a) Partial structures of **1** as assigned on the basis of COSY data. (b) Selected NOESY correlations of the aglycon in **1**. Several hydrogen atoms are omitted for clarity.

TABLE 2. ^{1}H and ^{13}C NMR Data for Sokodoside B (2)

position	^{13}C	^{1}H	position	^{13}C	^{1}H
1	35.0	1.77 dt (13.1, 3.8)	1′	103.8	4.33 d (6.5)
		1.17 dt (3.1, 13.1)	2′	75.7	3.77 dd (6.5, 8.8)
2	26.3	1.93 dq (13.5, 4.2)	3′	81.6	3.68 m
		1.62 br q (13)	4′	66.5	3.91 m
3	88.0	3.00 dd (4.2, 11.9)	5′	64.2	3.69 m
4	39.1				3.41 m
5	50.4	1.14 dd (1.0, 12.7)	1″	103.2	4.52 d (7.7)
6	17.7	1.71 br dd (7.7, 13.5)	2″	71.3	3.25 m
		1.53 m	3″	73.6	3.25 m
7	27.2	2.35 m	4″	67.8	3.67 m
		2.09 br dd (6.9, 16)	5″	74.8	3.29 m
8	122.3	-	6″	59.8	3.56 m
9	140.0	-			3.42 m
10	37.2	-	1‴	103.1	4.43 d (7.7)
11	20.7	2.19 m	2‴	70.2	3.42 m
		2.13 m	3‴	72.5	3.37 m
12	29.8	1.94 dd (5.0, 12.0)	4‴	69.8	3.92 dd (1.2, 3.1)
		1.03 m	5‴	73.8	4.12 d (1.2)
13	51.0	-	6‴	169.0	
14	154.5	-			
15	117.8	5.87 d (2.3)			
16	122.0	5.98 dd (1.0, 2.3)			
17	162.3	-			
18	19.4	0.88 (3H, s)			
19	21.2	1.05 (3H, s)			
20	25.9	2.48 m			
21	23.6	1.10 (3H, d, 6.9)			
22	24.0	1.04 (3H, d, 6.5)			
23	16.0	0.81 (3H, s)			
24	27.4	1.01 (3H, s)			

A standard sample of L-GalUA, which was not commercially available, was prepared by oxidation of L-Gal.[10] The analysis demonstrated that the absolute configuration of the sugar residues was L-Ara, D-GalUA, and L-Fuc. The absolute stereochemistry at C-3 was assigned to be *S* by application of the modified Mosher method[11] to the aglycon **3**, which was liberated by acid hydrolysis of **1**.

Sokodoside B (**2**) was analyzed for $C_{41}H_{62}O_{16}$ on the basis of HRFABMS and NMR data. The ^{1}H and ^{13}C NMR spectra of **2** suggested that this compound is also a related terpene glycoside (Table 2). Sokodoside B (**2**) had two olefinic protons (δ 5.87 and 5.97), which comprised the chromophore of **2** exhibiting the UV absorption at 309 nm.

Interpretation of the COSY, TOCSY, and HMQC data indicated the following partial structures: C-1 to C-3 (unit e); C-5 to C-7 (unit f); C-11 to C-12 (unit g); C-15 to C-16 (unit h); C-20 to C-21/C-22 (unit i) (Figure 2a). These partial structures and nonprotonated carbons were connected to each other on the basis of the following HMBC correlations: H-1/C-5, C-10, and C-19; H-3/C-4, C-5, C-23, and C-24;H-7β (δ 2.09)/C-8, C-9, and C-14; H-11/C-8, C-9 and C-13; H-15/C-13, C-14, C-16, and C-17, H_3-18/C-12, C-13, C-14, and C-17; $H_3$19/C-1, C-5, C-9, and C-10; H-20/C-13, C-16, and C-17; H_3-23/C-3, C-4 and C-5; and H_3-24/ C-3, C-4, and C-5. The data were consistent with a 4,4-dimethyl-$\Delta^{8,14,16}$ steroid skeleton having an isopropyl side chain. NOESY correlations, H-3/H-5, H-3/H_3-23; H_3-24/H_3-19; H_3-19/H-11β (δ 2.13); and H-11β/H_3-18, showed that H-3, H-5, and H_3-23 were on the same face of the molecule, while H_3-18, H_3-19, and H_3-24 were on the other (Figure 2b).

