Gracilarioside and Gracilamides from the Red Alga *Gracilaria asiatica*

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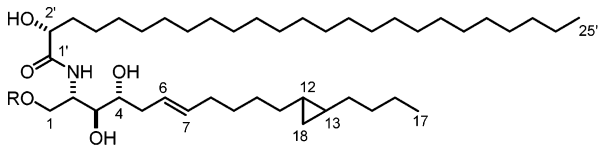
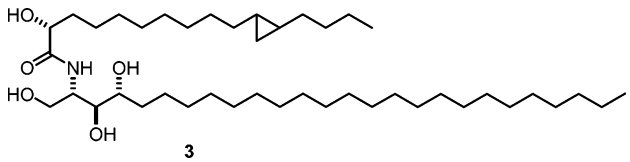
One gracilarioside and two gracilamides with unusual cyclopropane-containing alkyl chains were isolated from the red alga *Gracilaria asiatica*. Their structures were determined by spectroscopic methods and microscale chemical degradation. To our knowledge, no ceramides with a cyclopropane ring have been reported from marine organisms. These three compounds were mildly cytotoxic to the human A375-S2 melanoma cell line.

Many highly bioactive sphingolipids have been discovered from marine organisms able to act as biomediators of activation, cell agglutination, intracellular communication, and cell development.[1] Moreover, an agelasphin derivative, a glycosphingolipid isolated from the sea sponge *Agelas mauritiamus*, has been in phase I clinical trials for the treatment of cancer.[2] In a search for novel bioactive constituents from the marine red alga *Gracilaria asiatica* (C. F. Chang et B. M. Xia), one gracilarioside (**1**) and two gracilamides (**2**, **3**) bearing an unusual cyclopropane ring were isolated. Their structures were established by spectroscopic methods and chemical degradation. Herein, we describe the isolation, structure elucidation, and biological activities of these three compounds, named gracilarioside and gracilamides A and B. Cerebrosides with cyclopropane-containing chains are rare in nature. Although Fattorusso reported four cyclopropane-containing cerebrosides from two marine sponges,[3,4] no other such cerebrosides or ceramides have been discovered. In this paper, gracilamides A and B are reported here as the first ceramides from marine organisms with cyclopropane-containing alkyl chains.



1 R=1-β-D-glucosyl
2 R=H



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Gracilarioside (**1**), white amorphous powder, had a molecular formula of $C_{49}H_{93}NO_{10}$ obtained from its HRESIMS at *m*/*z* 856.6898 ($[M + H]^+$, calcd 856.6878), indicating four degrees of unsaturation. The ^{1}H NMR spectrum for **1** displayed resonances typical of an aliphatic chain (δ 1.26) and several multiplets from δ 3.98 to 5.24, which are reminiscent of a phytosphingosine-type cerebroside. Moreover, an anomeric carbon (δ 106.2) and a number of aliphatic carbons together with an amide function (δ 175.7 and 51.7) were deduced from ^{13}C NMR spectroscopic data, suggesting that **1** was a monocerebroside (Table 1).

The structure of **1** was assigned by the analysis of 2D NMR data, including HMQC and HMBC experimental methods. In addition, signals corresponding to a glucopyranose moiety were also identified from ^{1}H and ^{13}C NMR experiments (Table 1).[5] The anomeric proton doublet (δ 4.86, J = 7.7 Hz) indicated the β-orientation of the proton, and it was identified from its correlation peak with the relevant carbon C-1″ in the HMQC spectrum.

Examination of the HMBC spectrum showed that the amide methine C-2 was coupled with the NH doublet at δ 8.51, the oxymethylene protons at δ 4.74 and 4.48 (H-1), and the oxymethine proton at δ 4.30 (H-3). The HMBC correlations between H-3 and the carbon at δ 72.8 and 51.7 also showed that there was another hydroxyl function at C-4. In fact, HMBC correlations of the carbonyl at 175.7 to H-2′ confirmed the presence of a side chain of an α-hydroxyl fatty acid. The double bond in **1** was assigned at C-6 and C-7 in the sphingoid long chain base through which correlations in the HMBC were found between H-5 and δ 72.8, 132.8, and 128.3. The geometry of the C-6/C-7 double bond was determined to be *trans* by the large vicinal coupling constant (J = 14.8 Hz) displayed between H-6 and H-7.

