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Penasulfate A, a New α-Glucosidase Inhibitor from a Marine Sponge *Penares* sp.†

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A new α-glucosidase inhibitor, penasulfate A, has been isolated from a marine sponge *Penares* sp.[1] Its structure was elucidated by spectral and chemical methods to be a scalemic mixture of methyl pipecolates acylated with a novel sulfated fatty acid.

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] have become obvious through the recent advances in glycobiology.[2] α-Glucosidases are known to be involved in glycogenolysis and glycoprotein processing in the endoplasmic reticulum (ER). In addition to the application for treatment of diabetes, α-glucosidase inhibitors, which cause misfolding of viral glycoproteins and interfere with the viral life cycle, are expected to be antiviral drugs against HIV or HBV infection.[3]

Previously, we have reported penarolide sulfates A_1 (**1**) and A_2 (**2**), whose structures were assigned as proline-containing macrolide trisulfates, from a marine sponge *Penares* sp.[4] Further investigation of this sponge led to the isolation of a new inhibitor, penasulfate A (**3**).[5] In this paper we describe the isolation, structure elucidation, and biological activities of this compound.

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 flash chromatography on ODS with aqueous MeOH containing 0.1 M $NaClO_4$. The fraction eluted with 90% MeOH, which showed potent inhibition against α-glucosidase, was separated by repetitive reversed-phase HPLC on ODS to afford a peak and was further purified by two steps of recycling HPLC on ODS to yield an apparently homogeneous fraction (fr. 1).

Because NMR data of fr. 1 did not show any hint that it is a mixture,[6] we started structure elucidation using this sample. The IR spectrum (1240 cm^{-1}), the negative ion mode FABMS [at *m/z* 97 (HSO_4^-) and 80 (radical anion of SO_3^-)], and characteristic MS fragmentation pattern observed at *m/z* 776 $(M - Na)^-$, 754 $(M - 2Na + H)^-$, and 674 $(M - Na - NaO_3S + H)^-$ of fr. 1 indicated the presence of two sulfate groups. The composition of the ion peak at *m/z* 776 was determined as $[C_{36}H_{67}NO_{11}S_2Na]^-$ on the basis of HRFABMS [*m/z* 776.4019 $(M - Na)^-$, Δ −2.2 mmu].

The ^1H NMR, ^{13}C NMR, COSY, and HMQC[7] spectra exhibited the presence of a terminal (CH_3-28: δ_C 14.4; δ_H 0.89 t, $J = 6.9$ Hz), a branched (CH_3-29: δ_C 20.1; δ_H 0.85 d, $J = 6.5$), and an oxygenated (OCH_3-1′: δ_C 52.7; δ_H 3.72 s) methyl, a large methylene envelope (δ_H 1.3), lower field methylenes (δ_H 1.35−2.4), a nitrogenous methylene (CH_2-6′: δ_C 44.9; δ_H 3.87 and 3.21) and methine (CH-2′: δ_C 53.4; δ_H 5.25), and two oxygenated methines (CH-14 and 15: δ_C 81.3; δ_H 4.46, 2H), together with two carbonyl carbons (C-1 and 1′: δ_C 175.9 and 173.1, respectively).

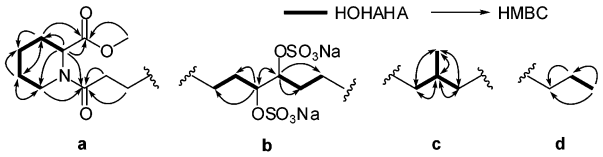

Figure 1. HOHAHA and HMBC correlations for partial structures **a**−**d**.

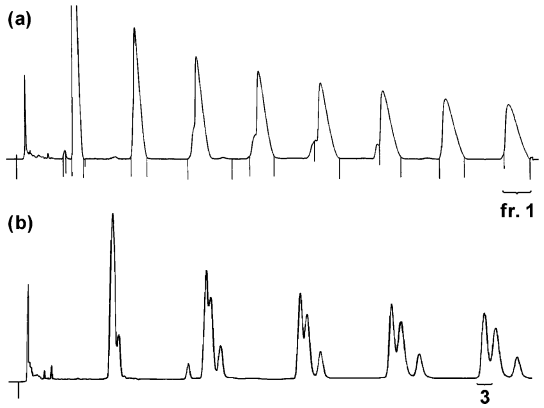

Figure 2. Recycling HPLC charts: (a) the chromatogram of the active fraction to afford fraction 1 [Inertsil ODS-3, *n*-PrOH/MeCN/H_2O (4:2:4) containing 0.2 M $NaClO_4$] and (b) the chromatogram of fr. 1 to afford penasulfate A (**3**) [Develosil C_{30}-UG-5, with *n*-PrOH/MeCN/H_2O (35:25:40) containing 0.2 M $NaClO_4$].

