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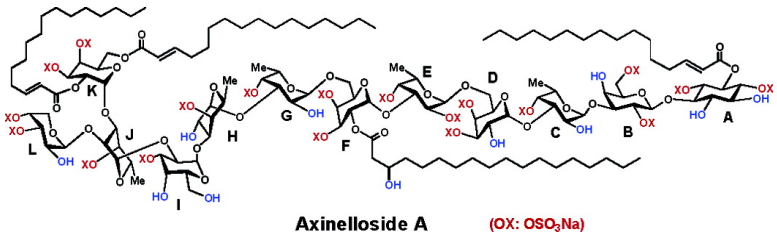

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Axinelloside A, an Unprecedented Highly Sulfated Lipopolysaccharide Inhibiting Telomerase, from the Marine Sponge, *Axinella infundibula*[1]

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Abstract: Axinelloside A was isolated from the lipophilic extract of the Japanese marine sponge *Axinella infundibula* as a strong human telomerase inhibitor (IC_{50} 2.0 μg/mL). It has the molecular weight of 4780.4 as the monoisotopic mass of the 19 sodium salt. The chemical structure was elucidated mainly by spectroscopic methods (2D NMR and MS). Axinelloside A consists of twelve sugars, e.g., a *scyllo*-inositol, a D-arabinose, 5 D-galactoses, and 5 L-fucoses, together with an (*R*)-3-hydroxy-octadecanoic acid, 3 (*E*)-2-hexadecenoic acids, and 19 sulfates.

Introduction

Telomerase is a ribonucleoprotein enzyme complex that extends telomere length by adding hexameric (TTAGGG) repeats onto the telomeric ends of chromosomes.[2] Telomerase activity is essential for the sustained proliferation of most immortal cells, including cancer cells.[3] Its activity is found in more than 90% of cancer cells, but not in most normal cells.[4] Thus, inhibitors of telomerase are a target for anticancer drug discovery.[5] In fact, some synthetic inhibitors based on the function of telomerase have been successful in clinical trials,[6] while some natural products have been reported to inhibit telomerase.[7] In the course of our continuing search for potential antitumor leads from Japanese marine invertebrates, we recently reported the isolation of the first telomerase-inhibitory marine natural products, dictyodendrines A−E from the marine sponge *Dyctyodendrilla verongiformis*,[8] which are sulfated alkaloids and completely inhibited the human telomerase activity at 50 μg/mL. Subsequently, we found significant telomerase-inhibitory activity in the lipophilic extract of the marine sponge *Axinella infundibula*, and bioassay-guided isolation afforded an unprecedented highly sulfated lipopolysaccharide named axinelloside A. This paper describes the isolation and structure elucidation of this novel metabolite.

Results and Discussion

Isolation and Characterization. The ether soluble portion of the extract of *A. infundibula* was subjected to solvent partitioning between 90% MeOH and *n*-hexane. The active *n*-hexane phase was fractionated by silica gel chromatography, followed by C_{18} reversed-phase HPLC. The final purification was carried out by recycling HPLC on a C_{30} reversed-phase column to yield axinelloside A (1.1×10^{-3}% of wet weight). Axinelloside A inhibited human telomerase with an IC_{50} value of 2.0 μg/mL.

Axinelloside A had a large molecular weight (more than 4 kDa) as suggested by NMR and MS data. The presence of a large number of sulfate groups was evident not only from fragment ion peaks of 80 mass units (SO_3^-) apart in the electrospray-ionization time-of-flight mass spectrometry (ESI-TOF-MS) but also those of 102 mass units ($NaSO_3 - H$) apart in the matrix-

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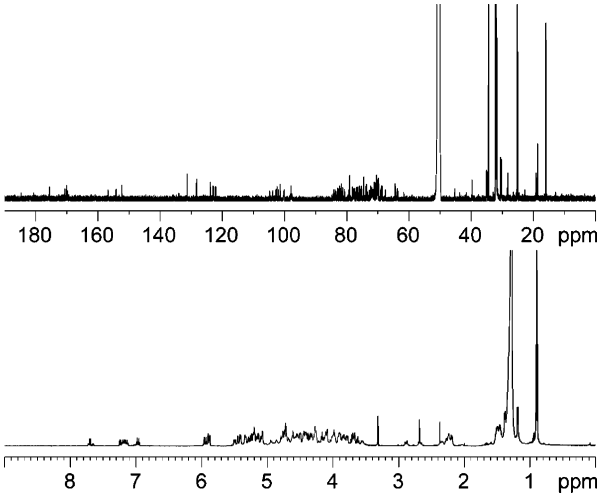

Figure 1. ^{1}H and ^{13}C NMR of axinelloside A.

assisted laser desorption ionization mass spectrometry (MALDI-TOF-MS),[9] which was also supported by an IR band at 1224 cm^{-1}. In fact, the acid hydrolysates of axinelloside A exhibited an intense sulfate ion peak as analyzed by ion chromatography on a Shim-pack IC-A3 (S) column, suggesting the presence of 10−20 sulfate groups in axinelloside A.[10]

NMR data showed many anomeric protons and carbons at δ_H 5.0−5.5 and δ_C 100.0, respectively (Figure 1). In fact, 11 anomeric signals were assigned from HMQC cross-peaks, δ_H 5.19/δ_C 101.5, δ_H 5.09/δ_C 104.8, δ_H 5.33/δ_C 102.8, δ_H 5.20/δ_C 100.3, δ_H 5.27/δ_C 101.4, δ_H 5.12/δ_C 101.9, δ_H 5.15/δ_C 103.9, δ_H 5.31/δ_C 102.5, δ_H 5.39/δ_C 98.1, δ_H 5.40/δ_C 100.2, and δ_H 5.44/δ_C 97.7. In addition, the ^{1}H and ^{13}C NMR spectra exhibited signals reminiscent of fatty acid residues (Figure 1). This was also supported by the ^{13}C NMR signals at around δ_C 170.0 and IR absorptions at 1711 and 1643 cm^{-1}. These results readily suggested that axinelloside A was a highly sulfated lipopolysaccharide. Due to the limited amount of sample, we carried out the structural study of axinelloside A by NMR and MS experiments, especially extensive 2D NMR.

Composition of Sugars. To determine sugar composition and the absolute stereochemistry of sugars, the acid hydrolysates of axinelloside A were converted into alditol acetates, which were analyzed by both GC and GC/MS.[11] The peaks due to heptaacetyl fucitol, heptaacetyl arabinitol, hexaacetyl galactitol, and hexaacetyl *scyllo*-inositol were observed in the ratio 5:1:5:1 in both GC and GC/MS analyses. These alditol acetates were identified by coinjection with authentic samples.

The absolute configuration of sugars was determined by Hara's method.[12] The sugars obtained by acid hydrolysis of axinelloside A were converted into the methyl (4*R*)-thiazolidine-4-carboxylate derivatives and analyzed by GC, which resulted in L-fucose, D-arabinose, and D-galactose.

Identification and Stereochemistry of Fatty Acids. Analyses of DQF-COSY, HOHAHA, HMQC, HMBC, NOESY, and HSQC-HOHAHA data of intact axinelloside A led to the identification of three α,β-unsaturated fatty acids and one β-hydroxy fatty acid. The $^{3}J_{H2,H3}$ values of 15.4, 15.4, and 15.6 Hz for α,β-unsaturated fatty acids readily indicated the *E*-geometry of all double bonds.

