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Nobiloside, a New Neuraminidase Inhibitory Triterpenoidal Saponin from the Marine Sponge *Erylus nobilis*[1]

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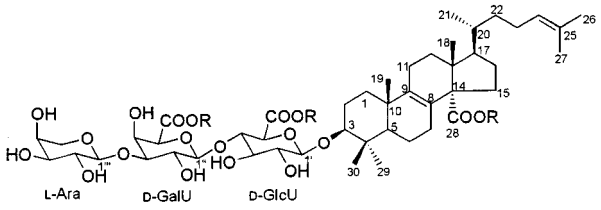
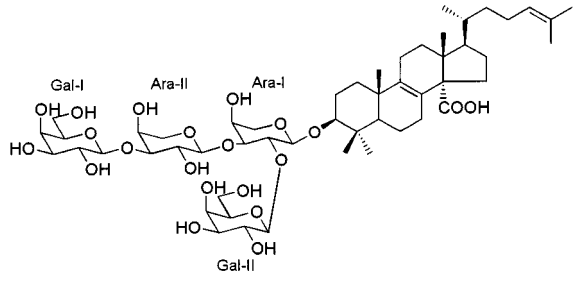
A neuraminidase inhibitor, nobiloside (**1**), was isolated from the marine sponge *Erylus nobilis* Thiele, 1903. Its structure was determined as a penasterol trisaccharide. The absolute configurations were determined by NMR and chiral GC analysis. It inhibited neuraminidase from the bacterium *Clostridium perfringens* with an IC_{50} value of 0.46 μg/mL.

Steroid or triterpenoid saponins are rarely found in marine sponges, except for astrophorid sponges. More than 10 unusual saponins, which are either antifungal, cytotoxic, ichthyotoxic, or immunosuppressive, have been reported from sponges of the genera *Asteropus* and *Erylus*.[2−12]

In our ongoing program of discovery of antineuraminidase metabolites from Japanese marine invertebrates,[13] we found marked activity in the hydrophilic extract of *Erylus nobilis* collected off Shikine-jima Island, 200 km south of Tokyo. Bioassay-guided separation afforded an active compound, nobiloside (**1**), whose structure was determined to be a triterpenoidal trisaccharide. This paper describes the isolation and structural elucidation of the saponin.



1: R = H
6: R = Me



2

The combined MeOH, EtOH, and acetone extracts of the frozen sponge were partitioned between Et_2O and H_2O, and the aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH extract was separated by ODS flash chromatography, followed by reversed-phase HPLC to yield nobiloside (**1**, 43.3 mg, 0.087% based on wet weight).

Nobiloside (**1**) has a molecular formula of $C_{47}H_{72}O_{19}$, which was determined by high-resolution FABMS [*m/z* 939.4580 (Δ −0.9 mmu)]. The IR spectrum exhibits characteristic bands attributable to hydroxyl (3382 cm^{-1}) and carboxyl (1688 cm^{-1}) groups. The ^1H NMR spectrum exhibits seven methyl signals (six singlets at δ 1.65, 1.57, 1.04, 1.03, 0.92, and 0.86, a doublet at δ 0.77), an olefinic proton (δ 5.08), and many oxymethines including three anomeric protons (δ 4.47, 4.47, and 4.42) (Table 1). The ^{13}C NMR spectrum displays three carbonyl carbons (δ 179.7, 171.7, and 171.0), four olefinic carbons (δ 141.0, 131.6, 128.6, and 125.9), and three anomeric carbons (δ 106.5, 106.1, and 106.1) (Table 1).

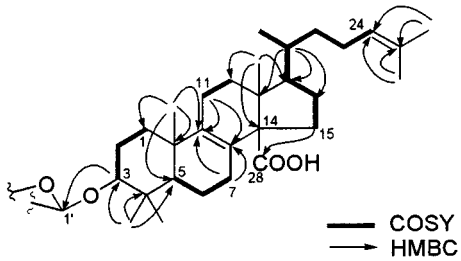


Figure 1. COSY and key HMBC correlations.

Two-dimensional NMR experiments including COSY, HOHAHA, and HMQC[14] indicated connectivities from C-1 to C-3, from C-5 to C-7, from C-11 to C-12, and from C-15 to C-24 (Figure 1). These partial structures were further connected by HMBC[15] correlations: H-19/C-1, C-5, C-9, and C-10; H-29/C-3, C-4, and C-5; H-30/C-3, C-4, and C-5; H-7/C-8 and C-9; H-11/C-8 and C-9; H-18/C-12, C-13, C-14, and C-17; and H-20/C-13, C-16, and C-17. An HMBC cross-peak H-15/C-28 placed the carboxyl group on C-14, while an HMBC correlation H-3/C-1′ linked the trisaccharide unit to C-3. The isobutenyl terminal of the side chain was straightforward from HMBC cross-peaks from two olefinic methyls to C-24 and C-25, as shown in Figure 1.