Interpretation of HOHAHA[8] and HMBC[9] data led to partial structures **a**−**d** (Figure 1). The connectivities from the methine at δ_H 5.25 (H-2′) to the methylene protons at δ_H 3.87 and 3.21 (H_2-6′) via three consecutive methylenes (H_2-3′, 4′, and 5′) were readily deduced from the HOHAHA spectrum. An HMBC correlation from H-2′ to C-6′ connected C-2′ and C-6′ via a nitrogen atom to form a piperidine unit. Further HMBC cross-peaks, C-1/H-2′ and H-6′, and C-1′/H-2′ and MeO-1′, secured the partial structure **a** as a methyl pipecolate in which the nitrogen atom was acylated.

Two sulfated methines showed identical resonances at δ_H 4.46/δ_C 81.3. HMBC correlations observed between the protons (δ_H 4.46) and carbons (δ_C 81.3) led to partial

† Dedicated to the late Dr. D. John Faulkner (Scripps) and the late Dr. Paul J. Scheuer (Hawaii) for their pioneering work on bioactive marine natural products.

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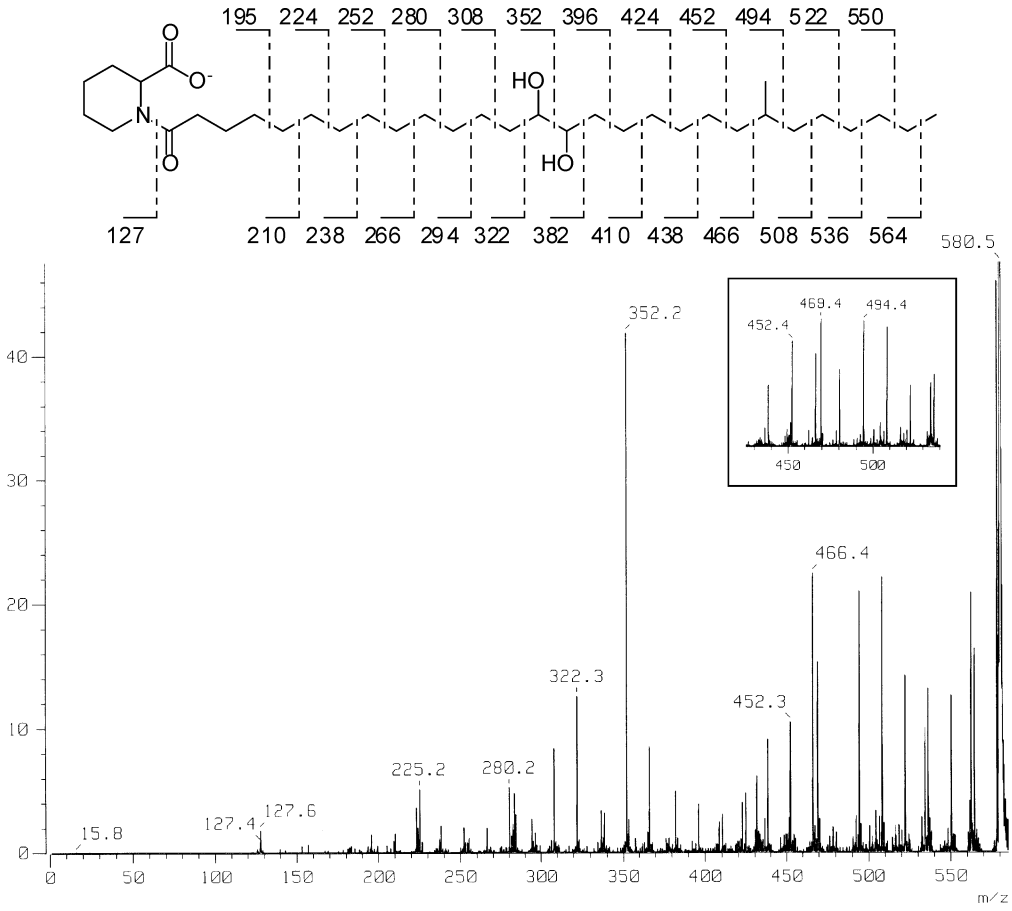
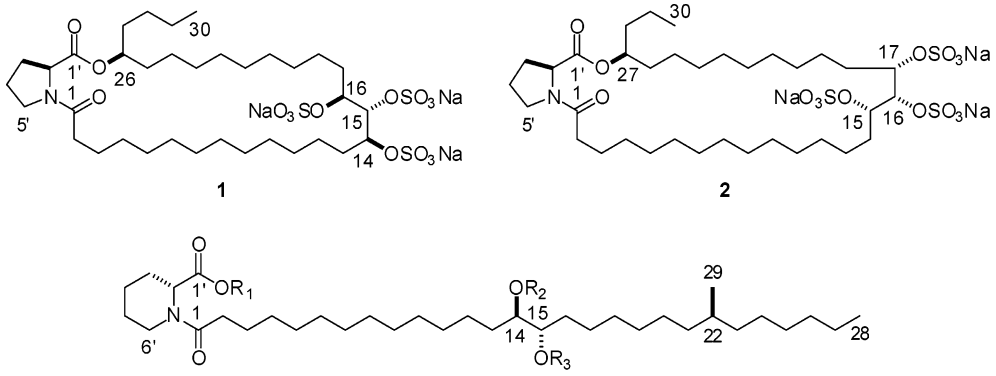