The alkali hydrolysates of axinelloside A were separated by solvent partitioning, followed by C_{18} reversed-phase HPLC to furnish an α,β-unsaturated fatty acid. Its negative mode FABMS spectrum exhibited an intense ion peak at *m*/*z* 253 $[M - H]^-$, while ^{1}H NMR data unambiguously assigned it as (*E*)-2-hexadecenoic acid. On the other hand, the acid hydrolysates of axinelloside A afforded a mixture of fatty acids which exhibited two intense $[M - H]^-$ ion peaks at *m*/*z* 253 and 299. Since the ion peak at *m*/*z* 253 $[M - H]^-$ was assignable to that of (*E*)-2-hexadecenoic acid, the other ion peak at *m*/*z* 299 $[M - H]^-$ was considered to be derived from β-hydroxy fatty acid. Therefore, the hydrolysates were methylated with Me_3SiCHN_2 and separated by silica gel chromatography to afford the methyl ester of β-hydroxy fatty acid; the positive mode FABMS spectrum exhibited two prominent ion peaks at *m*/*z* 315 $[M + H]^+$ and 337 $[M + Na]^+$. FABMS data and interpretation of the COSY spectrum were consistent with methyl 3-hydroxyoctadecanoate. The absolute configuration at C3 was determined to be *R* by the modified Mosher's method.[13]

Identification of Sugar Units. Analyses of the DQF-COSY, HOHAHA, HMQC, and HSQC-HOHAHA spectra of axinelloside A measured in CD_3OD/D_2O (9:1) led to one inositol (*a*: sugar **A**) and twenty-nine partial structures (*b*-*o*, *b*′-*k*′, and *m*′-*o*′) as shown in Figure 2. The connectivities of these partial structures were deduced by interpretation of HMBC and NOESY data, thereby identifying eleven sugars. NOESY cross-peaks, δ_H 5.19/δ_H 3.88, δ_H 3.78/δ_H 3.88, and δ_H 4.07/δ_H 4.27, connected partial structures *b* and *b*′ to construct sugar **B** (D-galactopyranose), while connectivity of the partial structures *c* and *c*′ was derived by HMBC cross-peaks, δ_H 5.09/δ_C 69.7, δ_H 4.75/δ_C 69.7, and δ_H 1.28/δ_C 83.0, and NOESY cross-peaks, δ_H 4.16/δ_H 4.40, δ_H 4.75/δ_H 4.40, and δ_H 4.75/δ_H 1.28, thus completing sugar **C** (L-fucose). The partial structures *d* and *d*′ were connected by an HMBC cross-peak, δ_H 5.33/δ_C 72.0, and NOESY cross-peaks, δ_H 5.06/δ_H 4.67, δ_H 5.06/δ_H 3.54, and δ_H 5.06/δ_H 3.98, resulting in sugar **D** (D-galactopyranose). HMBC cross-peaks, δ_H 5.20/δ_C 68.6 and δ_H 1.28/δ_C 83.6, and NOESY cross-peaks, δ_H 4.78/δ_H 4.31 and δ_H 4.78/δ_H 1.28, connected partial structures *e* and *e*′, thereby constructing sugar **E** (L-fucose). Partial structures *f* and *f*′ were linked by HMBC cross-peaks, δ_H 5.20/δ_C 68.6 and δ_H 4.78/δ_C 68.6, and NOESY cross-peaks, δ_H 4.24/δ_H 4.31, δ_H 4.78/δ_H 4.31, and δ_H 4.31/δ_H 1.28, which completed sugar **F** (D-galactopyranose). Connectivity of partial structures *g* and *g*′ established by HMBC cross-peaks, δ_H 5.12/δ_C 68.6 and δ_H 1.17/δ_C 82.6, and NOESY cross-peaks, δ_H 3.88/δ_H 4.11, δ_H 4.50/δ_H 4.11, and δ_H 4.50/δ_H 1.17, constructed sugar **G** (L-fucose). Similarly, HMBC cross-peaks, δ_H 5.15/δ_C 69.7, δ_H 4.55/δ_C 84.3, and δ_H 1.29/δ_C 84.3, and a NOESY cross-peak, δ_H 4.73/δ_H 4.55, correlated partial structures *h* and *h*′, completing sugar **H** (L-fucose). An HMBC cross-peak, δ_H 5.31/δ_C 73.8, and NOESY cross-peaks, δ_H 4.70/δ_H 4.44, δ_H 4.48/δ_H 3.68, and δ_H 4.48/δ_H 3.78, connected partial structures *i* and *i*′, completing sugar **I** (D-galactopyranose). Linkage of partial structures *j* and *j*′ was implied from HMBC correlations, δ_H 5.39/δ_C 69.9 and δ_H 1.37/δ_C 81.3, and NOESY cross-peaks,

(9) These fragment ion peaks were also consistent with the presence of phosphate groups, which were excluded by the absence of ^{31}P NMR signals.
(10) Ion chromatography showed 24% (w/w) of SO_4^{2-} present in axinelloside A.
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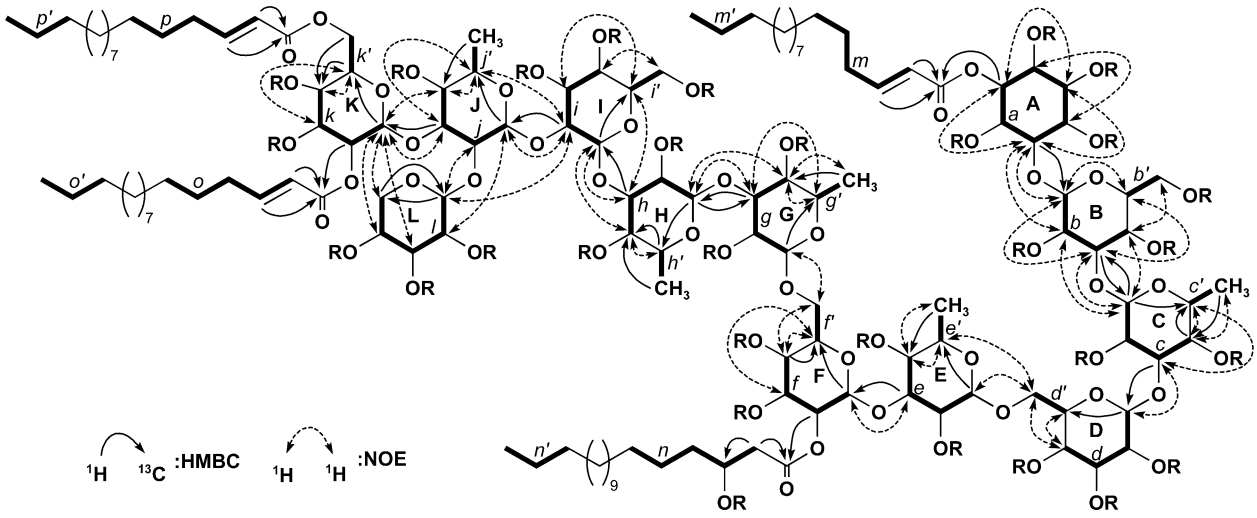

Figure 2. Partial structure and key HMBC and NOE correlations.

δ_H 4.53/δ_H 4.44 and δ_H 4.60/δ_H 4.44, resulting in sugar **J** (L-fucose). Similarly, HMBC cross-peaks, δ_H 5.40/δ_C 70.6, δ_H 4.58/δ_C 78.2, δ_H 4.40/δ_C 78.2, and δ_H 4.62/δ_C 78.2, and NOESY cross-peaks, δ_H 5.13/δ_H 4.58 and δ_H 5.19/δ_H 4.58, established the connection of partial structures *k* and *k*′, constructing sugar **K** (D-galactopyranose). Finally, sugar **L** (D-arabinopyranose) was established by intramolecular HMBC cross-peaks, δ_H 5.44/δ_C 63.6, δ_H 3.84/δ_C 97.7, and δ_H 4.02/δ_C 97.7, within partial structure *l*.

