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Penarolide Sulfates A_1 and A_2, New α-Glucosidase Inhibitors from a Marine Sponge *Penares* sp.

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Dedicated to Professor Paul J. Scheuer on the occasion of his 85th birthday

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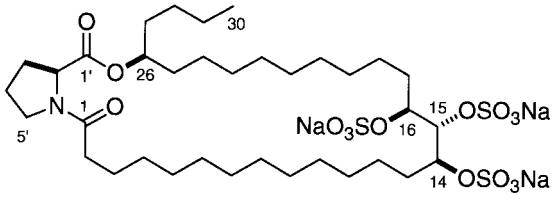
Abstract—Penarolide sulfates A_1 (**1**) and A_2 (**2**) have been isolated as α-glucosidase inhibitors from a marine sponge *Penares* sp.[1] Their structures including absolute stereochemistry were determined to be unique 30- and 31-membered macrolides encompassing a proline residue and three sulfate groups by interpretation of spectral data and chemical degradation. Penarolide sulfates A_1 (**1**) and A_2 (**2**) inhibit α-glucosidase with IC_{50} values of 1.2 and 1.5 μg/mL, respectively.

Introduction

Recent progresses in glycobiology have shed light on significant roles of glycosidases in various biological functions, including immune response, oncogenesis, metastasis of tumors, viral and bacterial infections, and differentiation of neural cells.[2] Specific inhibitors of glycosidases have potentials for treatment of a variety of diseases. α-Glucosidases are involved in glycoprotein processing and glycogenolysis and their inhibitors can be applied for treatment of diabetes, obesity, viral infections, and cancer.[3] In fact, several naturally-occurring inhibitors, e.g. acarbose and *N*-butyl-1-deoxynojirimycin which are sugar mimics, are used or tested in the treatment of diabetes and HIV infection.[3]

In our screening for α-glucosidase inhibitors from Japanese marine invertebrates, we encountered a marine sponge *Penares* sp. collected off Hachijo-jima Island whose hydrophilic extract was highly active. Bioassay-guided fractionation led to the isolation of two active compounds, penarolide sulfates A_1 and A_2 whose structures were assigned as proline-containing macrolide trisulfates. Here we describe the isolation, structure elucidation, and biological activities of these metabolites.



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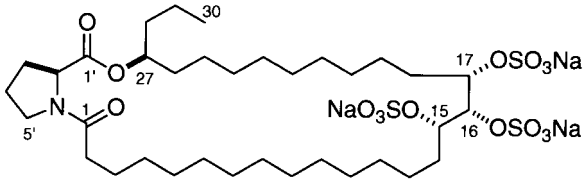

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Results and Discussion

The MeOH extract of the frozen sponge (150 g wet weight) was partitioned between CH_2Cl_2 and H_2O, and the aqueous layer was further extracted with *n*-BuOH. The *n*-BuOH layer was separated by ODS flash chromatography using aq MeOH containing 0.1 M $NaClO_4$ followed by repeated ODS HPLC to yield a major peak. Although the material

Keywords: enzyme inhibitors; macrolides; marine metabolites; mass spectrometry; sponge.

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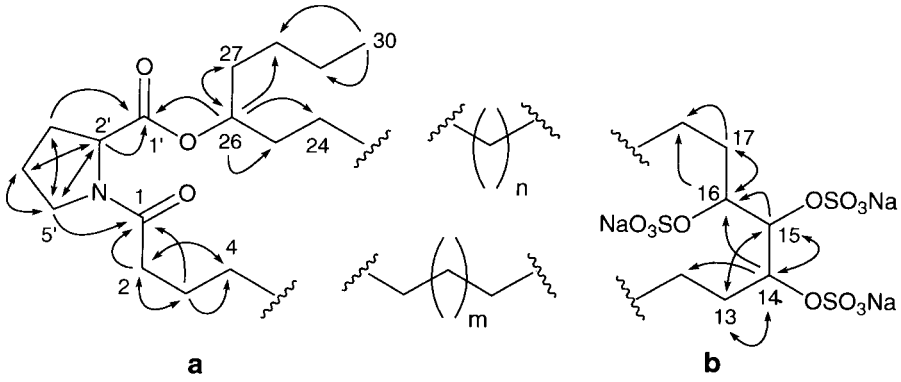


Figure 1. HMBC correlations and partial structures **a** and **b**.

gave a sharp and symmetric peak in ODS HPLC, it was shown to be a 3:2 mixture of two isomers on the basis of 2D NMR data and degradation studies. Further purification by 9 cycles of recycling HPLC afforded two pure compounds, penarolide sulfates A_1 (**1**) and A_2 (**2**) in yields of 6.2×10^{-2} and 4.1×10^{-2}% based on wet weight, respectively.

The molecular formula of penarolide sulfate A_1 (**1**) was determined to be $C_{35}H_{62}NO_{15}S_3Na_3$ on the basis of HR-FABMS and NMR data. Even after rigorous separation, the ^{1}H NMR spectrum contained a series of minor signals associated with the major signals. This was later shown to be due to the conformational isomerism. The ^{1}H NMR, ^{13}C NMR, COSY, and HMQC[4] spectra exhibited the presence of a terminal methyl, a large methylene envelop (δ 1.3), lower field methylenes (δ 1.35–2.4), one each of nitrogen-bearing methylene (δ 3.63, 2H) and methine (δ 4.39), and four oxygenated methines (δ 4.63, 4.66, 4.86, and 5.03) together with two carbonyls (δ 174.3 and 173.8).

Interpretation of 2D NMR spectra led to partial structures **a** and **b** (Fig. 1). Partial structure **a** contained a proline residue, which was straightforward from the ^{1}H and ^{13}C NMR shifts of the α-methine and δ-methylene interposed by two methylenes. Thus, the minor NMR signals were due to the isomerisation of the prolyl amide bond. HMBC[5] data led to the connectivity between the nitrogen of the proline unit and an acyl group through an amide bond. Interpretation of HMBC and HSQC-TOCSY[6] data resulted in connectivities from C1 to C4. On the other hand, the carboxyl group of the proline residue was esterified with an aliphatic secondary alcohol, which was implied by COSY and HMBC data; key HMBC crosspeaks were observed between H26/C1′, C24, C25, C27, C28.

The COSY spectrum also indicated the presence of three contiguous oxygenated methines, which were flanked by two or more methylenes (partial structure **b**). The carbon chemical shifts (δ 78.8, 79.2, and 80.8) were consistent with sulfated methines. The presence of the sulfate groups was also supported by an intense IR absorption at 1240 cm^{-1} and FABMS peaks at *m/z* 97 (HSO_4^-) and 80 (radical anion of SO_3^-). Unfortunately, the partial structure **b** could not be extended even by interpretation of HSQC-TOCSY data due to the degenerate methylene resonances.