Figure 3. FABMS/MS analysis of **4**. Inset: FABMS/MS of the acid hydrolysate of fr. 1.

Chart 1. Structures of Penarolide Sulfates A_1 (**1**) and A_2 (**2**) and Penasulfate A (**3**)

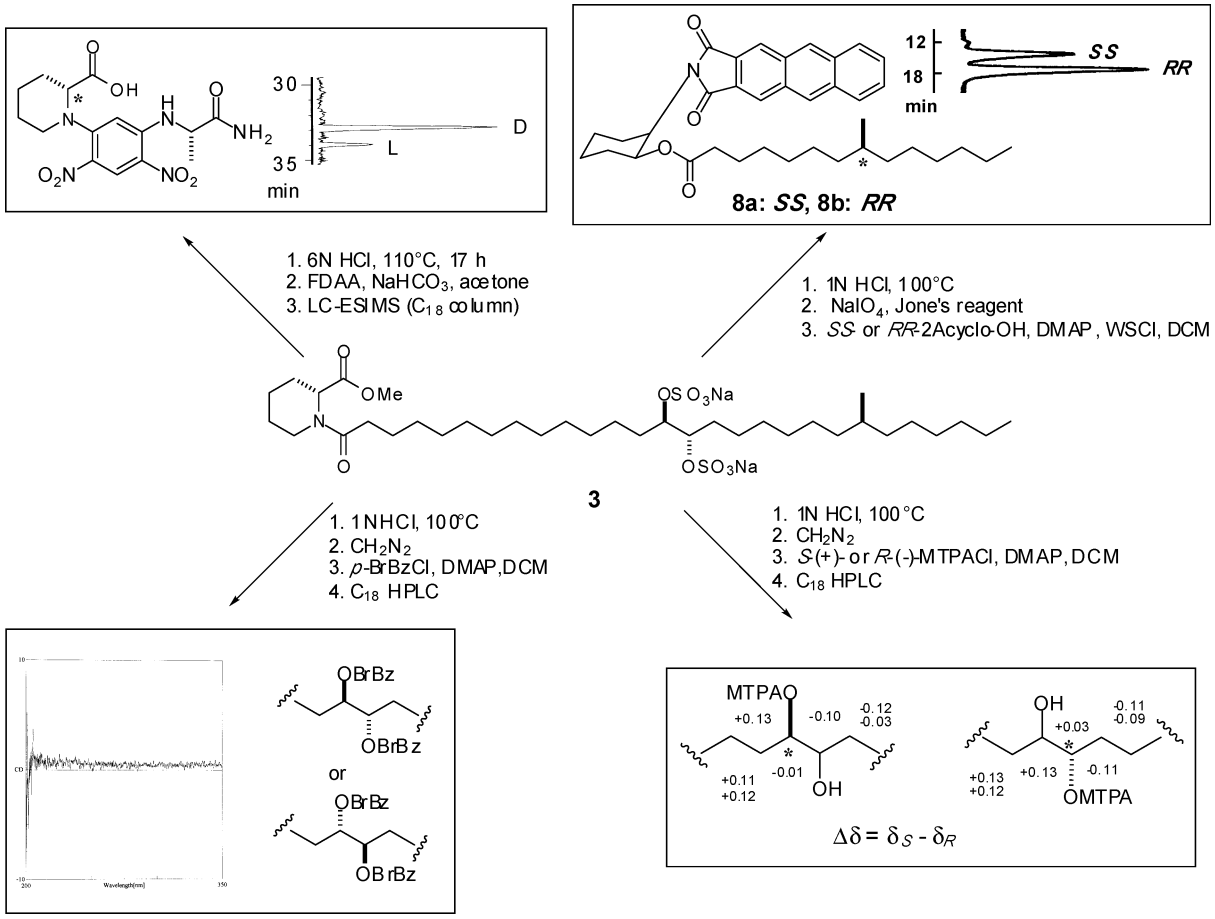


3: R_1 = Me, R_2 = R_3 = SO_3Na
4: R_1 = R_2 = R_3 = H
4a: R_1 = Me, R_2 = R_3 = H
5: R_1 = OH, R_2 = R_3 = *p*-BrBz
6a: R_1 = H, R_2 = *S*-(-)-MTPA, R_3 = H
6b: R_1 = H, R_2 = *R*-(+)-MTPA, R_3 = H
7a: R_1 = H, R_2 = H, R_3 = *S*-(-)-MTPA
7b: R_1 = H, R_2 = H, R_3 = *R*-(+)-MTPA

structure **b**. Partial structures **c** and **d** were determined in a straightforward manner from HOHAHA and HMBC data. Because these partial structures accounted for all the heteroatoms and unsaturations of the molecule, **a**, **b**, **c**, and **d** should be connected by methylene chains.

Although the gross structural feature of the sulfated fatty acid moiety was evident as mentioned above, the length of each alkyl chain connecting the partial structures was not determined by NMR data. To establish the length of each chain, FABMS/MS analysis was carried out on the acid hydrolysate of fr. 1, in which sulfate groups and the methyl ester were removed to generate the carboxylic acid, which is suitable for the analysis of charge remote fragmentation. In the negative ion mode FABMS/MS of the hydrolysate, a negative charge remains at the terminal carboxylate and the charge remote fragmentations were observed.[10] An intense ion peak at *m*/*z* 352, which arose from a cleavage between the vicinal diol, together with the ions at *m*/*z* 322 and 382, which were generated from the cleavages between C-13/C-14 and C-15/C-16, respectively, allowed the location of the diol to be assigned at C-14 and C-15. The position of the methyl substituent on the chain