The configuration of anomeric protons was determined from the three-bond coupling constant values between H1 and H2 ($^3J_{H1,H2}$), which were obtained either by the 1D ^{1}H NMR spectrum or by the DQF-COSY spectrum (Table 1). The $^3J_{H1,H2}$ value of 7.7 Hz of the anomeric proton at δ_H 5.19 in the sugar **B** indicated its β-anomeric nature, while the small $^3J_{H1,H2}$ values (ca. 4 Hz) for sugars **C** to **L**, the α-anomeric protons in these sugars. Other $^3J_{H,H}$ coupling constant values and NOESY correlation of each sugar further supported these results.

Glycosyl Sequence Analysis. Interpretation of the HMBC and NOESY spectra could determine not only the sequence of sugars but also the position of fatty acids as depicted in Figure 3A. Sequencing of sugars was started from sugar **A**, which was located at a terminus of axinelloside A. The sequence of sugars, **A**, **B**, **C**, and **D**, was deduced from a series of HMBC correlations between **A**-H5 and **B**-C1, **B**-H3 and **C**-C1, **C**-H1 and **B**-C3, and **C**-H3 and **D**-C1. NOESY correlations, **D**-H6s/**E**-H1 and **D**-H6s/**E**-H5, connected sugars **D** and **E**, while the connection of sugars **E** and **F** was established by an HMBC cross-peak between **E**-H3 and **F**-C1. A NOESY correlation, **F**-H6s/**G**-H1, linked sugars **F** and **G**. The sequence of sugars **G**, **H**, **I**, **J**, and **K** was established by a series of HMBC cross-peaks, **G**-H3/**H**-C1, **H**-H1/**G**-C3, **H**-H3/**I**-C1, **I**-H2/**J**-C1, and **J**-H3/**K**-C1. A NOESY correlation between **J**-H2 and **L**-H1 connected sugars **J** and **L**, thereby completing the full sugar sequence of axinelloside A.

Positions of Fatty Acids. HMBC correlations (Figure 3B) between **A**-H1 and δ_C 170.0, δ_H 5.88, and δ_C 170.0, and δ_H 6.95 and δ_C 170.0 placed an (*E*)-2-hexadecenoic acid at position C1 of sugar **A**, while those of **K**-H2/δ_C 169.8, δ_H 5.88/δ_C 169.8, and δ_H 7.18/δ_C 169.8 linked the second unsaturated fatty acid at C2 of sugar **K**. The position of 3-hydroxyoctadecanoic acid at C2 of sugar **F** was disclosed by HMBC cross-peaks, **F**-H2/

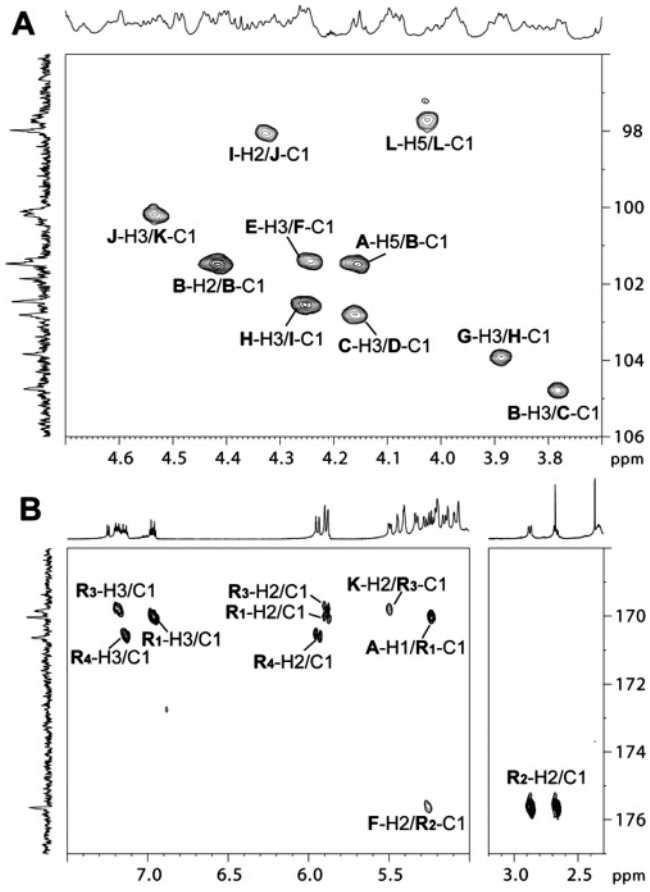

Figure 3. Parts of HMBC spectra of axinelloside A for glycosyl sequence analysis (A) and for fatty ester linkages (B).

δ_C 175.7, δ_H 2.67/δ_C 175.7, and δ_H 2.87/δ_C 175.7. The position of the remaining (*E*)-2-hexadecenoic acid could not be determined due to lack of informative HMBC and NOESY cross-peaks. However, the downfield shifts of **K**-H6s (δ_H 4.40 and δ_H 4.62) indicated the linkage of (*E*)-2-hexadecenoic acid to C6 of sugar **K**, thus completing determination of the sugar sequence and position of fatty acids in axinelloside A as shown in Figure 4.

Positions of Sulfate Groups. To determine the position of sulfate groups, the deuterium isotope shift experiment was

Table 1. ^{1}H and ^{13}C NMR Data of Axinelloside A in CD_3OD/D_2O (9:1) at 300 K[a]