In addition, the three upfield shifted multiplets at −0.21 (1H), 0.66 (1H), and 0.74 (2H) in the ^{1}H NMR and the relevant carbons assigned by the HMQC spectrum showed that **1** contained an uncommon cyclopropane ring (Table 1). Therefore, to confirm the location of the cyclopropane ring and establish the length of the alkyl chains, microscale chemical degradation of **1** was carried out using part of the method that Fattorusso reported.[3] We were fortunate to obtain the corresponding methyl fatty ester by starting from a trace amount of **1** (1 mg) via successive reduction, methanolysis, Lemieux oxidation, and methylation. The ester was submitted for GC-MS analysis (Scheme 1) to determine the correct position of the cyclopropane ring and the length of each chain. Thus, 1 mg of **1** was subjected to hydrogen reduction catalyzed by PtO_2 using acetic acid as a solvent at room temperature.[6] After the reduction of the double bond and the cleavage of the cyclopropane ring, the mixture of two methyl-branched alkyl chains and one unbranched chain obtained was treated with aqueous HCl/MeOH to produce methanolysis, giving the products α-hydroxyacid methyl ester (**4**), phytosphingosine (**5**), and a methyl glycoside, respectively.[7] Furthermore, the D configuration of the glucose unit was determined by HPLC with D- and L-glucose standard samples, which was derived from sugars and chiral α-methylbenzylamine (MBA) in the presence of sodium cyanoborohydride.[8] In order to identify the location of the cyclopropane ring, **4** and **5** were then determined on the basis of the ^{1}H NMR analysis. Two obvious additional branched methyl signals at δ 0.92 (d, J = 6.7 Hz) and 0.98 (d, J = 6.7 Hz) were observed on the long-chain bases (**5**), but there were no branched methyl signals of **4** in the ^{1}H NMR. Nevertheless, it was difficult to confirm the length of the chain, and the correct position of the branched methyl could only be obtained by ^{1}H NMR analysis. Hence, similar chemical degradations of **4** and **5** were then carried out. Following cleavage of the bonds C-3−C-4 of **5**

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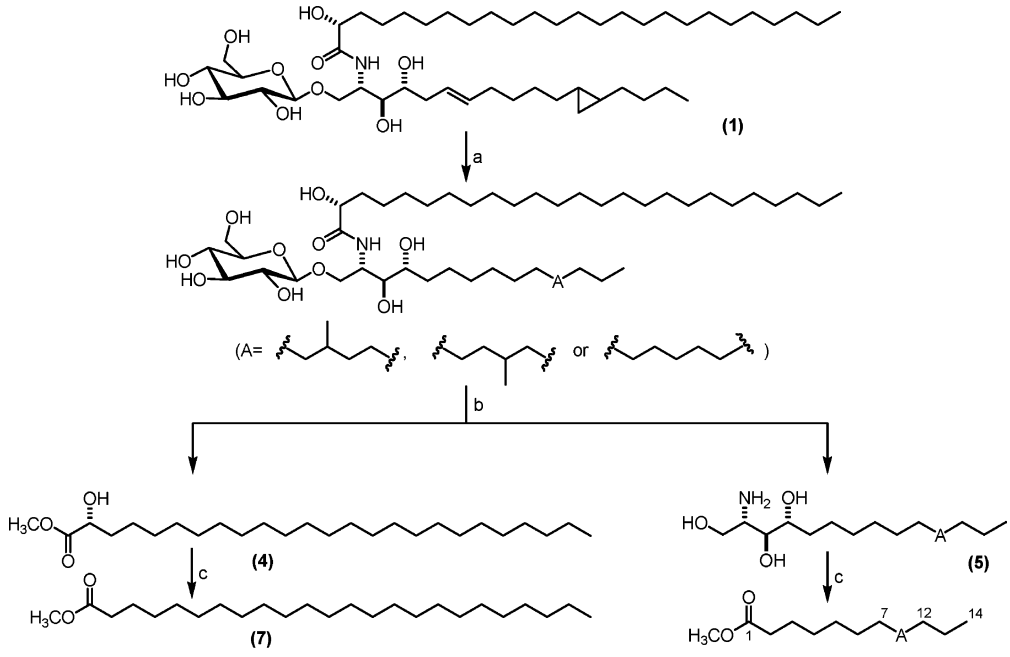
Table 1. NMR Spectroscopic Data for Gracilarioside (**1**) and Two Gracilamides (**2**, **3**) (C_5D_5N)[a]