Interpretation of the COSY and TOCSY data gave rise to spin systems for three monosaccharide units. Starting from the anomeric proton at δ 4.33, a pentopyranose structure was assigned to sugar A. NOESY cross-peaks, H-1′/H-3′, H-3′/H-5′, H-1′/H-5a′, and H-3′/H-4′, in conjunction with a large coupling between H-1′ and H-2′ showed this residue to be β-arabinopyranose (Ara). Sugar B was assigned as a hexose starting from the anomeric proton resonated at δ 4.52 and terminated with a pair of methylene protons (δ_H 3.42 and 3.56). HMBC cross-peaks disclosed the connectivity between C-1″ and C-5″ through an oxygen atom, indicating that this unit was hexopyranose. A large *J* value between H-1″ and H-2″ together with NOE correlations between H-1″/H-3″, H-1″/H-5″, H-4″/H-3″, and H-4″/H-5″ revealed that sugar B to be β-galactopyranose (Gal). Sugar C comprised five contiguous methines, reminiscent of sugar units A and B in sokodoside A. HMBC cross-peaks H-1‴/C-5‴ and H-5‴/C-1‴ revealed the connectivity of C-1‴ and C-5‴ through an oxygen, while an HMBC cross-peak from H-5‴ (δ 4.12) to a carbon at δ 169.0 disclosed the substitution of C-5‴ by a carboxylic acid. This unit was determined to be β-galactopyranosyluronic acid (GalUA) on the basis of a large coupling constant between H-1‴ and H-2‴ and the following NOE correlations: H-1‴/H-3‴, H-1‴/H-5‴, H-3‴/H-4‴, and H-4‴/H-5‴.

The connectivity of the trisaccharide portion was determined on the basis of the HMBC correlations, H-1′/C-3, H-1″/C-3′, and H1‴/C-2, which implied the sequence of β-galactopyranosyl-(1→2)-[β-galactopyranosyluronic acid-(1→3)]-β-arabinopy-

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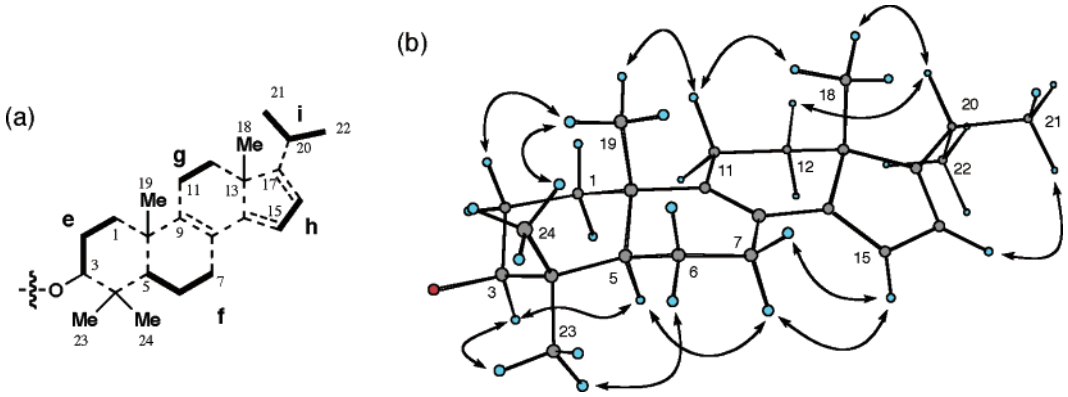

FIGURE 2. (a) Partial structures of **2** as assigned on the basis of COSY data. (b) Selected NOESY correlations of the aglycon in **2**. Several hydrogen atoms are omitted for clarity.

ranosyl. The trisaccharide moiety was attached at C-3 of the aglycon as demonstrated by the HMBC cross-peaks H-1′/C-3 and H-3/C-1′.