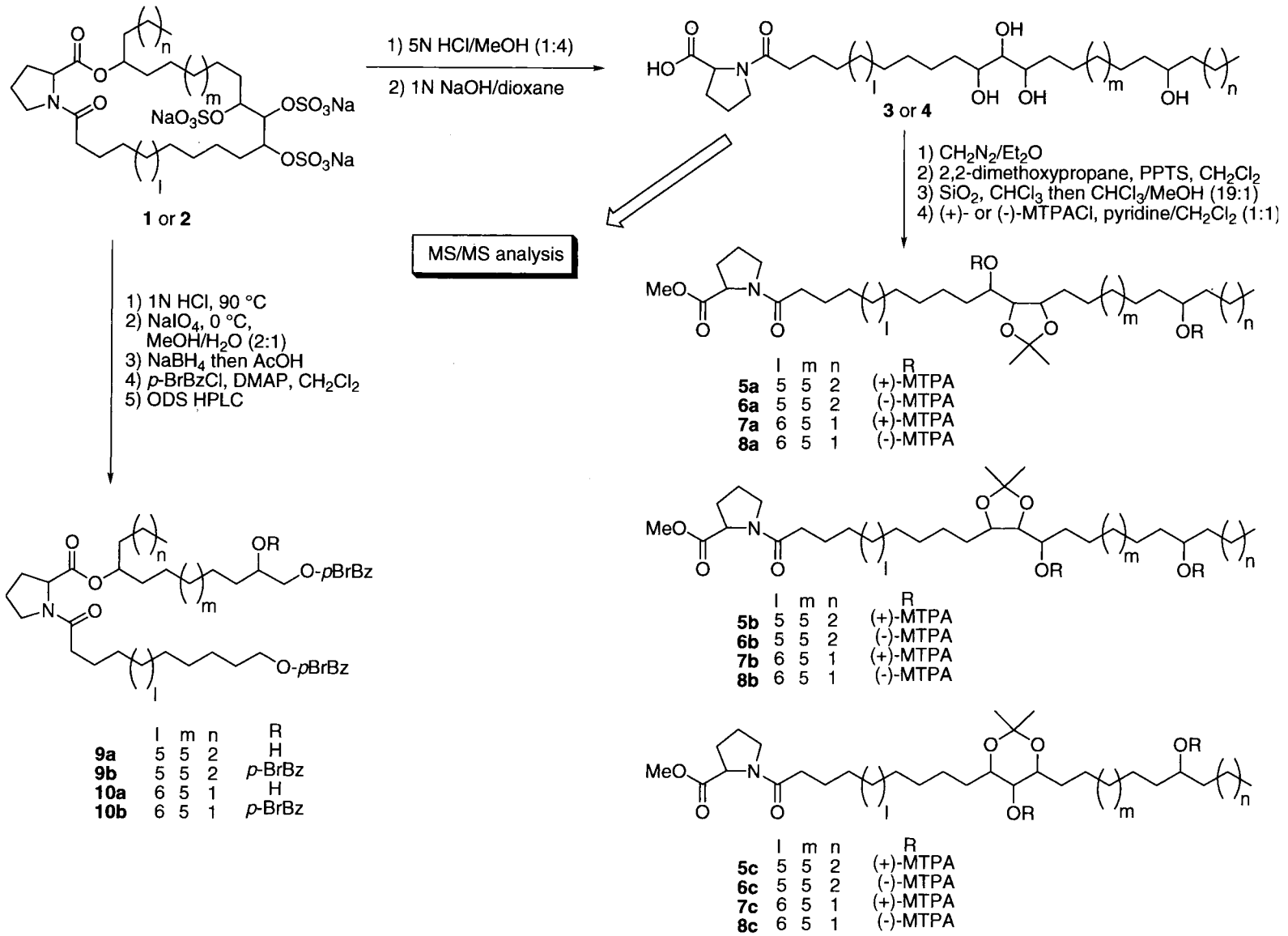
The remaining part of the molecule was composed of simple methylene chains, thus indicating that penarolide sulfate A_1 was bicyclic. Therefore, both ends of partial structures **a** and **b** must be connected through two methylene chains, whose ^{1}H NMR signals were all converged into the envelope.

The lengths of the interconnecting aliphatic chains were determined on the basis of MS/MS data. Penarolide sulfate A_1 was not amenable to MS/MS analysis due to the presence of three negatively-charged sulfate groups in the middle of the cyclic structure. In order to induce the charge-remote fragmentations,[7] it is preferred that a compound has a charged group near the end of a linear structure. Fortunately, the negative charges of the sulfate groups in penarolide sulfate A_1 were removable by acid hydrolysis and the ester group was located favorably for the introduction of a negatively charged carboxylate group by cleavage of the ester linkage (Scheme 1). Penarolide sulfate A_1 (**1**) was hydrolyzed with acid to afford the desulfated lactone as the major product, which was then treated with base followed by extraction with ether to afford the tetraol **3**.

In the negative ion FAB-MS/MS analysis, the $(M-H)^-$ ion peak at *m/z* 612 gave daughter ion peaks at *m/z* 554 and 524 which placed the isolated hydroxyl group at C26. The location of the contiguous triol unit was determined to be at C14, C15, and C16 on the basis of the ions at *m/z* 308, 338, 368, and 380, the first three of which were 30 mass units apart (Fig. 2). The ion at *m/z* 398 was very weak due to a facile dehydration to generate the ion at *m/z* 380. This assignment was confirmed by MS/MS data obtained after deuterium exchange which displayed the number of hydroxyl groups in the fragment ions.

Then we embarked on determination of the absolute stereochemistry of penarolide sulfate A_1, which was indeed challenging, particularly the three contiguous stereogenic centers at C14, C15, and C16. Our strategy was as follows: (1) establishing the orientation of the triol unit by protection with an acetonide followed by MS/MS analysis; (2) determination of the relative stereochemistry of the three chiral centers on the basis of NMR data of the acetonides; and (3) elucidation of the absolute stereochemistry by the modified Mosher's analysis[8] and CD exciton chirality method.

The tetraol **3** was converted to the methyl ester followed by treatment with 2,2-dimethoxypropane to afford a mixture of


1) 5N HCl/MeOH (1:4)
2) 1N NaOH/dioxane
1 or 2
3 or 4
MS/MS analysis
1) 1N HCl, 90 °C
2) NaIO4, 0 °C, MeOH/H2O (2:1)
3) NaBH4 then AcOH
4) p-BrBzCl, DMAP, CH2Cl2
5) ODS HPLC
1) CH2N2/Et2O
2) 2,2-dimethoxypropane, PPTS, CH2Cl2
3) SiO2, CHCl3 then CHCl3/MeOH (19:1)
4) (+)- or (-)-MTPACl, pyridine/CH2Cl2 (1:1)
9a 5 5 2 H
9b 5 5 2 p-BrBz
10a 6 5 1 H
10b 6 5 1 p-BrBz
5a 5 5 2 (+)-MTPA
6a 5 5 2 (-)-MTPA
7a 6 5 1 (+)-MTPA
8a 6 5 1 (-)-MTPA
5b 5 5 2 (+)-MTPA
6b 5 5 2 (-)-MTPA
7b 6 5 1 (+)-MTPA
8b 6 5 1 (-)-MTPA
5c 5 5 2 (+)-MTPA
6c 5 5 2 (-)-MTPA
7c 6 5 1 (+)-MTPA
8c 6 5 1 (-)-MTPA

Scheme 1.