	^{1}H	multiplicity	^{13}C		^{1}H	multiplicity	^{13}C
		ring A (scyllo-inositol)				ring G (α-L-fucose)	
1	5.23	dd, *J* = 9.7, 9.8	73.7	1	5.12	d, *J* = 4.0	101.9
2	4.17	dd, *J* = 9.4, 9.8	81.8	2	3.98	dd, *J* = 4.0, 10.5	70.0
3	3.71	dd, *J* = 9.4, 9.9	75.3	3	3.88	dd, *J* = 3.9, 10.5	81.4
4	3.61	dd, *J* = 9.4, 9.9	74.5	4	4.50	brd, *J* = 3.9	82.6
5	4.15	dd, *J* = 9.1, 9.4	78.0	5	4.11	brq, *J* = 6.5	68.6
6	4.71	dd, *J* = 9.1, 9.7	82.3	6	1.17	d, *J* = 6.5	18.6
		ring B (β-D-galactose)				ring H (α-L-fucose)	
1	5.19	d, *J* = 7.7	101.5	1	5.15	d, *J* = 4.2	103.9
2	4.41	dd, *J* = 7.7, 10.0	79.1	2	3.81	dd, *J* = 4.2, 10.6	71.1
3	3.78	dd, *J* = 3.1, 10.0	83.8	3	4.25	dd, *J* = 4.2, 10.6	77.8
4	4.07	brd, *J* = 3.1	72.3	4	4.73	brd, *J* = 4.2	84.3
5	3.88	brt, *J* = 7.2	76.0	5	4.55	brq, *J* = 6.8	69.7
6	4.27	*b*	70.0	6	1.29	d, *J* = 6.8	18.8
		ring C (α-L-fucose)				ring I (α-D-galactose)	
1	5.09	d, *J* = 4.2	104.8	1	5.31	d, *J* = 4.4	102.5
2	3.90	dd, *J* = 4.2, 10.5	71.9	2	4.32	dd, *J* = 4.4, 10.5	71.7
3	4.16	dd, *J* = 3.2, 10.5	76.9	3	4.70	dd, *J* = 4.0, 10.5	80.7
4	4.75	brd, *J* = 3.2	83.0	4	4.48	brd, *J* = 4.0	70.4
5	4.40	brq, *J* = 6.6	69.7	5	4.44	brdd, *J* = 4.2, 9.5	73.8
6	1.28	d, *J* = 6.6	19.1	6	3.68	dd, *J* = 4.2, 12.2	64.4
					3.78	dd, *J* = 9.5, 12.2	
		ring D (α-D-galactose)				ring J (α-L-fucose)	
1	5.33	d, *J* = 4.2	102.8	1	5.39	d, *J* = 4.0	98.1
2	3.97	dd, *J* = 4.2, 10.5	69.7	2	4.25	dd, *J* = 4.0, 10.0	73.6
3	4.76	dd, *J* = 3.2, 10.5	77.5	3	4.53	*b*	71.3
4	5.06	brd, *J* = 3.2	79.2	4	4.60	*b*	81.3
5	4.67	brt, *J* = 6.6	72.0	5	4.44	brq, *J* = 7.2	69.9
6	3.54	dd, *J* = 6.6, 12.1	70.3	6	1.37	d, *J* = 7.2	19.0
	3.98	dd, *J* = 6.6, 12.1					
		ring E (α-L-fucose)				ring K (α-D-galactose)	
1	5.20	d, *J* = 4.0	100.3	1	5.40	d, *J* = 3.9*	100.2
2	4.52	dd, *J* = 4.0, 10.0	76.9	2	5.48	dd, *J* = 3.9, 10.4*	70.4
3	4.24	dd, *J* = 4.2, 10.0	75.4	3	5.13	dd, *J* = 4.2, 10.4	75.7
4	4.78	brd, *J* = 4.2	83.6	4	5.19	brd, *J* = 4.2	78.2
5	4.31	brq, *J* = 6.5	68.6	5	4.58	*b*	70.6
6	1.28	d, *J* = 6.5	18.7	6	4.40	*b*	63.9
					4.62	*b*	
		ring F (α-D-galactose)				ring L (α-D-arabinose)	
1	5.27	d, *J* = 3.3	101.4	1	5.44	d, *J* = 3.9*	97.7
2	5.25	dd, *J* = 3.3, 10.6	70.7	2	4.09	dd, *J* = 3.9, 10.5	69.0
3	4.93	dd, *J* = 3.2, 10.6	74.5	3	4.35	dd, *J* = 4.1, 10.5	76.3
4	5.06	brd, *J* = 3.2	79.2	4	4.71	*c*	77.5
5	4.85	brt, *J* = 7.1	73.4	5	3.84	*c*	63.6
6	3.85	dd, *J* = 7.1, 12.8	67.5		4.02	*c*	
	3.98	dd, *J* = 7.1, 12.8					
		R_1 (2-hexadecenoic acid)				R_3 (2-hexadecenoic acid)	
1			170.0	1			169.8
2	5.88	d, *J* = 15.6*	123.8	2	5.88	d, *J* = 15.4*	122.2
3	6.95	td, *J* = 6.8, 15.6*	152.3	3	7.18	td, *J* = 6.8, 15.4*	156.6
4	2.18	m	34.8	4	2.25	m	35.0
					2.33	m	
5	1.45	m	30.6	5	1.48	m	30.3
6−13	1.37−1.25	*b*	32.9−24.5	6−13	1.37−1.25	*b*	32.9−24.5
14	1.26	m	34.5	14	1.26	m	34.5
15	1.29	m	25.0	15	1.29	m	25.0
16	0.88	t, *J* = 7.1*	15.8	16	0.88	t, *J* = 7.1*	15.8
		R_2 (2-hexadecenoic acid)				R_4 (3*R*-hydroxy-octadecanoic acid)	
1			175.7	1			170.6
2	2.67	dd, *J* = 9.8, 14.2*	45.4	2	5.93	d, *J* = 15.4*	122.9
	2.87	dd, *J* = 2.9, 14.2*					
3	4.09	m	71.2	3	7.13	td, *J* = 6.8, 15.4*	153.9
4	1.50	m	39.7	4	2.23	m	34.9
5	1.32	m	28.4	5	1.49	m	30.5
6−15	1.37−1.25	*b*	32.9−24.5	6−13	1.37−1.25	*b*	32.9−24.5
16	1.26	m	34.5	14	1.26	m	34.5
17	1.29	m	25.0	15	1.29	m	25.0
18	0.88	t, *J* = 7.1*	15.8	16	0.88	t, *J* = 7.1*	15.8

[a] The coupling constant values marked with an asterisk were determined from 1D ^{1}H spectrum, and others were determined from the DQF-COSY spectrum. [b]The coupling constant values could not be determined due to the signal overlappings. [c]No cross-peaks between H4 and H5s for ring L are observed in the DQF-COSY spectrum.

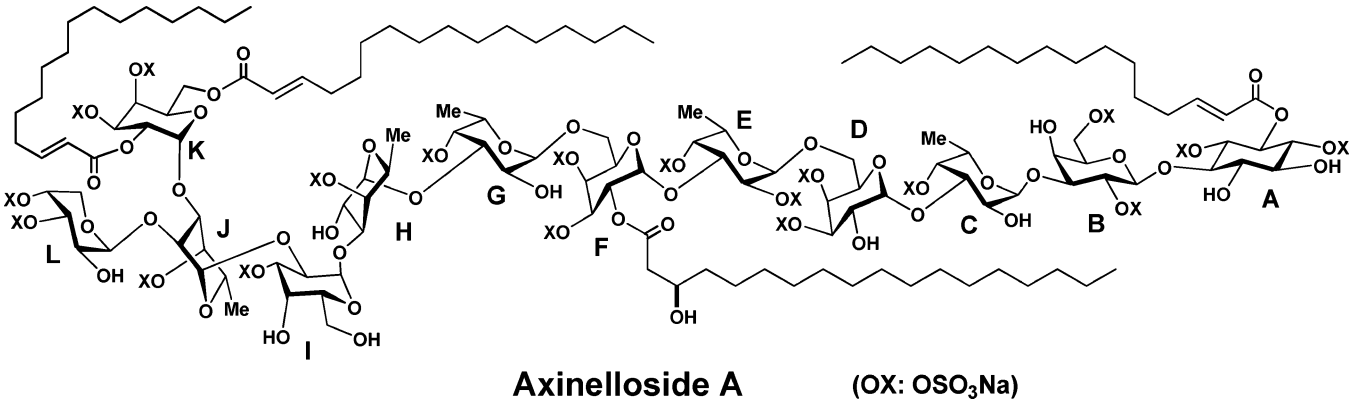

Figure 4. Chemical structure of axinelloside A.

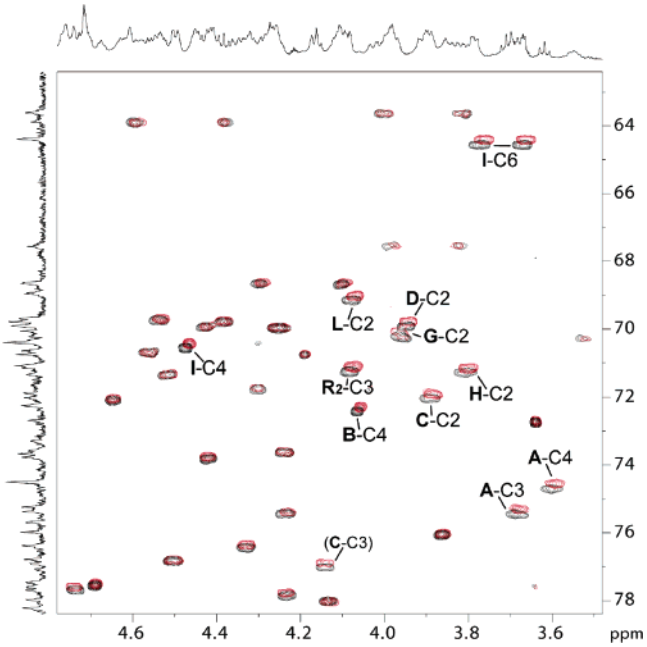

Figure 5. HMQC spectra of axinelloside A in CD_3OD/D_2O (9:1) (red) and in CD_3OH/H_2O (9:1) (black).