	gracilarioside (**1**)		gracilamide A (**2**)			gracilamide B (**3**)	
pos.	δ_H (m, *J*, Hz)	δ_C	δ_H (m, *J*, Hz)	δ_C	pos.	δ_H (m, *J*, Hz)	δ_C
1	4.74 (dd, 10.6, 6.4)	70.6	4.62 (dd, 10.5, 4.0)	61.8	1	4.52 (dd, 10.7, 4.6)	62.0
	4.48 (dd, 10.6, 4.2)		4.50 (dd, 10.5, 6.2)			4.43 (dd, 10.7, 5.0)	
2	5.24 (m)	51.7	5.10 (m)	52.7	2	5.14 (m)	53.0
3	4.30 (m)	75.4	4.33 (m)	76.1	3	4.35 (m)	76.8
4	4.23 (m)	72.8	4.30 (m)	73.1	4	4.28 (m)	73.0
5	2.99 (m)	37.5	3.06 (m)	37.5	5	1.93 (m)	34.1
	2.67 (m)		2.72 (m)			2.24 (m)	
6	5.95 (ddd, 14.8, 8.0, 6.9)	132.8	5.98 (ddd, 14.5, 8.1, 6.8)	132.6	6	1.91 (m)	26.7
7	5.72 (ddd, 14.8, 7.6, 6.6,)	128.3	5.73 (ddd, 14.5, 8.3, 7.1)	128.1	7−23, 5′−9′	1.26	29.9, 30.4
8	2.05 (m)	33.2	2.04 (m)	33.0	24, 14′	1.26	32.2
9−10	1.26	29.6	1.26	29.4	25, 15′	1.26	23.0
4′−22′	1.46	30.6	1.44	30.4			
11, 14	1.46	29.0	1.44	28.8	26, 16′	0.86	14.3
12, 13	0.74 (m)	16.2	0.72 (m)	16.0	1′		175.2
15, 23′	1.26	32.1	1.26	31.9	2′	4.62 (m)	72.5
16, 24′	1.26	22.9	1.26	22.7	3′	2.05 (m)	35.7
17, 25′	0.88 (t, 6.8)	14.3	0.85	14.0		2.21 (m)	
18	−0.21 (ddd, 5.3, 5.3, 4.3)	11.4	−0.20 (ddd, 5.1, 5.1, 3.8)	11.1	4′	1.69 (m)	25.8
	0.66 (ddd, 4.3, 3.9, 3.9)		0.65 (ddd, 3.8, 3.3, 3.3)			1.78 (m)	
1′		175.7		175.0	10′, 13′	1.43	29.0
2′	4.54 (m)	72.4	4.53 (m)	72.2	11′, 12′	0.73 (m)	16.2
3′	2.17 (m)	35.6	2.22 (m)	35.5	17′	−0.22 (ddd, 5.0, 5.0, 4.5)	11.4
	1.96 (m)		1.94 (m)				
1″	4.86 (d, 7.7)	106.2				0.68 (ddd, 4.5, 3.5, 3.5)	
2″	4.41 (t, 7.8)	72.6					
3″	4.10 (m)	75.2					
4″	4.50 (m)	70.3					
5″	3.98 (m)	77.1					
6″	4.43 (dd, 12.6, 5.0)	62.4					
	4.40 (dd, 12.6, 5.0)						
NH	8.50		8.58		NH	8.60	

[a] ^{1}H was recorded at 600 MHz; ^{13}C was recorded at 125 MHz; ^{1}H was recorded at 300 MHz; ^{13}C was recorded at 75 MHz.