NOESY correlations H-5/H-3 and H-29; H-18/H-19; H-18/H-20; and H-19/H-30 indicated that H-3, H-5, and H29 were on the same face of the molecule, while H-18, H-19, H-20, and H-30 were on the opposite face (Figure 2). The carboxyl group on C14 was *anti* to C-18, since ^{13}C NMR data for the aglycone of **1** agreed well with those of formoside (**2**).[9] Therefore, the aglycone was identified as penasterol.

Interpretation of the $^1H-^1H$ COSY and HOHAHA spectra gave rise to spin systems for three monosaccharides, which were assigned as α-arabinopyranose (Ara), β-galactopyranosyluronic acid (GalU), and β-glucopyranosyluronic acid (GluU) by analysis of HMQC, HMBC, and NOE data

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Table 1. NMR Data for Nobiloside in CD_3OD

	aglycone				trisaccharide		
	^1H	^{13}C	HMBC		^1H	^{13}C	HMBC
1a	1.74	36.2	C-2,3,5,10,19	1′	4.42, d (7.4)	106.5	C-3
1b	1.28		C-10,19	2′	3.33, t (7.4)	74.7	C-1′,3′
2a	1.90	27.3	C-10	3′	3.58, m	76.0	C-4′,5′
2b	1.68, m		C-10	4′	3.74, m	82.4	C-2′,3′,6′
3	3.18, dd (12.0, 7.0)	90.9	C-2,4,29,30,1′	5′	3.96, d (9.6)	74.7	C-1′,3′,4′,6′,1″
4		40.1		6′		171.7	
5	1.12, d (12.4)	51.4	C-4,6,9,10,19,29,30				
6a	1.68, m	19.1	C-5,7,8	1″	4.47, d (7.4)	103.9	C-4′
6b	1.52, m		C-5,7,10	2″	3.70, m	70.7	C-1″,3″
7a	2.10, m	28.6	C-5,8,9	3″	3.67, m	83.0	C-1″,2″,1‴
7b	1.94, m			4″	4.38, d (3.6)	70.4	C-2″,3″
8		128.6		5″	4.28, brs	74.7	C-1″,6″
9		141.0		6″		171.0	
10		38.2					
11a	2.19, m	23.1		1‴	4.47, d (6.6)	106.1	C-3″
11b	2.06, m		C-8,9	2‴	3.62, m	72.6	C-1‴,3‴
12a	2.20, m	32.5	C-11,13,18	3‴	3.54, m	73.7	C-2‴
12b	1.67, m		C-9,11,13,14	4‴	3.82, brs	69.3	C-2‴
13		47.9		5‴a	3.87, dd (12.5, 2.5)	66.8	C-1‴,3‴,4‴
14		63.6		5‴b	3.56, m		C-1‴,4‴
15a	2.05, m	28.7	C-8,13,14,17,28				
15b	1.55, m		C-14,16,28				
16a	2.06, m	30.1	C-13,14,17				
16b	1.36, m		C-14,17,20				
17	1.52, m	51.9	C-13,15,18,20				
18	0.77, s	18.2	C-12,13,14,17				
19	1.03, s	20.0	C-1,5,9,10				
20	1.43, s	36.7	C-17,21				
21	0.92, d (6.2)	19.0	C-17,20,22				
22a	1.42, m	37.0	C-17,20,21,23				
22b	1.02, m		C-21,23,24				
23a	2.00, m	25.6	C-24				
23b	1.85, m		C-22,24,25				
24	5.08, t (7.0)	125.9	C-26,27				
25		131.6					
26	1.65, m	26.0	C-24,25,27				
27	1.57, m	17.8	C-24,25,26				
28		179.7					
29	1.04,s	28.2	C-3,4,5,30				
30	0.86,s	16.8	C-3,4,5,29				

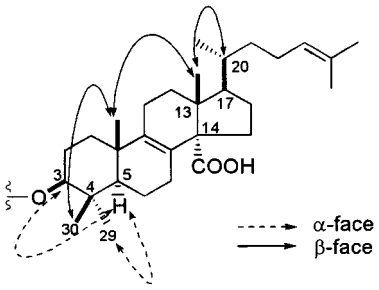

Figure 2. Key NOESY correlations.

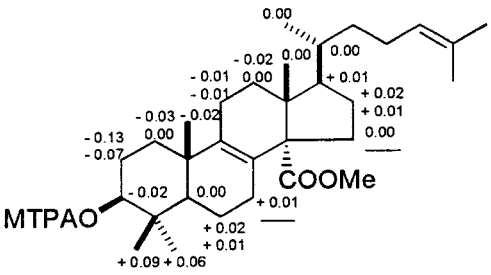

Figure 3. Distribution of $\Delta\delta$ values for the MTPA esters of **3**.

as well as by $^1H-^1H$ coupling constant values.[9,16,17] HMBC correlations H-1′/C-3, H-1″/C-4′, and H-1‴/C-3″implied the sequence of α-arabinopyranosyl-(1→3)-*β*-galacturonopyranosyl-(1→4)-*β*-glucuronopyranoside.