carried out. In case of carbohydrates, ^{13}C chemical shift values of carbons possessing free hydroxyl groups are downfield shifted in the range −0.2 to −0.1 ppm when measured in D_2O, comparing with those measured in H_2O.[14] The isotope shift experiment of axisinelloside A revealed several significant isotope shifts as determined by HMQC data measured in CD_3OD/D_2O (9:1) and in CD_3OH/H_2O (9:1), revealing the presence of 11 carbons bearing hydroxyl groups (Figure 5): **A**-C3 ($\Delta\delta_C = \delta_{COD} - \delta_{COH} = -0.17$), **A**-C4 (−0.17), **B**-C4 (−0.15), **C**-C2 (−0.16), **D**-C2 (−0.14), **G**-C2 (−0.14), **H**-C2 (−0.14), **I**-C4 (−0.14), **I**-C6 (−0.17), **L**-C2 (−0.14), and **R2**−C3 (−0.17). Although the glycosylated carbon of **C**-C3 also showed downfield shift ($\Delta\delta_C = -0.12$), the small value for positive $\Delta\delta_H$ ($\delta_D - \delta_H$) for **C**-H3 proton (<+0.01) indicated that this was not a result of deuterium isotope effects. Therefore, the remaining 19 oxygen-linked carbons which were neither glycosylated nor esterified by fatty acids were concluded to be sulfated; e.g., C2 and C6 of the ring **A**, C2 and C6 of the ring **B**, C4 of the ring **C**, C3 and C4 of the ring **D**, C2 and C4 of the ring **E**, C3 and C4 of the ring **F**, C4 of the ring **G**, C4 of the ring **H**, C3 of the ring **I**, C4 of the ring **J**, C3 and C4 of the ring **K**, and C3 and C4 of the ring **L** were found to be sulfated. C4 positions in all five α-L-fucose residues, C3 positions in all four α-D-galactose residues, and C4 positions in three out of four α-D-galactose residues were sulfated, while several other positions in the sugar units were irregularly sulfated, which makes the chemical structure very complex. The lowfield ^1H and ^{13}C chemical shifts of carbons possessing sulfate groups also supported the assignment made by the deuterium isotope shift experiment of axinelloside A.

Molecular Weight and Structural Determination by MS Analyses. Although the complete structure could be proposed by extensive 2D-NMR analyses, the exact molecular weight remained to be determined. In the case of highly sulfated polysaccharides, it is difficult to obtain their molecular ion peaks due to the labile nature of sulfate groups.[15] To overcome this problem, various attempts have been made; particularly effective is ESI mass measurements with excess quaternary ammonium salts.[16] In fact, we could observe a molecular ion peak of axinelloside A in the electrospray-ionization quadrupole ion trap mass spectrum (ESI-QIT-MS) when measured with excess ammonium counterions. To ensure the quality of mass spectroscopy, metal cation contaminants, such as Na^+, K^+, and Ca^{2+}, were removed by treatment with Dowex 50WX8 resin, followed by neutralization with ammonium hydroxide. The negative ion mode ESI-MS spectra of axinelloside A tetramethylammonium salt (Figure 6A) showed average mass signals in the triple (*m*/*z* 1844), quadruple (*m*/*z* 1365), pentuple (*m*/*z* 1077), and hextuple charge states (*m*/*z* 885), corresponding to the molecular ion at *m*/*z* 5755 (molecular weight 5756.13 calcd from $C_{213}H_{447}O_{117}S_{19}N_{19}$), which was consistent with the presence 19 sulfate groups in axinelloside A as established by the deuterium shift experiment. This was also supported by the negative ion mode MALDI-TOF-MS (Figure 6B) measured with 6-aza-2-thiothymine (ATT) as a matrix and sodium counterions, which revealed a weak peak due to a molecular ion at *m*/*z* 4761 $(M - Na)^-$, consistent with the molecular weight of 4780.4 as the monoisotopic mass of the 19 sodium salt. Thus, the molecular formula of axinelloside A is $C_{137}H_{219}O_{117}S_{19}Na_{19}$.

Furthermore, mass fragmentation analysis of axinelloside A was carried out using MALDI-TOF-MS method to substantiate the molecular structure assigned. Several MS/MS experiments

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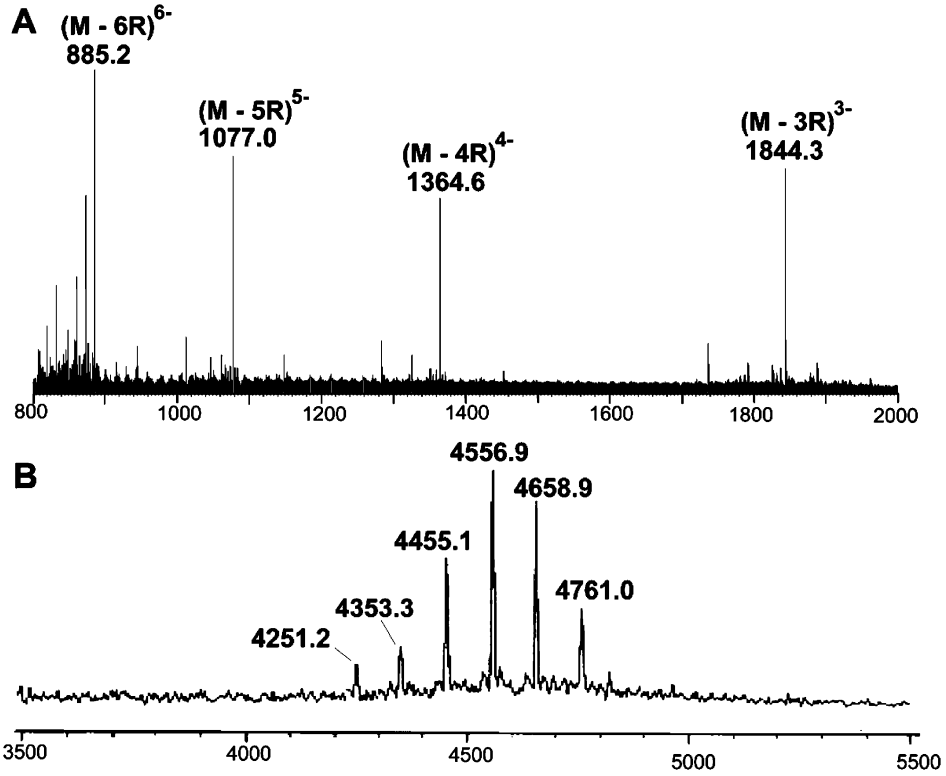

Figure 6. Negative-ion mode mass spectra of axinelloside A. (A) ESI-QIT mass spectrum obtained with excess tetramethylammonium salt, where M is the molecular weight of axinelloside A 19 tetramethylammonium salt ($C_{213}H_{447}O_{117}S_{19}N_{19}$) and R is the mass of the tetramethylammonium (NMe_4) ion. (B) MALDI-TOF mass spectrum obtained using 6-aza-2-thiothymine as a matrix in the presence of NaCl.