Scheme 1. Determination of Stereochemistry of **3**



should also be determinable under charge remote fragmentation. Cleavage of the carbon−carbon bonds on either side of the $CH(CH_3)$ carbon should result in two ions differing by 28 amu, whereas cleavage around and within isomeric CH_2CH_2 fragments should generate a continuous series of ions 14 amu apart. The actual MS (Figure 3, inset) was consistent with the latter pattern, but since there was no doubt fr. 1 contained a branched chain, we concluded that fr. 1 was still a mixture of positional isomers of the methyl group.

Further separation of fr. 1 with the recycling HPLC using a C_{30} column yielded several peaks, in which the most abundant constituent was designated as penasulfate A (**3**). By this procedure 0.7 mg of **3** was obtained in total (1.6×10^{-4} % yield based on wet weight). The MS and NMR data of **3** were indistinguishable from those of fr. 1. However, the negative mode FABMS/MS of the diol **4** prepared from **3** exhibited (Figure 3) fragment ions at *m*/*z* 466/494 and 322/352/382, which demonstrated the purity and planar structure of **3**.

The stereochemistry of penasulfate A (**3**) was determined as shown in Scheme 1. Diol **4** was converted to bis-*p*-bromobenzoate **5**, whose CD spectrum exhibited no Cotton effect, indicating the 1,2-*anti* relationship of the diol.[11] Diol **4** was next treated with diazomethane, followed by treatment with MTPACl (DMAP, CH_2Cl_2) to furnish mono-MTPA esters (**6a**,**b** and **7a**,**b**). The $\Delta\delta$ values of the MTPA esters indicated the 14*R*,15*S* configuration,[12] which was in agreement with the CD data.