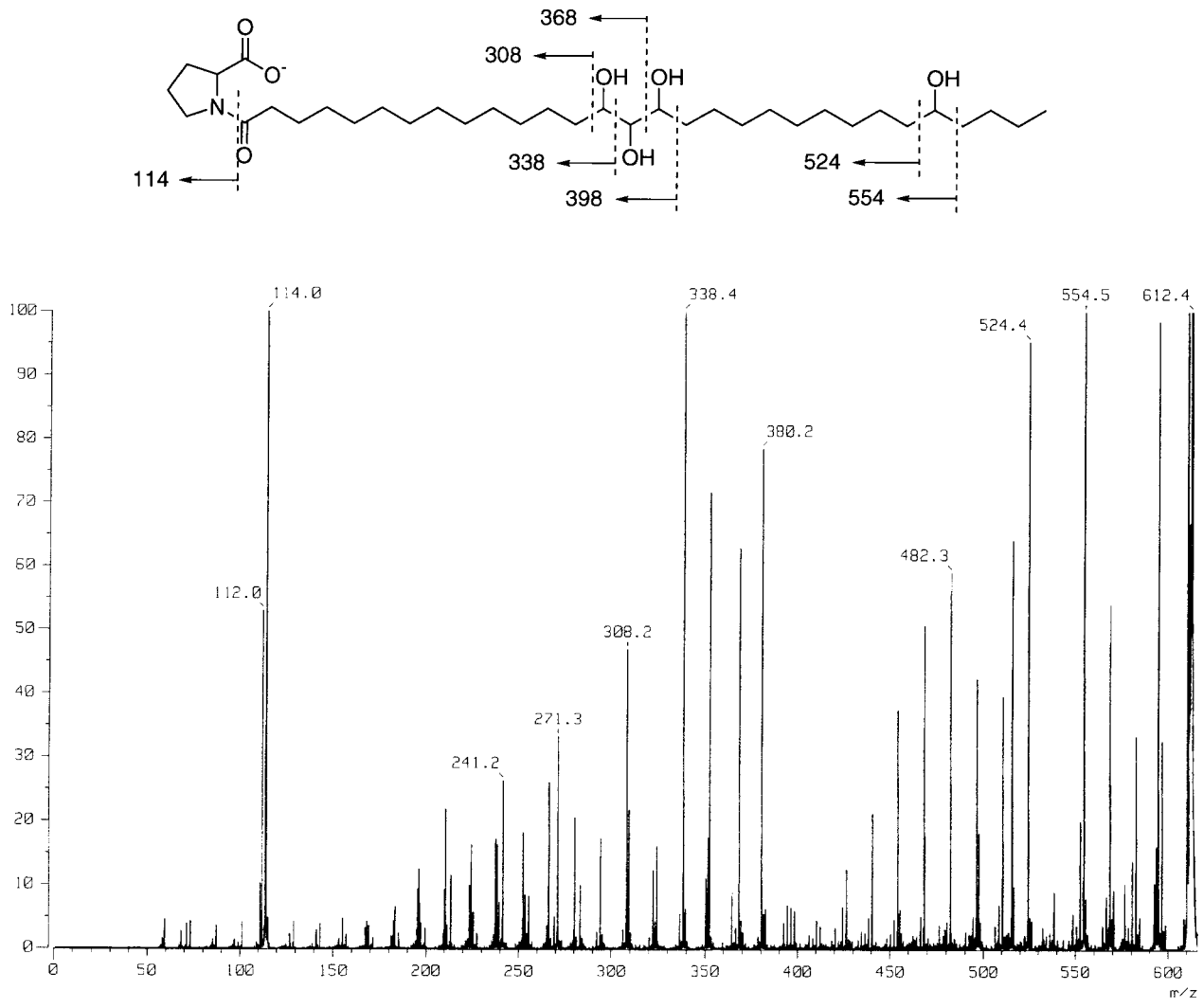


Figure 2. FAB-MS/MS of **3**.

three acetonides. Because of the facile migration of the acetonide groups after separation and for the advantage in HPLC detection, the mixture was esterified with (+)- or (−)-MTPACl to yield mixtures of **5a**–**5c** and **6a**–**6c**, respectively. After HPLC separation, each product was analyzed by COSY experiments, which revealed that **5a/6a**, **5b/6b**, and **5c/6c** had the same substitution patterns.

Inspection of ^{1}H and ^{13}C NMR data for **5a**, **5b**, and **5c** indicated that **5a** and **5b** were 1,2-diol acetonide, while **5c** was 1,3-diol acetonide. In order to determine the location of the acetonide group in **5a** and **5b**, we carried out FAB-MS/MS analysis of **5a** and **5b**. By addition of NaCl, we were able to obtain an intense $(M+Na)^{+}$ ion at *m/z* 1122, which was used as the precursor. Na^{+} ion is thought to reside near the proline residue so that the charge-remote fragmentations were expected to take place (Fig. 3). We compared the data for the three compounds by using the data for **5c** as a reference. Large fragment ions were observed by sequential losses of two MTPA groups and an acetonide (*m/z* 888, 830, 654, and 596). Albeit in lower intensities, fragment ions arising from the cleavage of bonds around the acetonide group were observed. Although many of them were observed in common, a few characteristic ions were observed in a particular compound. The ion at *m/z* 446 was observed only in **5b**, which corresponded to the cleavage between C15 and C16 of the C14, C15-acetonide. On the other hand, the ion at *m/z* 622 was observed only in **5a**, corresponding to a cleavage between C15 and C16 with the retention of the MTPA group on C14 and the oxygen atom on C15. These characteristic ions were also observed in the counterparts **6a** and **6b**. Therefore, **5a** was the C14, C15-acetonide and **5b** was the C15, C16-acetonide.

The relative stereochemistry of the three contiguous chiral centers was assigned on the basis of NOE data for **5a** and **5b**. In **5a**, the acetonide methyls were observed at δ 1.37 and 1.22, only the latter of which exhibited NOE's with H15 and H16, demonstrating that **5a** had a *cis*-acetonide. In **5b**, the acetonide methyls resonated at δ 1.25 and 1.31. The former signal gave NOE with H14, while the latter with H15, indicating that **5b** had a *trans*-acetonide (Fig. 4). This information was in agreement with an *anti*-relationship of the C14, C16-acetonide **5c**, which was evident from the carbon chemical shifts for the acetonide methyls (δ 24.7 and 24.2).[9]

The absolute stereochemistries at C14 and C16 were determined to be 14*S*, 16*S* by comparing the ^{1}H NMR data of **5a**