The absolute configuration of the sugar residues was determined to be D-GlcU, D-GalU, and L-Ara by chiral GC analysis.

The absolute configuration of the aglycone was determined by the modified Mosher's method.[18] Nobiloside (**1**) was treated with 10% HCl/MeOH to afford penasterol methyl ester, which was converted to the MTPA esters. The distribution of $\Delta\delta$ values secured the 3*S* configuration (Figure 3), thus determining 3S, 5*R*, 10*S*, 13*R*, 14*R*, 17*R*, and 20*R* stereochemistry.

Nobiloside inhibited neuraminidase from the bacterium *Clostridium perfrigens* with an IC_{50} value of 0.46 μg/mL. Generally, it is said that a carboxyl group in many neuraminidase inhibitors plays an important role to inhibit the enzyme. To confirm this, nobiloside (**1**) was converted to nobiloside trimethyl ester (**6**) with diazomethane. As expected, the methyl ester was not active at 100 μg/mL, thus indicating the importance of carboxyl groups for the activity.

Nobiloside is a rare example of marine saponins that contain 14-carboxylanosterol derivatives as an aglycone. The other examples are formoside (**2**)[9] and erylosides C−J[6,8,11,12] from the Caribbean Choristida sponge *Erylus formosus*. It should be noted that marine saponins containing uronic acids have not been reported to date.

Experimental Section

General Experimental Procedures. ^1H and ^{13}C NMR spectra were recorded on a JEOL A600 NMR spectrometer.

^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peaks δ_H 3.30 and δ_C 49.0 for CD_3OD and δ_H 7.24 and δ_C 77.0 for $CDCl_3$. Optical rotation was measured on a JASCO DIP-1000 digital polarimeter in MeOH. FAB mass spectra were obtained on a JEOL SX102/SX102 tandem mass spectrometer using triethanolamine or *m*-nitrobenzyl alcohol as the matrixes. Negative mode HR-FABMS were obtained at a resolution of 5000 using PEG 1000 sulfate as a marker. Chiral GC analysis was carried out on a Shimadzu GC-9A using a Chirasil-L-Val capillary column (25 m × 0.25 mm, i.d.): detection, FID; initial temperature 60 °C for 6 min, final temperature 160 °C for 1 min, temperature raised at 4 °C min^{-1}; carrier gas, He. IR spectra were recorded on a JASCO FT/IR-5300 spectrometer. Fluorescence for inhibition assay was measured with a Molecular Device Spectra MAX GEMINI apparatus.

Collection and Isolation. The sponge was collected by hand using scuba at depths of 15−20 m off Shikine-jima Island, 200 km south of Tokyo, immediately frozen, and kept frozen at −20 °C until processed. The sponge was identified as *Erylus nobilis* Thiele, 1903 (order Astrophorida, class Geodiidae). A voucher specimen was deposited at the Zoological Museum of the University of Amsterdam, (ZMA 16719).

A 50 g portion of the frozen specimen was homogenized and extracted with MeOH (250 mL × 3), EtOH (250 mL × 2), and acetone (250 mL × 2). The extracts were combined, concentrated, and partitioned between H_2O and Et_2O. The aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH layer was evaporated under reduced pressure and separated by ODS flash chromatography with aqueous MeOH systems followed by reversed-phase HPLC (Develosil C-30 UG-5) with 95% MeOH containing 0.1% TFA to afford nobiloside (**1**: 43.3 mg, 0.087% wet weight).

Nobiloside (1): white powder; $[\alpha]^{24}_D$ −41.6° (*c* 0.10, MeOH); IR (film) 3382, 1731, 1688, 1374, 1257, 1154, 1082, 1011 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRFABMS (neg) *m*/*z* 939.4580 (M − H)$^-$ [$C_{47}H_{71}O_{19}$ (Δ −0.9 mmu)].

Methanolysis of Nobiloside. A portion of **1** (8.0 mg) was dissolved in 10% HCl/MeOH and kept at 100 °C for 2 h. The solvent was removed in a stream of N_2, and the residue was further dried under reduced pressure. The product was partitioned between H_2O (2 mL) and $CHCl_3$ (2 mL × 3). The organic phase yielded penasterol methyl ester (**3**), and the aqueous layer furnished a mixture of methylated monosaccharides.