The absolute stereochemistry at C-22 was deduced as *S* according to Ohrui's method.[13] Diol **4** was cleaved with $NaIO_4$, oxidized with Jones reagent, and reacted with *RR*- or *SS*-2Acyclo-OH (DMAP, WSCI, CH_2Cl_2, overnight). The products were purified by SiO_2 PTLC (Merck 1.05715, developed with $CHCl_3$) and were analyzed by HPLC on C_{18}, in which the *SS*-2Acyclo derivative (**8a**) moved faster than the *RR*-derivative (**8b**). Since the methyl group of **8** is located on the carbon at even number (C-8), the eluting order of **8a** (*SS*)/**8b** (*RR*) was indicative of *S* configuration at C-22 following the empirical rule of Ohrui's method.[13]

The stereochemistry of the pipecolic acid residue was determined by Marfey's method[14] using ESIMS. Acid hydrolysate of **3** was derivatized with FDAA and analyzed by LC-ESIMS on an ODS column. MS monitoring of the eluent at *m*/*z* 382 [$(M + H)^+$, corresponding to the FDAA derivative of pipecolic acid] showed peaks of D- and L-pipecolic acid derivatives in a ratio of 4:1, showing that **3** was a scalemic mixture of 14*R*,15*S*,22*S*,2′*R* and 14*R*,15*S*,-22*S*,2′*S* isomers.

Initially, fr. 1 showed an inhibitory activity against α-glucosidase (G7256, Sigma, St. Louis, MO) about 10 times more potent (IC_{50} 0.14 μg/mL) than those of penarolide sulfates A_1 (**1**) and B_1 (**2**) (IC_{50} 1.2 and 1.5 μg/mL, respectively). Unfortunately, as G7256 glucosidase was no longer available, the activity of the pure penasulfate A (**3**) had to be determined by using an α-glucosidase of the different source (G0660, Sigma). To our disappointment, it showed activity (IC_{50} 3.5 μg/mL) that was comparable to those of **1** and **2**. The reduced activity may be due to the usage of the enzyme from a different source or a disappearance of synergism that had been evoked by the mixture.

Experimental Section

General Experimental Procedures. Optical rotation was measured on a JASCO DIP-1000 digital polarimeter. The CD

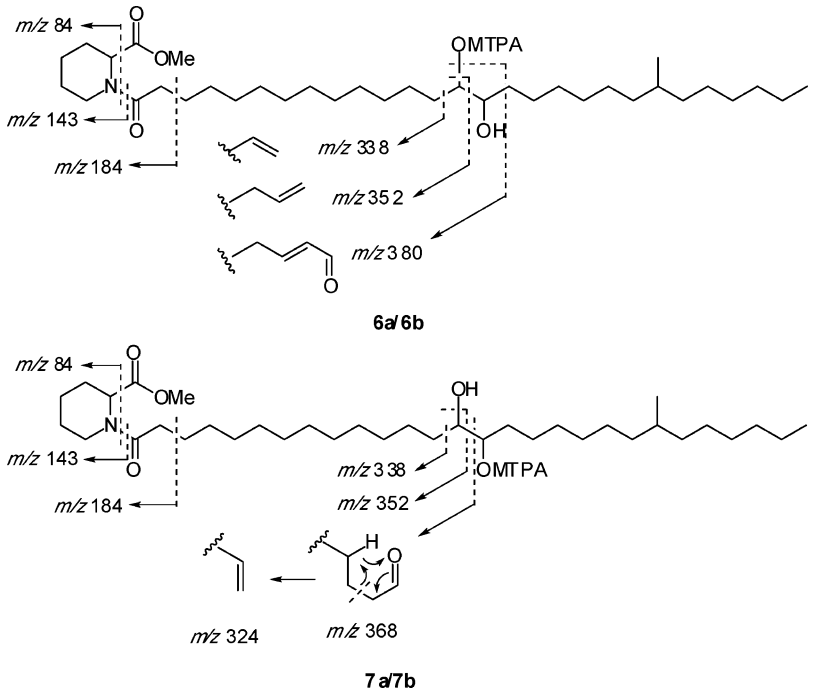


Figure 4. FABMS/MS analyses of MTPA esters **6a**,**b** and **7a**,**b**.