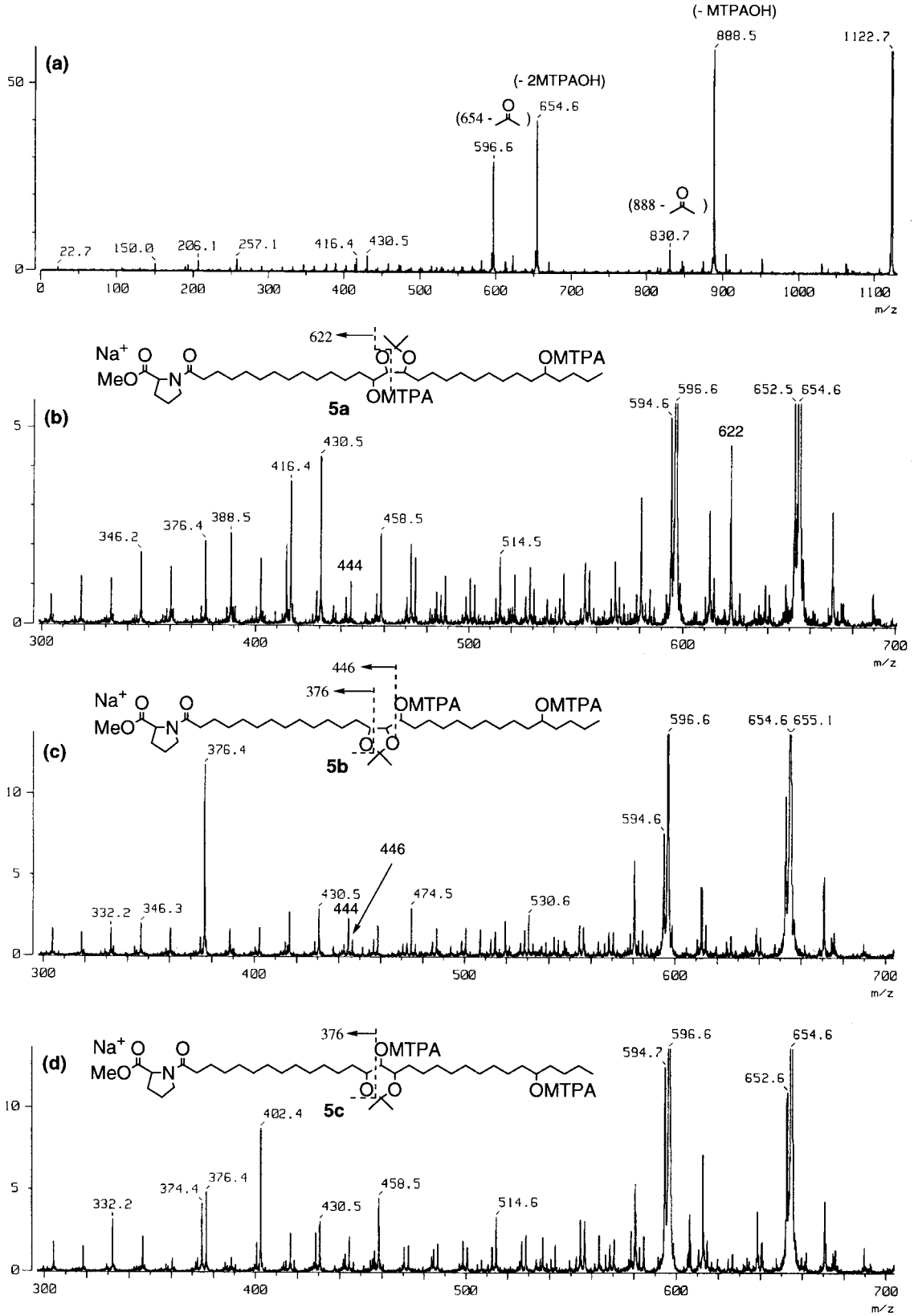


Figure 3. FAB-MS/MS of MTPA Esters **5a**–**c**. (a) FAB-MS/MS of **5a**. (b) FAB-MS/MS of **5a** (expansion). (c) FAB-MS/MS of **5b** (expansion). (d) FAB-MS/MS of **5c** (expansion).

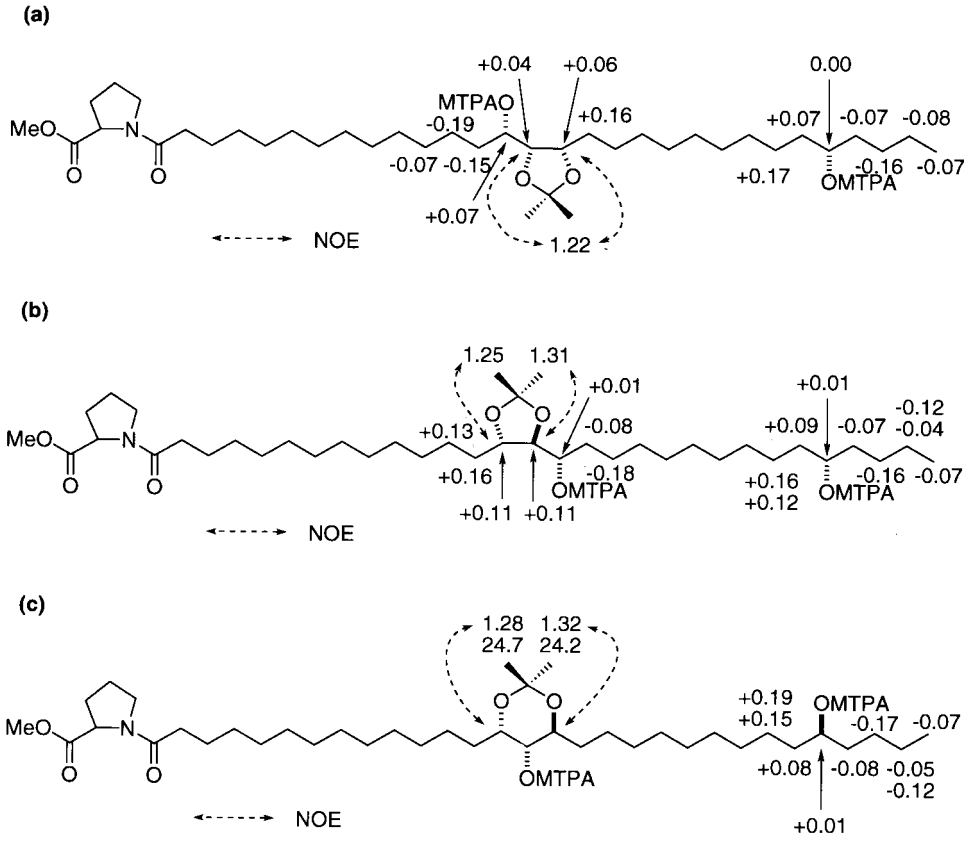


Figure 4. $\Delta\delta$ Values for MTPA esters and NOE's. (a) $\Delta\delta=\delta_{6a}-\delta_{5a}$, NOE's for **5a**. (b) $\Delta\delta=\delta_{6b}-\delta_{5b}$, NOE's for **5b**. (c) $\Delta\delta=\delta_{6c}-\delta_{5c}$, NOE's for **5c**.

and **6a** and those of **5b** and **6b**, respectively (Fig. 4). Considering the relative stereochemistry of the acetonides **5a** and **5b**, the absolute configuration at C15 was assigned as 15*R*.

This assignment was substantiated by the CD exciton chirality method. A 3:2 mixture of **1** and **2** was subjected to a series of reactions [(1) acid hydrolysis, (2) oxidation with $NaIO_4$, (3) reduction with $NaBH_4$, and (4) esterification with *p*-BrBzCl] to afford a mixture of bis-*p*-bromobenzoates (**9a** and **10a**) and tris-*p*-bromobenzoates (**9b** and **10b**), which were separated by HPLC. The FABMS of the four compounds gave characteristic fragment ions arising from the cleavage of the ester bond, thus enabling us to assign which compound was derived from **1** or **2**. The cleavage of the triol moiety preferentially took place between C14 and C15 in **1** and C15 and C16 in **2**. This tendency is in accordance with those reported for $NaIO_4$ oxidation of palytoxin, in which *syn*-diols were cleaved faster than *anti*-diols.[10] The positively split CD curve of **9b** at 253/237 nm led to the 16*S* stereochemistry for **1**.[11]

The absolute stereochemistry at C26 was determined to be *S* by the modified Mosher's method for the acetonides. Stereochemistry of the Pro residue was determined to be L by Marfey analysis of the acid hydrolysate.[12]

Penarolide sulfate A_2 (**2**) had a molecular formula identical to that of **1**, as determined by the HR-FABMS and NMR data. The ^1H and ^{13}C NMR spectra of **2** were almost superimposable on those of **1**, except for the chemical shifts for C28 and C29, which indicated the difference in the site of esterification. Interpretation of 2D NMR data indicated that **2** had the same sets of functional groups as **1**, with an esterification at C27.