Penasterol methyl ester (3): ^{1}H NMR (CD_3OD) δ 5.07 (1H, t), 3.34 (3H, s), 3.15 (1H, dd), 1.66 (3H, s), 1.59 (3H, s), 1.03 (3H, s), 0.97 (3H, s), 0.92 (3H, d), 0.80 (3H, s), 0.79 (3H, s); FABMS *m*/*z* 469 (M − H)$^-$, 455 (neg).

(*R*)-MTPA Ester of 3. To a half portion of the organic phase dissolved in pyridine was added (*S*)-(+)-MTPACl. After 30 min, H_2O was added, and the mixture was extracted with $CHCl_3$ (2 mL × 3). The $CHCl_3$ layer was chromatographed on SiO_2 with $CHCl_3$ to furnish (*R*)-MTPA ester **4**.

4: ^{1}H NMR ($CDCl_3$) δ 7.48, 7.37, 4.72 (dd, H-3), 2.18 (dd, H-15), 2.13 (m, H-11), 2.05 (m, H-12), 2.04 (m, H-7), 2.02 (m, H-16), 2.01 (m, H-11), 1.85 (m, H-2), 1.79 (m, H-1), 1.76 (m, H-2), 1.67 (m, H-12), 1.63 (m, H-7), 1.48 (m, H-6), 1.36 (m, H-1), 1.36 (m, H-17), 1.33 (m, H-16), 1.33 (m, H-20), 1.27 (m, H-6), 1.15 (dd, H-5), 1.01 (s, H-19), 0.87 (d, H-21), 0.79 (s, H-30), 0.75 (s, H-29), 0.72 (s, H-18); FABMS *m*/*z* 687 (M + H)$^+$, 627 (pos).

(S)-MTPA Ester of 3. (*S*)-MTPA ester **5** was prepared as described above.

5: ^{1}H NMR ($CDCl_3$) δ 7.48, 7.37, 4.69 (dd, H-3), 2.18 (dd, H-15), 2.12 (m, H-11), 2.05 (m, H-7), 2.03 (m, H-12), 2.02 (m, H-16), 2.00 (m, H-11), 1.78 (m, H-2), 1.76 (m, H-1), 1.63 (m, H-2), 1.67 (m, H-12), 1.50 (m, H-6), 1.39 (m, H-15), 1.36 (m, H-1), 1.37 (m, H-17), 1.33 (m, H-16), 1.35 (m, H-20), 1.28 (m, H-6), 1.15 (dd, H-5), 0.99 (s, H-19), 0.87 (d, H-21), 0.88 (s, H-30), 0.81 (s, H-29), 0.72 (s, H-18); FABMS *m*/*z* 687 (M + H)$^+$, 627 (pos).

Chiral GC Analysis. A 0.1 mg portion of the fraction of the methanolysis product of **1** was dissolved in a mixture of dry CH_2Cl_2 (50 μL) and trifluoroacetic anhydride (50 μL) and kept at 100 °C for 10 min. The mixture was dried and redissolved in CH_2Cl_2. Retention times: standard, D-GlcU (19.19, 23.20, and 25.35 min), L-GlcU (18.85, 23.47, and 25.46 min), D-GalU (15.90, 20.48, 25.62 min), L-GalU (15.69, 20.28, and 26.10 min), D-Ara (9.37, 10.12, and 14.07 min), and L-Ara (9.39, 9.95, 13.47 min); products from nobiloside, 9.37, 9.93, 13.45, 15.90, 19.17, 20.47, 23.17, 25.32, and 25.58 min. Product peaks from nobiloside were identified by co-injection with the standard samples of L-Ara, D-GlcU, and D-GalU.

Nobiloside Trimethyl Ester (6). A portion of **1** (4.0 mg) was dissolved in MeOH (1 mL) and treated with diazomethane in Et_2O (1 mL). The solvent was dried under reduced pressure. The product was partitioned between H_2O (2 mL) and $CHCl_3$ (2 mL × 3). The organic phase yielded nobiloside methyl ester (**6**) (3.7 mg): ^{1}H NMR (CD_3OD) δ 5.07 (1H, t), 4.48 (1H, d), 4,45 (1H, d), 4,40 (1H, d), 4.38 (1H, m), 4.02 (1H, d), 3.87 (1H, dd), 3,81 (1H, m), 3.77 (3H, s), 3.77 (3H, s), 3.72 (1H, m), 3.59 (3H, s), 3.54 (1H, d), 3,53 (1H, d), 3.16 (1H, dd), 1.66 (3H, s), 1.59 (3H, s), 1.04 (3H, s), 1.03 (3H, s), 0.92 (3H, d), 0.87 (3H, s), 0.79 (3H, s); FABMS *m*/*z* 981 (M − H)$^-$ (neg).

Enzyme Inhibition Assay. Neuraminidase inhibitory activity was examined using the enzyme from the bacterium *Clostridium perfringens* (Sigma N-2876)[19,20] as described previously.[13]

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