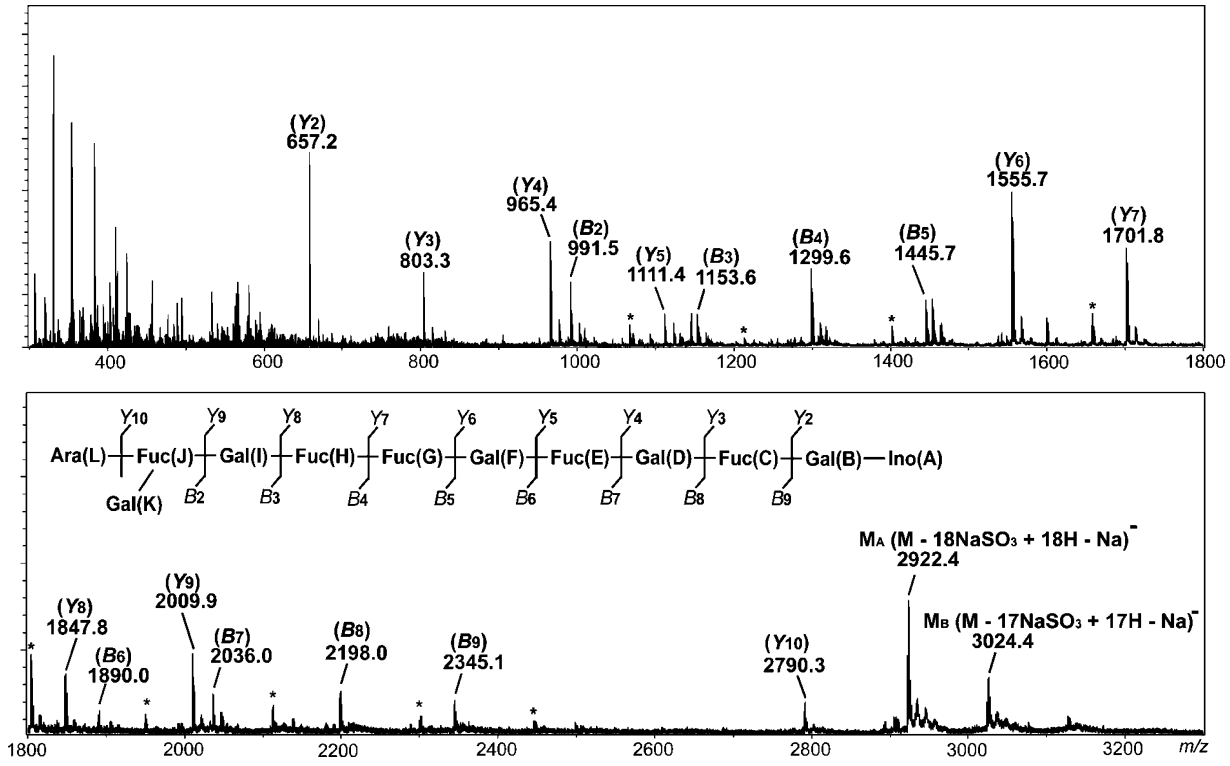

Figure 7. Negative-ion mode MALDI-TOF mass spectrum of the H-exchanged axinelloside A obtained using DHBA. *Y*- and *B*-Cleavages of M_A ($M - 18NaSO_3 + 18H - Na)^-$ were assigned in the spectrum, where M is the molecular weight of axinelloside A 19 sodium salt ($C_{137}H_{219}O_{117}S_{19}Na_{19}$). Peaks with an asterisk in the spectrum correspond to the fragment peaks of M_B ($M - 17NaSO_3 + 17H - Na)^-$.

and in-source decay fragmentation of intact axinelloside A were unsuccessful due to poor fragmentation. The mass spectrum of the proton-exchanged sample obtained by treatment with Dowex 50WX8 resin showed a strong fragment peak (M_A in Figure 7) at *m*/*z* 2922 ($C_{137}H_{237}O_{63}S$), corresponding to the monosulfate fragment of axinelloside A, in addition to several major prominent signals between *m*/*z* 600 and 2800, presumably generated by glycosyl linkage cleavages of the *Y*- and *B*-types

from the M_A ion (Figure 7). All these *Y*- and *B*-cleavages were observed at most of the glycosyl linkages, which unambiguously supported the glycosyl sequence. Interestingly, each prominent peak was observed as an adduct with one sulfate group, which suggested that the sulfate group was localized randomly at one of the sulfated positions in intact axinelloside A, thereby causing the fragmentations from both termini of the molecule.

Concluding Remarks. In this paper, we show that axinelloside A, a potent telomerase inhibitor from the marine sponge *A. infundibula*, is a highly sulfated, unusual lipopolysaccharide of the molecular weight 4780.4. The sugar backbone was comprised of 12 sugars, e.g., a *scyllo*-inositol, a D-arabinose, 5 D-galactoses, and 5 L-fucoses, to which one (*R*)-3-hydroxyoctadecanoic acid, 3 (*E*)-2-hexadecenoic acids, and 19 sulfates were attached. It should be noted that axinelloside A shows some similar structural features to sulfate polysaccharides from the marine sponge *Chondrilla nucula* and *Dysidea fragilis*,[17] which are composed of fucose, arabinose, and galactose, together with sulfated hexuronic acids (approximately two sulfate groups per sugar residue).

Scyllo-inositol is a rare polyol in natural products; phosphatidyl *scyllo*-inositols and a sialylated *scyllo*-inositol galactoside were reported from plant seeds[18] and normal human urine,[19] respectively, although *scyllo*-inositol is widely contained in human brains. (*R*)-3-Hydroxy fatty acids are typically found in bacterial lipopolysaccharide;[20] in fact, (*R*)-3-hydroxyoctadecanoic acid was contained in *Helicobacter pylori* lipid A.[21] Although (*E*)-2-hexadecenoic acid was not reported from natural sources, it is analogous to the most common fatty acids (usually $C_{10}-C_{16}$) in lipid A. To the best of our knowledge, this is the first report of a fatty acid-contained sulfated polysaccharide from a marine sponge. It is likely that axinelloside A is produced by symbiotic bacteria, and its function in the sponge is an interesting subject.

Axinelloside A strongly inhibited the activity of human telomerase with an IC_{50} value of 0.4 μM (2.0 μg/mL). Perhaps the sulfate groups in axinelloside A play an important role for the activity, since the dictyodendrins lost all telomerase inhibitory activity when sulfate groups were removed.[8] On the other hand, it was reported that oleic acid (i.e., C_{18} fatty acid) competitively inhibited the activity of telomerase (K_i = 3.1 μM).[22] The mechanism of action of axinelloside A is an interesting subject.

Experimental Section

General Procedures. For NMR spectroscopy, a sample is dissolved at the concentration of 1 mM in CD_3OD/D_2O (9:1). NMR spectra were recorded on a Bruker Avance 600 or an Avance 800 spectrometer, and data processing was performed with XWINNMR software (Bruker) running on Silicon Graphics O_2 workstations. ^1H and ^{13}C chemical shifts were referenced to solvent peaks at δ_H 3.302 and δ_C 50.695 for CD_3OD/D_2O (9:1) and at δ_H 3.310 and δ_C 50.806 for CD_3OH/H_2O (9:1) related to DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid)-trimethyl singlet resonance (0 ppm for proton and carbon) as the standard. One-dimensional ^1H and ^{13}C spectra were recorded at 300 K with 32K and 64K data points, respectively. The DQF-COSY spectra were measured in phase-sensitive mode using 8K (t2) by 1K (t1) data points. The mixing time in NOESY experiments was 300 ms, and the spin-locking time in HOHAHA was 80 ms. HMBC and HMQC spectra were recorded essentially as described in the literature. For the deuterium isotope shift experiment, 1 mM of axinelloside A in CD_3OH/H_2O (9:1) was used for HMQC experiment. HOHAHA-HSQC spectra were also measured with the mixing time of 60 ms. A usual data size for 2D measurements was 2K (t2) by 1K points (t1) with a phase sensitive mode except for those of DQF-COSY. FABMS spectra were acquired on a JEOL JMX-SX102/SX102 tandem mass spectrometer at an accelerating voltage of 10 kV. After the sample dissolved in *n*-PrOH/MeCN/H_2O (2:3:3) and 5 mM ammonium acetate, ESI-MS analysis was performed in a negative ionization mode, using a Q-Tof2 mass spectrometer equipped with a nanospray ionization source (Micromass UK Limited, Manchester, UK). The negative and linear or reflector mode MALDI-TOF-MS was performed using an Ultraflex II TOF/TOF instrument (Bruker Daltonik GmbH, Bremen, Germany) with 1 μL of a 9:1 (v/v) mixture of a matrix (α-cyano-4-hydroxy cinnamic acid, sinapic acid, 2,5-dihydroxybenzoic acid, or 6-aza-2-thiothymine) and a counterion (0.1% TFA or 0.2 M NaCl). The negative ion mode ESI-MS analysis of the tetramethylammonium salt of the sample was performed using an LTQ mass spectrometer (Thermo Electron, San Jose, CA) fitted with a nano-ESI probe. IR spectrum was recorded on a JASCO FT/IR-5300 spectrophotometer. Optical rotation was measured on a JASCO DIP-1000 digital polarimeter. UV spectra were recorded on a Shimadzu Bio Spc-1600 spectrophotometer.