The structure of penarolide sulfate A_2 (**2**) was determined as in the case of **1** (Scheme 1). The acid and base hydrolysis afforded the tetraol **4**, which was analyzed by FAB-MS/MS using the $(M-H)^-$ ion peak at *m/z* 612 as a precursor. The daughter ion peaks at *m/z* 568 and 538 were consistent with the location of the oxygenation at C27, while the positions of the triol unit was shown to be at C15, C16, and C17 on the basis of the ion peaks at *m/z* 322, 352, 382, and 394 (Fig. 5). The MTPA esters of the acetonides were prepared and analyzed as described above, which resulted in the 15*S*, 16*R*, 17*S*, 27*S* stereochemistry for **2**. The proline unit was in the L-form as determined by the Marfey analysis of the acid hydrolysate.

From marine sponges belonging to the genus *Penares*, two types of aliphatic nitrogenous compounds are known; penaresidins A and B are sphingosine-derived azetidines isolated as potent actomyosin ATPase-activators from an Okinawan *Penares* sp.,[13] while penaramides are polyamines isolated from *Penares* aff. *incrustans* which inhibit binding of ω-conotoxin GVIA to N-type Ca^{2+} channels.[14] Penarolide sulfates A_1 (**1**) and A_2 (**2**) are the unique 30- and 31-membered macrolides encompassing a proline residue and three sulfate groups. Recently, architectually related

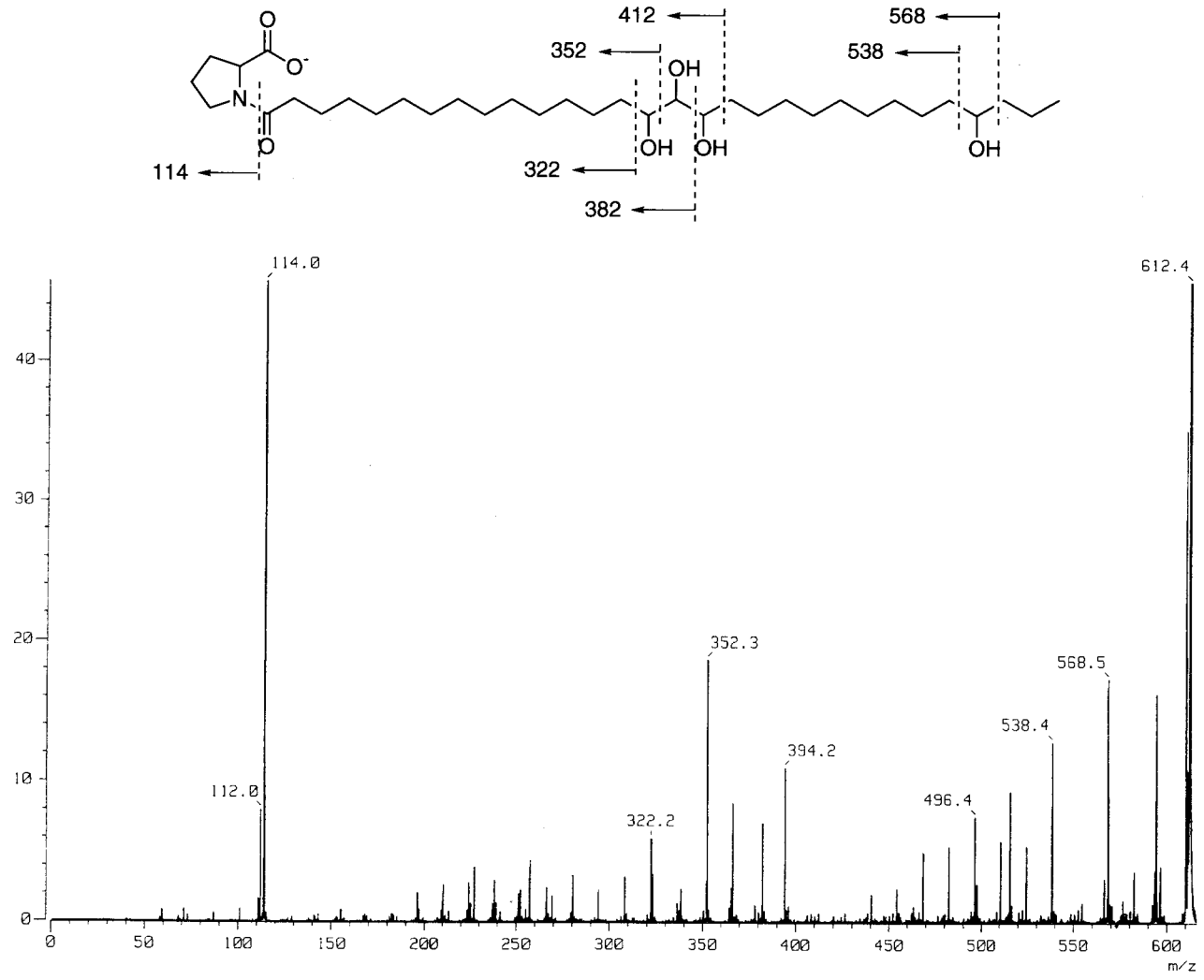


Figure 5. FAB-MS/MS of **4**.

compound, cyclodidemniserinol trisulfate was reported from the Palauan ascidian *Didemnum guttatum*.[15]

Penarolide sulfates A_1 (**1**) and A_2 (**2**) inhibited α-glucosidase with IC_{50} values of 1.2 and 1.5 μg/mL, respectively; 30–40 times more potent than 1-deoxynojirimycin (Table 1). Interestingly, they were only marginally active against β-glucosidase at a concentration of 100 μg/mL; castanospermine and 1-deoxynojirimycin showed IC_{50} values of 15 and 96 μg/mL, respectively. All four compounds did not inhibit β-galactosidase at 100 μg/mL. Because penarolide sulfates inhibited thrombin as did other sulfated compounds,[16] it is of interest to examine whether the sulfate groups in these compounds are indispensable for their enzyme inhibitory activities.

Table 1. Enzyme inhibitory activity of penarolide sulfates A_1 (**1**), A_2 (**2**) and known glucosidase inhibitors (IC_{50}, μg/mL)

	1	**2**	1-Deoxynojirimycin	Castanospermine
α-glucosidase	1.2	1.5	48	N/A[a]
β-glucosidase	19%[b]	16%[b]	96	15
β-galactosidase	N/A[a]	N/A[a]	N/A[a]	N/A[a]
thrombin	3.7	4.2	1.4	16%[b]

[a] N/A: not active at 100 μg/mL.
[b] Percent inhibition at 100 μg/mL.