Table 1. NMR Data for Fr. 1 (Mixture) in CD_3OD

position	δ_C	δ_H	mult. *J* (Hz)	HMBC
1	175.9			
2	34.3	2.41	t 7.7	C: 1
3	26.4	1.57		C: 1
4	30.5	1.3		
5−11	30.7	1.3		
12	26.4	1.40		
13	31.2	1.76		C: 12
		1.57		
14	81.3	4.46		C: 12, 15, 16
15	81.3	4.46		C: 13, 14, 17
16	31.2	1.76		C: 17
		1.57		
17	26.4	1.40		
18, 19	30.7	1.3		
20	28.2	1.3		
21	38.2	1.29		
		1.10		C: 20, 29
22	33.9	1.36		
23	38.2	1.29		C: 24, 29
		1.10		
24	31.1	1.3		
25	30.7	1.3		
26	33.1	1.28		
27	23.7	1.30		
28	14.4	0.89	t 6.9	C: 26, 27
29	20.1	0.85	d 6.5	C: 21, 22, 23
1′	173.1			
2′	53.4 (57.6)[a]	5.25 (4.82)[a]	d 5.0	C: 1′, 2′, 4′, 6′
3′	27.6 (28.1)[a]	2.24 (2.30)[a]		
		1.64 (1.70)[a]		C: 1′, 4′, 5′
4′	21.8	1.71		
		1.35		
5′	26.4 (25.7)[a]	1.72 (1.67)[a]		
		1.48 (1.35)[a]		
6′	44.9 (40.6)[a]	3.87 (4.45)[a]	d 13.5 (d 12.7)[a]	
		3.21 (2.60)[a]	dt 13.1, 3.1 (dt 13.0, 3.0)[a]	C: 1, 4′, 5′
OMe	52.7 (53.0)[a]	3.72 (3.75)[a]	s	C: 1′

[a] Signals for the minor conformer.

spectrum was recorded in MeOH using a JASCO J-820 spectropolarimeter. UV spectra were recorded on a Hitachi 330 spectrophotometer. IR spectra were obtained using a JASCO FT/IR-230 Fourier transform infrared spectrometer. NMR spectra were recorded on a JEOL α-600 spectrometer in CD_3OD. FABMS data were obtained using a JEOL SX102 mass spectrometer using triethanolamine (negative ion mode) or *p*-nitrobenzyl alcohol+NaCl (positive ion mode) as matrixes. Negative mode HRFABMS spectra were obtained at a resolution of 5000 using PEG sulfate 1000 as a marker. FABMS/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%. ESI- and LCESI-MS were measured on a JEOL AccuTOF (JMS-T100LC).

Sponge Sample. The sponge specimens were collected at a depth of 20 m off Hachijo-jima Island, 300 km south of Tokyo, and were identified as *Penares* sp. as described previously.[4]

Extraction and Isolation. A part of 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 aqueous 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 three-step ODS HPLC [COSMOSIL-5C_{18}ARII; first step with 30% *n*-PrOH containing 0.2 M $NaClO_4$; second with 38% *n*-PrOH containing 0.2 M $NaClO_4$; third on COSMOSIL-5C_{18}MS with *n*-PrOH/MeCN/H_2O (3:3:4) containing 0.2 M $NaClO_4$] to afford a broad peak. This was further separated by eight cycles of recycling ODS HPLC [Inertsil ODS-3, *n*-PrOH/MeCN/H_2O (4:2:4) containing 0.2 M $NaClO_4$] to yield 2.1 mg of a single peak fraction (fr. 1). Separation of this fraction by recycling HPLC on a C_{30} column [Develosil C_{30}-UG-5, with *n*-PrOH/MeCN/H_2O (35:25:40) containing 0.2 M $NaClO_4$] yielded four peaks, of which the major peak was designated as penasulfate A (**3**). Processing of the remaining portion of this sponge sample (280 g) yielded 0.7 mg of **3**.

Penasulfate A (3): colorless amorphous solid; $[\alpha]^{29}_D$ +10° (*c* 0.03, MeOH); UV (MeOH) 203 nm (ϵ 9700); IR (film) 1745, 1650, 1245, 1220 cm^{-1}; HRFABMS $(M - Na)^-$ *m*/*z* 776.4019 for $C_{36}H_{67}NO_{11}S_2Na$ (Δ −3.5 mmu).