The absolute stereochemistry of the component sugars in **2** was determined as L-Ara, D-GalUA, and D-Gal by GC analysis of the methanolysis product. The absolute stereochemistry of the aglycon of **2** was not determined because it decomposed by acid hydrolysis.

Structural features of the steroid nucleus of sokodosides A and B partially coincide with those of other sponge-derived triterpene glycosides: they have 4,4-dimethyl group and unsaturation(s) in the C and D rings of steroid nucleus.[2−8] However, the isopropyl side chain of sokodosides was unprecedented among this class of metabolites. Several sterols with an isopropyl side chain have been reported from marine invertebrates as minor constituents: 20-methylpregn-5-en-3β-ol was detected by GC−MS in the extracts of the sponge *Petrosia ficiformis*[11] and the hydrozoan *Obelia longissima*,[12] whereas 20-methyl-5α-pregnane-2β,3α,6α-triol triacetate was isolated from the acetylation products of the acid hydrolysate of a polar steroid fraction of *Trachyopsis halichondroides*.[13] Samandinine, a minor steroid alkaloid from the salamander *Salamandra maculosa*, also has an isopropyl group as the side chain.[14] Sokodosides are the first examples with a 4,4-dimethylsteroid nucleus that possesses isopropyl side chain.[15] The $\Delta^{8,14,16}$-triene system observed in sokodoside B is very rare among natural products and has only been reported in cardiac steroids from terrestrial plants, *Nerium* spp.[16]

Sokodosides A and B showed moderate growth-inhibitory activity against the fungus *Mortierella ramanniana* and the yeast *Saccharomyces cerevisiae* with and without mutations (cdc28, act1-1, and erg6) (Table 3). No antibacterial activity was detected against *Escherichia coli* and *Staphylococcus aureus*. Additionally, sokodosides A and B exhibited cytotoxic activity against P388 cells with IC_{50} values of 100 and 50 μg/mL, respectively.

TABLE 3. Antimicrobial Activity of Sokodosides

	sokodoside A (**1**)		sokodoside B (**2**)	
microbial strain	50 μg[a]	100 μg	50 μg	100 μg
S. cerevisiae W303-1B[b]	14[c]	18	16	18
S. cerevisiae 1907[d]	9	10	11	14
S. cerevisiae act1-1[e]	8	8	13	15
S. cerevisiae YAT2285[f]	10	11	12	14
M. ramanniana	10	12	10	11

[a] Amount of the sample loaded on a 6 mm ϕ thin paper disk. [b] Wild type. [c] Diameter of inhibitory zone in mm. [d] Strain with a mutation in cdc28 gene. [e] Strain with a mutation in act1-1 gene. [f] Strain with a mutation in erg6 gene.

Experimental Section

Biological Material. The sponge was collected off Hachijo Island in 1994 and identified as *E. placenta*. The voucher specimen was deposited at the Zoological Museum, University of Amsterdam, with the collection number ZMARPOR19091. The sponge was frozen after collection and kept frozen until extraction.

Isolation of Sokodosides. A 100 g portion of the frozen sponge *E. placenta* was homogenized and extracted with *n*-PrOH/H_2O (3:1, 500 mL × 3). The extracts were combined, concentrated, and partitioned between H_2O and $CHCl_3$, and the resulting H_2O layer was partitioned between *n*-BuOH and H_2O. The 1-BuOH fraction was separated by ODS flash chromatography with *n*-PrOH/H_2O (1:9, 3:7, 5:5, and 8:2) and $CHCl_3$/MeOH/H_2O (6:4:1). Antifungal fractions were combined and separated by ODS HPLC with *n*-PrOH/H_2O/TFA (45:55:0.05) followed by HPLC with a phenylhexyl column [*n*-PrOH/H_2O/TFA (38:62:0.05)] to afford sokodoside A (**1**, 15.8 mg) and sokodoside B (**2**, 26.9 mg).

Sokodoside A (1): colorless powder; $[\alpha]^{23}_D$ +14.3 (*c* 0.1, *n*-PrOH/H_2O = 1:1); ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS *m*/*z* 973.4652 (M − H)$^-$ (calcd for $C_{47}H_{73}O_{21}$ 973.4644).