Experimental

General

NMR spectra were recorded on a JEOL α-600 spectrometer in CD_3OD. FAB-MS data were obtained using a JEOL SX102 mass spectrometer using triethanolamine (negative ion mode) or *p*-nitrobenzylalcohol+NaCl (positive ion mode) as matrices. Negative mode HR-FABMS spectra were obtained at resolution of 5000 using PEG sulfate 1000 as a marker. FAB-MS/MS experiments were performed on a JEOL SX102/SX102 tandem mass spectrometer. The geometry of the spectrometer was BEBE with an accelerating voltage of 10 kV. A collision cell located in the third field-free region was not floated. Helium was introduced to cause the dissociation at a pressure that reduced the intensity of precursor ions to 10%. Optical rotation was measured on a JASCO DIP-1000 digital polarimeter. UV spectra were recorded on a Hitachi 330 spectrophotometer. CD spectra were recorded in MeOH using JASCO J-820

spectropolarimeter. IR spectra were obtained using JASCO FT/IR-230 Fourier transform infrared spectrometer.

Sponge sample

The sponge specimens were collected at a depth of 20 m off Hachijo-jima Island, 300 km south of Tokyo. The sponge is a globular mass of approximately 8×4×4 cm with a mottled surface due to encrusting patches of hydroids, colonial tunicates, and coralline algae. Colour reddish or dark brown, interior whitish beige. Consistency hard, incompressible, compact. Surface skeleton consists of a crust of 150–200 μm thickness, made up of small and intermediate-sized oxeas arranged in all directions, but predominantly tangentially. The crust is carried by radiating columns of large oxeas of 350–500 μm in diameter, which fan out at the surface to form supporting spicule bundles and single spicules. Aster microscleres are rare in the surface crust, but common along interior columns and lining the canals and cavities. Spicules: smooth fusiform oxeas in three distinct categories, large choanosomal oxeas, frequently with stylotes ending, 750–1100×18–28 μm, intermediate oxeas of the surface crust, 220–300×10–14 μm, and small, often centrotylote oxeas of the surface crust, 60–120×3–9 μm. Tylasters of a single category but variable in size and ray number, occasionally appearing to be oxyasters, 9–15 μm in diameter, with 8–12 rays. This combination of characters is found only in the genus *Penares* (Class Demospongiae, Order Astrophorida, family Ancorinidae), but the apparently complete lack of triaenes is unusual and prevents definite identification to a described species. A voucher fragment is incorporated in the collection of the Zoological Museum Amsterdam, under number POR. 11061.

Extraction and isolation

The frozen sponge (150 g wet weight) was extracted with MeOH, and the concentrated extract was partitioned between CH_2Cl_2 and H_2O. The aqueous layer was further extracted with *n*-BuOH; the *n*-BuOH layer was separated by ODS flash chromatography using aq MeOH containing 0.1 M $NaClO_4$ as the mobile phase. The active fraction eluted with 90% MeOH containing 0.1 M $NaClO_4$ was separated by ODS HPLC (COSMOSIL-5C_{18}ARII; 30% *n*-PrOH containing 0.2 M $NaClO_4$) followed by ODS HPLC on the same column with *n*-PrOH/MeCN/H_2O (20:25:60) containing 0.2 M $NaClO_4$ to give a major peak (277.4 mg). This was further separated by ODS HPLC [COSMOSIL-5C_{18}MS, *n*-PrOH/MeCN/H_2O (2:3:6) containing 0.2 M $NaClO_4$] to afford a single peak (188.9 mg). As this single peak fraction (=fr. 1) turned out to be a mixture of two compounds, a 40 mg portion of fr. 1 was finally separated by nine cycles of recycling HPLC on ODS column [Inertsil ODS-3, *n*-PrOH/MeCN/H_2O (2:3:6) containing 0.2 M $NaClO_4$] to yield penarolide sulfate A_1 (**1**; 19.6 mg) and penarolide sulfate A_2 (**2**; 12.9 mg).

Penarolide sulfate A_1 (1). Colorless amorphous solid; HR-FABMS $(M-Na)^-$ *m/z* 878.3056 for $C_{35}H_{62}NO_{15}S_3Na_2$ (Δ −2.1 mmu); $[\alpha]_D^{29}=-25.7°$ (*c* 0.5, MeOH); UV (MeOH) 204 nm (ε 9,100); IR (film) 1730, 1640, 1240 cm^{-1}; ^1H and ^{13}C NMR, see Table 2.

Penarolide sulfate A_2 (2). Colorless amorphous solid; HR-FABMS $(M-Na)^-$ *m/z* 878.3058 for $C_{35}H_{62}NO_{15}S_3Na_s$ (Δ −1.9 mmu); $[\alpha]_D^{29}=-25.2°$ (*c* 0.3, MeOH); UV (MeOH) 205 nm (ε 9,000); IR (film) 1730, 1640, 1240 cm^{-1}; ^1H and ^{13}C NMR, see Table 2.

Tetraol 3 and 4

Penarolide sulfates A_1 (**1**; 8.1 mg) was dissolved in 1 mL of 5N HCl/MeOH (1:4) and heated at 100°C for 1 h in a sealed vial. The reaction mixture was neutralized with 1N NaOH, dried in a stream of N_2. The residue was dissolved in 4 mL of 1N NaOH/dioxane (1:1) and heated at 60°C for 1 h. After cooling to rt, the solution was neutralized with 1.1 mL of 6N HCl and extracted with Et_2O. The Et_2O extract was washed with H_2O and evaporated to yield the tetraol **3**. Penarolide sulfate A_2 (2; 2.2 mg) was treated in the same manner to afford **4**.

MTPA esters

The tetraol **3** was treated with diazomethane and evaporated to afford the methyl ester. This material was dissolved in 2 mL of CH_2Cl_2 and 2,2-dimethoxypropane containing catalytic amounts of PPTS, and the mixture was stirred for 1 h at 0°C. The reaction mixture was passed through a short SiO_2 column [$CHCl_3$ and $CHCl_3$/MeOH (19:1)] to remove PPTS. The fraction containing a mixture of acetonides was separated in two portions. To each portion dissolved in 0.1 mL of C_5H_5N/CH_2Cl_2 (1:1) was added 7.5 μl of (+)- or (−)-MTPACl at room temperature, the reaction proceeded immediately and the product was separated by ODS HPLC (COSMOSIL 5C_{18}-ARII, linear gradient of 95–100% MeOH) to afford **5a**–**5c** and **6a**–**6c**, respectively. The tetraol **4** was reacted in the same manner to give **7a**–**7c** and **8a**–**8c** by reaction with (+)- or (−)-MTPACl, respectively.

5a. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.29 (H11), 1.38 (H12), 1.66 (H13), 5.19 (H14), 4.11 (H15), 4.08 (H16), 1.40 (H17a), 1.27 (H17b), 1.15 (H24), 1.56 (H25), 5.09 (H26), 1.64 (H27), 1.32 (H28), 1.32 (H29), 0.90 (H30), 1.37 (3H s, CH_3 of acetonide), 1.22 (3H s, CH_3 of acetonide).

5b. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.25 (H11), 1.16 (H12), 1.35 (H13), 3.73 (H14 and 15), 5.17 (H16), 1.76 (H17), 1.35 (H18), 1.27 (H19), 1.35 (H23), 1.20 (H24a), 1.16 (H24b), 1.56 (H25), 5.08 (H26), 1.64 (H27), 1.32 (H28), 1.32 (H29), 0.90 (H30), 1.31 (3H s, CH_3 of acetonide), 1.25 (3H s, CH_3 of acetonide).