Diol 4. Penasulfate B_1 (**3**; 0.3 mg) was dissolved in 0.36 mL of 6 N HCl/MeOH (1:5) and heated at 100 °C for 1 h in a sealed vial. After cooling to room temperature, the solution was extracted with $CHCl_3$. The $CHCl_3$ extract was evaporated to yield diol **4**: FABMS (matrix: triethanolamine) *m*/*z* 580 $(M - H)^-$.

Bis-*p*-bromobenzoate 5. Diol **4** was treated with CH_2N_2 to yield methyl ester **4a**. One-third portion of **4a**, 2 mg of DMAP, and 2 mg of BrBzCl were dissolved in 0.5 mL of CH_2Cl_2, and the mixture was stirred for 1 h at 50 °C. The reaction mixture was passed through a short SiO_2 column [$CHCl_3$/MeOH (1:0, 19:1, and 9:1)], and the fraction eluted with $CHCl_3$ was separated by ODS HPLC [COSMOSIL-5C_{18}ARII; 95 to 100% MeOH linear gradient] to afford bis-*p*-bromobenzoate **5**, as well as mono-*p*-bromobenzoate. **5**: ESI-MS *m*/*z* 960/962/964 (1:2:1 cluster, $M + H)^+$.

MTPA Esters 6a,b and 7a,b. Each half of the remaining **4a** was dissolved in 38 μL of CH_2Cl_2 containing 0.38 mg of DMAP, to which was added *R*-(−)- or *S*-(+)-MTPACl (2 μL); the reaction was stirred for 1 h. The reaction mixture was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ layer was separated on a SiO_2 short column (1 × 3 cm) with $CHCl_3$/MeOH (10:0, 19:1, and 9:1) and on ODS (Develosil C_{30}-UG-5) with 97% MeOH to afford mono-*S*-(−)-MTPA esters **6a**/**7a** (retention time: 26.8 and 29.2 min, respectively). Mono-*R*-(+)-MTPA esters eluted in the opposite elution order of **7b**/**6b** (retention time: 27.4 and 29.6 min, respectively). Location of the MTPA ester was determined by FABMS/MS analyses of **6a**,**b** and **7a**,**b**. **6a**: ESIMS *m*/*z* 812 $(M + H)^+$; FABMS/MS *m*/*z* 812 (parent ion), 780 (−OMe), 578 (−MTPAOH), 560 (578 − H_2O), 546, 380, 352, 338, 188, 143, 83; ^{1}H NMR (CD_3OD) δ 2.44 (t, H-2), 1.60 (H-3), 1.38, 1.22 (H-12), 1.73 (H-13), 5.02 (H-14), 3.57 (H-15), 1.45, 1.25 (H-16), 1.33 (H-17), 1.40 (H-22), 0.91 (t, H-28), 0.86 (d, H-29), 5.27 (H-2′), 2.26, 1.65 (H-3′), 1.73, 1.35 (H-4′), 1.73, 1.49 (H-5′), 3.87, 3.22 (H-6′), 3.73 (3H s, MeO1′). **6b**: ESIMS *m*/*z* 812 $(M + H)^+$; FABMS/MS *m*/*z* 812 (parent ion), 780 (−OMe), 578 (−MTPAOH), 560 (578-H_2O), 546, 380, 352, 338, 188, 143, 83; ^{1}H NMR (CD_3OD) δ 2.42 (t, H-2), 1.60 (H-3), 1.27, 1.20 (H-12), 1.60 (H-13), 5.03 (H-14), 3.67 (H-15), 1.37 (H-16), 1.48, 1.30 (H-17), 1.37 (H-22), 0.90 (t, H-28), 0.86 (d, H-29), 5.25 (H-2′), 2.24, 1.63 (H-3′), 1.71, 1.33 (H-4′), 1.71, 1.47 (H-5′), 3.87, 3.21 (H-6′), 3.71 (3H s, MeO1′). **7a**: ESIMS *m*/*z* 812 $(M + H)^+$; FABMS/MS *m*/*z* 812 (parent ion), 780 (−OMe), 578 (−MTPAOH), 560 (578 − H_2O), 546, 368, 324, 188, 143, 83; ^{1}H NMR (CD_3OD) δ 2.42 (t, H-2), 1.59 (H-3), 1.30 (H-12), 1.50, 1.35 (H-13), 3.67 (H-14), 5.03 (H-15), 1.60 (H-16), 1.25, 1.20 (H-17), 1.37 (H-22), 0.89 (t, H-28), 0.85 (d, H-29), 5.26 (H-2′), 2.24, 1.63 (H-3′), 1.71, 1.34 (H-4′), 1.71, 1.48 (H-5′), 3.86, 3.21 (H-6′), 3.72 (3H s, MeO1′). **7b**: ESIMS *m*/*z* 812 $(M + H)^+$; FABMS/MS *m*/*z* 812 (parent ion), 780 (−OMe), 578 (−MTPAOH), 560 (578 − H_2O), 546, 368, 352, 338, 336, 324, 258, 188, 143, 83; ^{1}H NMR (CD_3OD) δ 2.42 (t, H-2), 1.59 (H-3), 1.43, 1.31 (H-12), 1.23 (H-13), 3.54 (H-14), 5.00 (H-15), 1.71 (H-16), 1.36, 1.29 (H-17), 1.38 (H-22), 0.89 (t, H-28), 0.85 (d, H-29), 5.25 (H-2′), 2.24, 1.63 (H-3′), 1.71, 1.34 (H-4′), 1.71, 1.47 (H-5′), 3.86, 3.20 (H-6′), 3.72 (3H s, MeO1′).