Animal Material. The sponge was collected by hand using Scuba diving at 15−20 m depths off Shikine-jima Island (32°18′ N, 139°12′ E), the Izu Islands, and kept frozen until extraction. It was identified as *Axinella infundibula*, and the voucher specimen was deposited at the Zoological Museum of University of Amsterdam (ZMA POR 15731).

Telomerase Inhibition Assay. A pellet of 2×10^6 cells of telomerase positive HeLa cells was extracted with 200 μL of CHAPS buffer (1 mM $MgCl_2$, 1 mM EGTA, 10 mM Tris-HCl, pH 7.5, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS, and 10% glycerol). Telomerase activity was measured by the telomeric repeat amplification protocol (TRAP) method with a slight modification.[3,23] A 0.5 μL portion of the test solution was added to a mixture of 8.3 μL of TRAP buffer solution[3] and 0.5 μL of the cell extract, and the mixture was preincubated on ice for 20 min. After addition 0.7 μL of a solution that contained 25 ng of TS primer and 0.5 nmol of dNTP mixture,[3] the reaction mixture was incubated at 30 °C for 40 min and then heated at 80 °C for 10 min in order to inactivate telomerase. After the reaction mixture was cooled to room temperature, additions of 0.15 U of TaKaRa Ex Taq (Takara Bio Inc., Japan) and 20 μL of a solution prepared by mixing 96 μL of TaKaRa Ex Taq buffer, 19.2 μL of dNTP solution (2.5 mM), 2.59 μL of TS primer solution (1 μg/μL), 2.59 μL of ACX primer solution (1 μg/μL), 1.20 μL of NT primer solution (0.5 μg/μL), 12.0 μL of TSNT solution (0.01 μmol),[23] and 826.5 μL of water were made; PCR was then conducted for 30 cycles consisting of 3 cycles (94 °C for 30 s, 66 °C for 30 s, and 72 °C for 30 s), 3 cycles (94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s), and 24 cycles (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s). The PCR products were analyzed by SDS PAGE on a 7%/15% polyacrylamide gel in Tris-borate buffer (2.22 mM Tris-HCl, 2.22 mM H_3BO_3, and 0.055 mM $EDTANa_2$) at 150 V for 90 min. The gel was stained with Gel Star and photographed under a UV lamp.

Isolation of Axinelloside A. A 1.2 kg portion of the frozen sponge (3.9 kg) was successively extracted with MeOH (4.4 L), EtOH (2.9 L), and acetone (3.0 L), while a 2.7 kg portion was also successively extracted with MeOH (6.0 L), EtOH (3.3 L), and acetone (3.0 L). The

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extracts were combined and partitioned between water (2.0 L) and Et_2O (4.0 L). The Et_2O layer was partitioned between 90% aqueous MeOH (1.0 L) and *n*-hexane (2.1 L). A 7.2 g portion of the active *n*-hexane fraction (12.2 g) was separated by silica gel column chromatography using stepwise elution with $CHCl_3$, $CHCl_3$/MeOH (9:1), $CHCl_3$/MeOH/H_2O (9:1:0.1), $CHCl_3$/MeOH/H_2O (3:2:0.5), MeOH, and *n*-BuOH/AcOH/H_2O (4:1:2), while the remaining portion of the *n*-hexane fraction was separated by silica gel column chromatography using stepwise elution of $CHCl_3$, $CHCl_3$/MeOH (9:1), $CHCl_3$/MeOH (7:3), $CHCl_3$/MeOH (3:2), $CHCl_3$/MeOH/H_2O (3:2:0.5), $CHCl_3$/MeOH/H_2O (3:2:0.75), MeOH, to *n*-BuOH/AcOH/H_2O (4:1:2). A 696.1 mg portion of the active fraction (1035.6 mg) was separated by C_{18} reversed-phase HPLC on COSMOSIL AR-II (Nacalai Tesque) using a gradient elution from 40% MeCN containing 50 mM $AcONH_4$ to 60% MeCN containing 50 mM $AcONH_4$. The active fraction (129.1 mg) was purified by reversed-phase HPLC on Develosil C30-UG-5 (Nomura Chemical Co.) with *n*-PrOH/MeCN/H_2O (3:1:2) containing 100 mM $AcONH_4$. The active fraction (95.0 mg) was further purified by recycling reversed-phase HPLC on Develosil C30 eluted with *n*-PrOH/MeCN/H_2O (3:1:2) containing 100 mM $AcONH_4$ to afford axinelloside A (27.6 mg, 0.0011% based on wet weight).

Axinelloside A: Colorless amorphous solid; $[\alpha]^{26}_D$ −10.0° (*c* 0.10, MeOH); UV (MeOH) λ_{max} 210 nm; IR (film) ν_{max} 3464, 3144, 3045, 2926, 2847, 1711, 1643, 1406, 1224, 1049 cm^{-1}.

Alditol Acetate Analysis of Axinelloside A.[11] A 0.1 mg portion of axinelloside A was hydrolyzed with 90% aqueous HCOOH/10% aqueous TFA (1:1, 0.5 mL) at 70 °C for 18 h. The hydrolysates were dried by centrifugal evaporation and then in vacuo. The hydrolysates were partitioned between water (300 μL) and *n*-hexane (300 μL × 3). To the aqueous layer were added 30 μL of 1 M aqueous NH_3 and 300 μL of DMSO solution of $NaBH_4$ (20 mg/mL). After being kept at 40 °C for 90 min, the reaction was quenched by addition of 60 μL of AcOH in an ice bath. The reaction mixture was dried by centrifugal evaporation and then in vacuo. To the residue was added AcOH/dry MeOH (1:99, 500 μL × 5), and then the solvents were removed in a stream of N_2 gas in order to blow boric acid away as methyl borate. 200 μL of dry pyridine and 200 μL of Ac_2O were added to the mixture. After being kept at 100 °C for 50 min, the reaction mixture was cooled to room temperature and then dried in a stream of N_2 gas. The alditol acetates were dissolved in acetone, and aliquots of the solution were applied to GC on DB-1701 (J & W SCIENTIFIC, ϕ 0.25 mm × 30 m) using the temperature program from 150 to 250 °C at a rate of 3 °C/min. Retention times (min): fucose (21.1), arabinose (22.2), galactose (30.4), *scyllo*-inositol (32.5). Fucose, arabinose, galactose, and *scyllo*-inositol were detected in the ratio of 5:1:5:1.