5c. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.30 (H11), 1.26 (H12a), 1.18 (H12b), 1.14 (H13a), 1.13 (H13b), 3.89 (H14), 5.01 (H 15), 3.64 (H16), 1.60 (H17a), 1.44 (H17b), 1.27 (H18a), 1.26 (H18b), 1.20 (H24a), 1.15 (H24b), 1.56 (H25), 5.08 (H26), 1.65 (H27), 1.32 (H29), 0.90 (H30), 1.32 (3H s, CH_3 of acetonide), 1.28 (3H s, CH_3 of acetonide).

6a. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.21 (H11), 1.19 (H12), 1.51 (H13), 5.26 (H14), 4.15 (H15), 4.14 (H16), 1.56 (H17), 1.30 (H18), 1.34 (H19), 1.30 (H23), 1.32 (H24), 1.63 (H25), 5.09 (H26), 1.57

Table 2. NMR data for penarolide sulfates A_1 (**1**) and A_2 (**2**) in CD_3OD

1				**2**			
#	^{13}C	^{1}H mult. J (Hz)	HMBC	#	^{13}C	^{1}H mult. J (Hz)	HMBC
1	174.3			1	174.3		
2	35.2	2.43 dt 15.0, 7.7	C: 1, 3, 4	2	35.1	2.42 dt 15.0, 7.5	C: 1, 3, 4
		2.25	C: 1, 3, 4			2.28	C: 1, 3, 4
3	26.1	1.64	C: 2	3	26.0	1.65	C: 1, 2, 4
		1.57	C: 1, 2, 4			1.57	C: 1, 2, 4
4	30.5	1.39	C: 2	4	30.1	1.3[a]	
		1.32	C: 2				
5–11	30[a]	1.3[a]		5–12	30[a]	1.3[a]	
12	26.1	1.60					
		1.50					
13	31.3	1.81	C: 15	13	25.8		
		1.52	C: 14				
14	78.8	4.63 q 4.2	C: 12, 13, 15, 16	14	31.5	1.87	
						1.57	C: 15
15	80.8	5.03 d 5.4	C: 13, 14, 16	15	78.6	4.67 q 5.9	C: 13, 14, 16, 17
16	79.2	4.66 d 9.6	C: 17, 18	16	80.6	4.96 d 5.8	C: 14, 15, 17, 18
17	31.7	1.82	C: 16, 18, 19	17	79.5	4.70 bd 6.9	C: 15, 18, 19
		1.56	C: 18, 19				
18	26.4	1.55		18	31.9	1.82	C: 16, 17
		1.45				1.59	C: 16
19	30.5	1.3[a]		19	26.5		
20–23	30[a]	1.3[a]		20–24	30[a]	1.3[a]	
24	26.1						
25	35.2	1.50		25	26.0	1.24	
		1.47					
26	76.3	4.86 quin. 6.2	C: 24, 25, 27, 28, 1′	26	35.1	1.50	
27	34.7	1.55	C: 26	27	76.0	4.88 quin. 6.2	C: 25, 26, 28, 29, 1′
		1.52	C: 26				
28	28.6	1.34		28	37.4	1.55	C: 26, 27, 29, 30
		1.26				1.48	C: 26, 27, 29, 30
29	23.5	1.33		29	19.6	1.35	C: 27, 28, 30
		1.28				1.29	C: 27, 28, 30
30	14.3	0.90 t 6.9	C: 28, 29	30	14.3	0.92 t 7.5	C: 28, 29
1′	173.8			1′	173.7		
2′	61.1	4.39 dd 8.9, 4.6	C: 1′, 3′, 4′, 5′	2′	61.0	4.39 dd 8.5, 4.2	C: 1′, 4′
3′	30.4	2.26	C: 1′, 2′, 5′	3′	30.4	2.26	C: 1′, 2′, 4′, 5′
		1.95	C: 1′, 2′, 5′			1.95	C: 1′, 2′, 4′, 5′
4′	25.8	2.07	C: 2′, 5′	4′	25.8	2.05	C: 2′, 3′, 5′
		2.06	C: 2′, 5′			2.02	C: 2′, 3′, 5′
5′	48.5	3.63 t 7.7	C: 1, 2′, 3′, 4′	5′	48.5	3.63	C: 2′, 3′, 4′

[a] Overlap of methylene signals.

(H27), 1.16 (H28), 1.24 (H29), 0.83 (H30), 1.40 (3H s, CH_3 of acetonide), 1.31 (3H s, CH_3 of acetonide).

6b. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.29 (H12), 1.51 (H13), 3.84 (H14 and 15), 5.18 (H16), 1.68 (H17), 1.17 (H18), 1.32 (H24), 1.65 (H25), 5.09 (H26), 1.57 (H27), 1.16 (H28), 1.28 (H29a), 1.20 (H29b), 0.83 (H30), 1.36 (3H s, CH_3 of acetonide), 1.34 (3H s, CH_3 of acetonide).

6c. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.26 (H11), 1.37 (H12a), 1.25 (H12b), 1.52 (H13), 3.47 (H14), 4.98 (H 15), 3.93 (H16), 1.40 (H17a), 1.36 (H17b), 1.27 (H18), 1.32 (H24), 1.64 (H25), 5.09 (H26), 1.56 (H27), 1.15 (H28), 1.27 (H29a), 1.20 (H29b), 0.83 (H30), 1.28 (3H s, CH_3 of acetonide), 1.23 (3H s, CH_3 of acetonide).

7a. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.29 (H12), 1.38 (H13), 1.66 (H14), 5.20 (H15), 4.10 (H16), 4.08 (H17), 1.39 (H18), 1.21 (H19), 1.21 (H25), 1.63 (H26), 5.10 (H27), 1.56 (H28), 1.36 (H29), 0.93 (H30), 1.37 (3H s, CH_3 of acetonide), 1.22 (3H s, CH_3 of acetonide).

7b. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.26 (H13a), 1.17 (H13b), 1.36 (H14), 3.73 (H15 and 16), 5.17 (H17), 1.74 (H18), 1.34 (H19), 1.31 (H20), 1.20 (H25), 1.64 (H26), 5.09 (H27), 1.57 (H28), 1.37 (H29), 0.93 (H30), 1.31 (3H s, CH_3 of acetonide), 1.25 (3H s, CH_3 of acetonide).

7c. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.29 (H13), 1.14 (H14), 3.89 (H15), 5.00 (H 16), 3.63 (H17), 1.60 (H18), 1.27 (H19), 1.19 (H25), 1.65 (H26), 5.09 (H27), 1.58 (H28), 1.36 (H29), 0.93 (H30), 1.32 (3H s, CH_3 of acetonide), 1.29 (3H s, CH_3 of acetonide).

8a. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.21 (H12), 1.19 (H13), 1.51 (H14), 5.26 (H15), 4.15 (H16), 4.14 (H17), 1.55 (H18), 1.29 (H19), 1.33 (H24), 1.29 (H25), 1.63 (H26), 5.10 (H27), 1.55 (H28), 1.19 (H29), 0.81 (H30), 1.40 (3H s, CH_3 of acetonide), 1.31 (3H s, CH_3 of acetonide).