Determination of Absolute Stereochemistry at C22. One hundred micrograms of **3** was hydrolyzed in 6 N HCl/MeOH (1:5) at 100 °C for 1 h. After cooling to room temperature, the hydrolysate was dried in a stream of N_2. The residue was dissolved in 100 μL of MeOH, to which 20 μL of $NaIO_4$ solution (20 mg/mL in MeOH) was added. The mixture was left to stand at room temperature for 1 h. After evaporation to dryness, the reaction mixture was mixed with 100 μL of acetone and 2 μL of Jones reagent. The mixture was allowed to stand at 0 °C for 1 h. The reaction was quenched by addition of excess amounts of acetone, followed by evaporation and separation by SiO_2 chromatography [5 × 25 mm, $CHCl_3$/MeOH (10:0, 19:1, 9:1)]. The $CHCl_3$/MeOH (19:1) eluting fraction was divided in two parts; each part was reacted with 100 μg of *SS*- or *RR*-2Acyclo-OH, respectively (in 650 μL of CH_2Cl_2, with 100 μg of DMAP and 40 μg of WSCI, rt, 22 h). The reaction mixtures were separated by preparative TLC on SiO_2 with $CHCl_3$ to yield *S*,*S*- or *R*,*R*-2Acyclo ester **8a**,**b**, respectively. In HPLC analysis [Inertosil ODS-3 (2 × 150 mm), MeCN/THF/*n*-hexane (100:100:5), 80 μL/min, −47 °C] using fluorescence detection (λ_{ex} 298 nm, λ_{em} 462 nm), **8a** eluted faster (retention time: 14.4 min) than **8b** (retention time: 17.4 min).

Marfey Analysis. A 100 μg portion of penasulfate A was dissolved in 400 μL of 6 N HCl, and the mixture was heated at 110 °C for 17 h in a sealed tube. After cooling to room temperature, the hydrolysate was dried in 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 0.2 N HCl and diluted with 50 μL of 50% MeCN containing 0.05% TFA. A portion of this solution was subjected to LC-ESIMS analysis on ODS (COSMOSIL 5C_{18}-MS, 20% MeCN containing 50 mM NH_4OAc) monitoring absorption at 340 nm. Ion chromatogram, monitoring ions at *m*/*z* 382, showed ion peaks in a ratio of 4:1 at 32.8 and 34.0 min, respectively, which were identified as D- and L-pipecolic acid, respectively, by comparison with the standard samples.

Enzyme Inhibition Assays. Enzyme inhibition assays were performed as described previously.[4]

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Supporting Information Available: NMR spectra for fr. 1. Comparison of ^{1}H NMR spectra of **3** and fr. 1. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

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(6) The ^{1}H NMR spectrum of **3** revealed a series of minor signals associated with the major signals; perhaps these signals were not due to the contamination by a minor component but to conformational isomerism at the amide bond, which was also seen in the case of penarolide sulfates A_1 (**1**) and A_2 (**2**).
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