Determination of the Absolute Configurations of Sugars.[12] A 0.5 mg portion of axinelloside A was hydrolyzed in 90% aqueous HCOOH/10% aqueous TFA (1:1, 500 μL) at 70 °C overnight. The hydrolysates were dried by centrifugal evaporation and then in vacuo. The reaction mixture was partitioned between water (400 μL) and *n*-hexane (400 μL). The aqueous layer was further extracted with $CHCl_3$ (400 μL). To the water-soluble fraction was added 0.9 mg of L-cysteine methyl ester hydrochloride in 100 μL of dry pyridine, and the mixture was kept at 60 °C for 1 h. After cooled to room temperature, 100 μL of trimethylsilyl imidazole were added to the reaction mixture, which was warmed at 60 °C for 30 min. The trimethylsilyl ethers of the methyl (4*R*)-thiazolidine-4-carboxylate derivatives were analyzed by GC on DB-1701 using the temperature program from 180 to 250 °C at a rate of 3 °C/min. Retention times (min): D-arabinose (23.4), L-fucose (26.2), D-galactose (36.5).

Isolation of (*E*)-2-Hexadecenoic Acid from Axinelloside A. A 2.2 mg portion of axinelloside A was hydrolyzed in a mixture of MeOH (300 μL) and of 4 N aqueous LiOH (99 μL) at room temperature for 12 h. The reaction mixture was diluted with MeOH (1 mL) and then was neutralized with 1 N aqueous HCl. The mixture was dried by centrifugal evaporation. The hydrolysates were partitioned between water (1 mL) and AcOEt (1 mL × 4). The organic layer was dried over anhydrous Na_2SO_4 and concentrated to afford 0.6 mg of fatty acids. A 5.2 mg portion of axinelloside A was hydrolyzed in a mixture of MeOH (675 μL) and 4 N aqueous LiOH (225 μL) at room temperature for 17 h. The reaction mixture was diluted with MeOH (1 mL) and then was neutralized with 1 N aqueous HCl. After dilution with water (2 mL), the mixture was dried by centrifugal evaporation and then lyophilized. The hydrolysate was partitioned between water (1.5 mL) and AcOEt (1.5 mL × 4). The organic layer was dried over anhydrous Na_2SO_4 and concentrated to yield 1.1 mg of fatty acids. The AcOEt-soluble fractions were combined and purified by C_{18} reversed-phase HPLC on COSMOSIL AR-II using a gradient elution from 90% aqueous MeOH containing 200 mM $NaClO_4$ to 97% aqueous MeOH containing 200 mM $NaClO_4$ to afford 1.0 mg of (*E*)-2-hexadecenoic acid. FABMS (matrix: triethanolamine) *m*/*z* 253 $[M - H]^-$; ^1H NMR (600 MHz, CD_3OD) δ 6.91 (1H, dt, J = 6.9, 15.6 Hz), 5.78 (1H, dt, J = 1.5, 15.6 Hz), 2.20 (2H, ddt, J = 1.5, 6.9, 7.1 Hz), 1.46 (2H, tt, J = 7.1, 7.3 Hz), 1.28 (20H, m), 0.98 (3H, t, J = 6.9 Hz).

Isolation of 3-Hydroxyoctadecanoic Acid Methyl Ester from Axinelloside A. A 0.5 mg portion of axinelloside A was hydrolyzed with 90% aqueous HCOOH/10% aqueous TFA (1:1, 500 μL). The mixture was heated at 70 °C overnight, and the reaction mixture was dried by centrifugal evaporation. The hydrolysate was partitioned between water (400 μL) and *n*-hexane (400 μL × 3). The aqueous layer was further extracted with $CHCl_3$ (400 μL). The *n*-hexane and the $CHCl_3$ layers were combined to yield 0.1 mg of a mixture of fatty acids. Additionally, a 1.5 mg portion of axinelloside A was hydrolyzed in a similar manner to that mentioned above. The hydrolysate was partitioned between water (300 μL) and *n*-hexane (300 μL × 3). The aqueous layer was further extracted with AcOEt (300 μL × 2). The *n*-hexane and AcOEt layers were combined to furnish 0.3 mg of a mixture of fatty acids. The two fatty acids-containing preparations were combined and then dissolved in a mixture of 600 μL of toluene/MeOH (7:2) and 6 μL of 2 N aqueous HCl. To this solution were added 50 μL of 2 M trimethylsilyl diazomethane in *n*-hexane. The mixture was kept at room temperature for 1 h and concentrated in a stream of N_2 gas to yield 0.6 mg of the crude methyl esters, which was purified by silica gel chromatography using stepwise elution with *n*-hexane, *n*-hexane/AcOEt (95:5), *n*-hexane/AcOEt (9:1), *n*-hexane/AcOEt (8:2), and *n*-hexane/AcOEt (7:3) to afford 0.1 mg of the methyl ester of 3-hydroxyoctadecanoic acid. FABMS (matrix: NBA) *m*/*z* 315 $[M + H]^+$ and 337 $[M + Na]^+$; ^1H NMR (CD_3OD, 600 MHz) δ 3.96 (1H, m), 3.66 (3H, s), 2.47 (1H, dd, J = 4.2, 15.0 Hz), 2.38 (1H, dd, J = 8.5, 15.0 Hz), 1.45 (2H, m), 1.28 (26H, m), 0.89 (3H, t, J = 6.9 Hz).

Determination of the Absolute Configuration of Methyl 3-Hydroxyoctadecanoate.[13] To a 50 μg portion of methyl 3-hydroxyoctadecanoate, a 100 μL portion of a mixture composed of pyridine (250 μL), CH_2Cl_2 (250 μL), DMAP/pyridine (5 μL, 2.3 mg/mL), and (−)-MTPACl (5 μL) was added. The mixture was kept at room temperature for 2 h. The reaction was quenched by addition of saturated aqueous $NaHCO_3$ (200 μL), and the mixture was extracted with AcOEt (200 μL × 3). The AcOEt layer was dried over anhydrous Na_2SO_4. After filtration, the AcOEt layer was concentrated in a stream of N_2 gas. The residue was separated by silica gel column chromatography using stepwise elution with *n*-hexane, *n*-hexane/AcOEt (95:5), *n*-hexane/AcOEt (9:1), and *n*-hexane/AcOEt (8:2) to yield the (*S*)-MTPA ester of methyl 3-hydroxyoctadecanoate.

A 50 μg portion of methyl 3-hydroxyoctadecanoate was similarly reacted with (+)-MTPACl to yield the (*R*)-MTPA ester of methyl 3-hydroxyoctadecanoate. $\Delta\delta$ values of H2, H3, and H4 were obtained from both ^1H NMR and COSY spectra.

Determination of Sulfate Contents by Ion Chromatography. A 55 μg portion of axinelloside A was hydrolyzed at 70 °C overnight with 90% aqueous HCOOH/10% aqueous TFA (1:1, 500 μL). The reaction mixture was concentrated by centrifugal evaporation. The residue was resolved in 400 μL of water, and 10 μL aliquots of the

solution were analyzed by ion chromatography on a Shim-pack IC-A3 (S) (Shimadzu Co., Kyoto, Japan) with IC-MA3-1 (Shimadzu Co.). The quantity of sulfate ion was estimated from the calibration curve using an authentic sample.

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Supporting Information Available: (1) isolation scheme and a chart for recycling HPLC of axinelloside A, (2) GC data of alditol acetate and methyl (4*R*)-thiazolidine-4-carboxylate derivatives of axinelloside A, (3) spectral data for fatty acids in axinelloside A, (4) $\Delta\delta$ values for MTPA ester of methyl 3-hydroxyoctadecanoate, and (5) spectral data of axinelloside A (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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