8b. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.57 (H14a), 1.52 (H14b), 3.83 (H15 and 16), 5.18 (H17), 1.69 (H18), 1.22 (H19), 1.26 (H20), 1.31 (H24), 1.26 (H25), 1.63 (H26), 5.12 (H27), 1.56 (H28), 1.19 (H29), 0.85 (H30), 1.36 (3H s, CH_3 of acetonide), 1.34 (3H s, CH_3 of acetonide).

8c. FAB-MS (matrix; NBA+NaCl) *m/z* 1122 $(M+Na)^+$; ^1H NMR (CD_3OD) δ 1.26 (H13), 1.52 (H14), 3.47 (H15), 4.98 (H 16), 3.93 (H17), 1.39 (H18a), 1.38 (H18b), 1.27 (H19), 1.33 (H24), 1.27 (H25), 1.63 (H26), 5.10 (H27), 1.55 (H28), 1.19 (H29), 0.85 (H30), 1.28 (3H s, CH_3 of acetonide), 1.23 (3H s, CH_3 of acetonide).

Preparation of bis- and tris-*p*-bromobenzoates 9a,b and 10a,b

A portion (10 mg) of fr. 1 was hydrolyzed with 1N HCl at 90°C for 1 h, followed by extraction with $CHCl_3$. A part (5 mg) of the $CHCl_3$ extract was dissolved in 3 mL of H_2O/MeOH (1:2), to which was added with 2.7 mg (1.5 equiv.) of $NaIO_4$, and the mixture was stirred for 15 min at 0°C. The reaction was terminated by adding excess $NaBH_4$. After quenching by addition of 50 μl of AcOH, the reaction mixture was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was dried and dissolved in 1 mL of CH_2Cl_2/pyridine (1:1). To this solution, *p*-bromobenzoylchloride (in CH_2Cl_2) and a catalytic amount of DMAP were added, and the mixture was stirred until a spot of starting material disappeared in TLC. The reaction mixture was then partitioned beteween H_2O and $CHCl_3$. The $CHCl_3$ layer was evaporated and separated by ODS HPLC to furnish bis-*p*-bromobenzoates **9a** (0.3 mg) and **10a** (0.3 mg), and tris-*p*-bromobenzoates **9b** (0.9 mg) and **10b** (0.8 mg).

9a. FAB-MS (matrix; NBA+NaCl) *m/z* 988/986/984 $(M+Na)^+$, 966/964/962 $(M+H)^+$, 526/524 $(M+H-C_{23}H_{35}BrO_3)^+$; ^1H NMR (CD_3OD) δ 7.95 (2H, d, 8.8), 7.90 (2H, d, 8.8), 7.65 (4H, d, 8.5), 4.40 (1H, dd, 8.9, 4.2; H1′), 4.30 (2H, t, 6.5; H14), 4.29 (1H, dd, 11.2, 3.9; H15a), 4.20 (1H, dd, 11.2, 6.5; H15b), 3.88 (1H, m; H16).

9b. FAB-MS (matrix; NBA+NaCl) *m/z* 1172/1170/1168/1166 $(M+Na)^+$, 1150/1148/1146/1144 $(M+H)^+$, 526/524 $(M+H-C_{30}H_{38}Br_2O_4)^+$; ^1H NMR (CD_3OD) δ 7.89 (4H, d, 8.5), 7.83 (2H, d, 8.5), 7.64 (4H, d, 8.5), 7.61 (2H, d, 8.5), 5.46 (1H, m; H16), 4.60 (1H, dd, 11.9, 2.7; H15a), 4.44 (1H, dd, 11.9, 7.3; H15b), 4.39 (1H, dd, 8.8, 4.2; H1′), 4.29 (2H, t, 6.5; H14); UV (MeOH) 244 nm (ϵ 48,000); CD (MeOH) λ_{ext} 253 nm ($\Delta\epsilon$ +8.6), 237 (−4.6).

10a. FAB-MS (matrix; NBA+NaCl) *m/z* 988/986/984 $(M+Na)^+$, 966/964/962 $(M+H)^+$, 540/538 $(M+H-C_{22}H_{33}BrO_3)^+$; ^1H NMR (CD_3OD) δ 7.95 (2H, d, 8.8), 7.90 (2H, d, 8.8), 7.65 (4H, d, 8.5), 4.40 (1H, dd, 8.5, 3.9; H1′), 4.30 (2H, t, 6.5; H15), 4.27 (1H, dd, 11.2, 3.8; H16a), 4.20 (1H, dd, 11.2, 6.5; H16b), 3.88 (1H, m; H17).

10b. FAB-MS (matrix; NBA+NaCl) *m/z* 1172/1170/1168/1166 $(M+Na)^+$, 1150/1148/1146/1144 $(M+H)^+$, 540/538 $(M+H-C_{30}H_{38}Br_2O_4)^+$; ^1H NMR (CD_3OD) δ 7.89 (4H, d, 8.5), 7.83 (2H, d, 8.9), 7.64 (4H, d, 8.8), 7.61 (2H, d, 8.8), 5.46 (1H, m; H17), 4.60 (1H, dd, 11.9, 3.1; H16a), 4.44 (1H, dd, 11.9, 7.3; H16b), 4.39 (1H, dd, 8.8, 4.2; H1′), 4.29 (2H, t, 6.5; H15); UV (MeOH) 244 nm (δ 29,000); CD (MeOH) λ_{ext} 254 nm ($\Delta\epsilon$ +6.0), 237 (−3.3).

Marfey analysis

A 300 μg portion of penarolide sulfates A_1 was dissolved in 400 μL of 12N HCl/MeOH (1:1) and the mixture was heated at 110°C for 17 h in a sealed tube. After cooling to rt, the hydrolysate was diluted with 300 μL of H_2O and washed with EtOAc. The aqueous layer was dried under a stream of N_2 and dissolved in 100 μL of 0.1 M $NaHCO_3$. To the solution was added a 50 μL portion of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) in acetone (1 mg/mL), and the mixture was kept at 80°C for 10 min. The reaction mixture was neutralized with 50 μL of 0.2N HCl and diluted with 50 μL of 50% MeCN containing 0.05% TFA. A portion of this solution was subjected to ODS HPLC (COSMOSIL $5C_{18}$-MS, 20% MeCN containing 50 mM NH_4OAc). The peak at 12.8 min was identified as L-Pro by comparison with the standard Pro.

Enzyme inhibition assays

Enzyme inhibition assays were performed following the modified method of Cannell et al.[17] α-Glucosidase (Type III from Yeast, G7256) and the substrate, *p*-nitrophenyl α-D-glucopyranoside (N-1377) were purchased from Sigma Chemical Co (St. Louis, MO, USA). To each well of microtiter plates were added 20 μL of test solution, 80 μL of H_2O, 25 μL of buffer solution (0.5 M potasium phosphate, 16 mM $MgCl_2$, pH 6.8), and 50 μL of enzyme solution (0.5 U/mL). After preincubated at 37°C for 30 min, 50 μL of substrate solution (5 μmol/mL) was added to each well. The absorbance at 410 nm was measured after the incubation at 37°C for 30 min, using TOSOH MPR-A4i II micro plate reader. IC_{50} value is defined as the concentration of α-glucosidase inhibitor to inhibit 50% of its activity under the assayed conditions.

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