Sokodoside B (2): yellowish powder; $[\alpha]^{23}_D$ +64.7 (*c* 0.1, *n*-PrOH/H_2O = 1:1), UV (*n*-PrOH/H_2O = 1:1) λ_{max} 310 nm (ϵ 6000); ^{1}H and ^{13}C NMR data, see Table 2; HRFABMS *m*/*z* 809.3959 (M − H)$^-$ (calcd for $C_{41}H_{61}O_{16}$ 809.3960).

Methanolysis of 1 and 2. A 0.2 mg portion of either **1** or **2** was dissolved in 10% HCl−MeOH (0.5 mL) and heated at 100 °C for 2 h. The reaction mixture was concentrated under a stream of N_2 and partitioned between $CHCl_3$ and H_2O. The H_2O layer was concentrated under a stream of N_2 to afford a mixture of methyl glycosides.

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Preparation of L-GalUA. L-Gal (1 mg) was treated with 10% HCl−MeOH (0.5 mL) at 100 °C for 2 h, and the solution was neutralized with a solution of $NaHCO_3$ (pH 8). To the mixture were added PtO_2 (1 mg) and 10% Pd−C (1 mg) under H_2 atmosphere at rt. After 2 h, the atmosphere was replaced by O_2 and stirred for 36 h at rt. The reaction mixture was filtered with Celite and dried under reduced pressure to give methyl glycosides of L-GalUA.

Derivatization of the Hydrolysate for GC Analysis. The methanolysis product was dissolved in a mixture of dry CH_2Cl_2 and trifluoroacetic anhydride and kept at 100 °C for 10 min. The mixture was dried and redissolved in CH_2Cl_2.

Chiral GC Analysis. GC analysis was carried out on a Chirasil-L-Val capillary column (25 m × 0.25 mm, i.d.): detection, FID; initial temperature 50 °C for 6 min, final temperature 160 °C for 1 min, temperature raised at 4 °C min^{-1}. Retention times: standard D-GalUA (23.47, 28.56, 33.65 min), L-GalUA (23.62, 28.68, 33.76 min), D-Gal (26.61, 31.77 min), L-Gal (27.09, 32.30 min), d-Fuc (17.09, 18.01, 22.26 min), L-Fuc (17.04, 18.41, 23.17 min) products from sokodoside A (**1**), 18.04, 21.18, 22.90, 23.45, 28.49, and 33.63 min; products from sokodoside B (**2**), 17.96, 21.17, 23.45, 26.61, 28.49, 31.79, and 33.64 min. Because retention times fluctuated, identity of the peaks was confirmed by injection of a mixture of the sample and standards. Absolute configuration of GalUA and Gal in the methanolysate were assigned as D, while Ara and Fuc was assigned as L.

Acid Hydrolysis of Sokodoside A (1). A 4.8 mg portion of sokodoside A (**1**) was dissolved in a mixture of concentrated HCl−toluene−EtOH (1:1:48) and kept at 65 °C for 1 h.[4] The reaction mixture was neutralized by the addition of $NaHCO_3$. The filtrate was evaporated and purified by preparative TLC developed with $CHCl_3$−MeOH (9:1) to afford 1.2 mg of the aglycon **3**.

Compound 3: ^{1}H NMR (CD_3OD), see Table S1; HRFABMS *m*/*z* 345.3141 $(M + H)^+$ (calcd for $C_{24}H_{40}O$ 345.3157).

Esterification of 3. To the solution of **3** (0.4 mg) in pyridine (100 μL) was added 7 μL of (−)-MTPACl, and the mixture was kept at rt for 10 min. The reaction mixture was diluted with EtOH, evaporated, and purified by preparative silica gel TLC developed with *n*-hexane−diethyl ether (9:1) to afford the (*S*)-MTPA ester **4**. The (*R*)-MTPA ester **5** was prepared in the same manner. For the ^{1}H NMR data of compounds **4** and **5**, see Table S1 (Supporting Information).

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Supporting Information Available: General experimental procedures, 2D-NMR spectra of **1** and **2**, and NMR data for **3** and its MTPA esters. This material is available free of charge via the Internet at http://pubs.acs.org.

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