Scheme 1. Reagents and Conditions for the Microscale Degradation of Gracilarioside

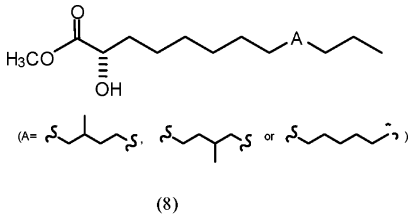


and C-1′−C-2′ of **4** via oxidation using $KMnO_4$ and $NaIO_4$,[3,9] the resulting fatty acids were then methylated with CH_2N_2 to give four methyl esters (**6**, **7**). Furthermore, it was concluded that the cyclopropane ring was on the phytosphingosines (**5**) by GC-MS analysis, in agreement with the ^{1}H NMR results. It was analyzed by examining the retention time and mass spectrum compared with standard samples, and **6** was a mixture of methyl pentadecanoate and two inseparable methyl-branched methyl myristates following degradation,[6] whereas **7** was a methyl lignocerate. Strong fragment ion peaks at *m*/*z* 185, 157, and 71 showed α-cleavage from either side of the tertiary carbon of one methyl-branched methyl myristate. Moreover, rearrangement of the peak at *m*/*z* 157 resulted in the diagnostic peaks at 158 and 159. The subsequent loss of methanol and H_2O at *m*/*z* 185 also showed characteristic peaks at *m*/*z* 153 and 135. Similarly, the other methyl-branched ester gave peaks at *m*/*z* 199, 173, 172, 171, 167, 149, and 57, respectively. Therefore,

it was concluded that the cyclopropane ring was located on the sphingosine chain at positions 9 and 10.

Gracilamide A (**2**) was concluded to have the formula $C_{43}H_{83}NO_5$ from the HRESIMS ($[M + H^+]$ *m/z* 694.6361), indicating three degrees of unsaturation. By comparing the ^1H NMR, ^{13}C NMR, and 2D NMR data, and the HRMS data of **2** with the data derived from **1**, it was found that both structures were almost identical except for the absent glucose of **2** (Table 1). The similar chemical degradation of **2** (also starting from 1 mg) further confirmed that the length of the chain and the location of the cyclopropane ring were the same as in **1**.

The HRESIMS spectrum of gracilamide B (**3**) also gave an ion peak at 696.6490 ($[M + H]^+$) corresponding to a molecular formula of $C_{43}H_{85}NO_5$. There was no double bond in the structure of **3** compared with the NMR of the above two compounds (Table 1). Chemical degradation and GC-MS analysis were again used to determine the location of its cyclopropane ring. After reduction and methanolysis, the products α-hydroxyacid methyl ester (**8**) and the long-chain base (**9**) produced were also analyzed by ^1H NMR.



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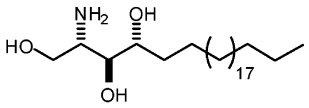

(9)

There were two obvious additional branched methyl signals at 1.01 (d, $J = 7.2$ Hz) and 0.93 (d, $J = 7.2$ Hz) for the fatty acid methyl esters (**8**), but no additional signals for the long-chain base. Moreover, the ring of **3** was located on the methyl-branched fatty acid on the basis of MS, and two methyl-branched fatty acid methyl esters were also eluted together and identified from their mass spectra with peaks at *m/z* 213, 187, 186, 185, 181, 163, 57 and 199, 173, 172, 171, 167, 149, 71, respectively. Therefore, it was concluded that the cyclopropane ring was located at positions 10′ and 11′ on the fatty acid chain.

The absolute configuration at C-2′ of **1**−**3** was determined as *R* because the CD spectrum of the α-hydroxyacid methyl esters exhibited a negative Cotton effect.[10] Also the stereochemistry of the ceramide moieties was inferred by comparison of the ^1H NMR and the physical data of **1**−**3** with the analogues as reported in the literature in terms of the signals due to 1-H to 4-H, which were 2*S*, 3*S*, 4*R*.[11]

The cytotoxicities of these three compounds were evaluated against the A375-S2 melanoma cell line using the MTT assay. Gracilarioside showed moderate cytotoxicity and induced 18.2% cell death at 20.0 μg/mL. The other two compounds exhibited a weak cytotoxicity of 11.7% cell death at 30.0 μg/mL.

Experimental Section

General Experimental Procedures. The optical rotation was measured with a Perkin-Elmer 241MC polarimeter. CD spectra were determined on a Jasco J-810 CD spectropolarimeter. UV spectra were measured on a Shimadzu UV-1601. 1D and 2D NMR spectra were recorded on a Bruker-ARX-600 and ARX-300 spectrometer. The chemical shifts were quoted relative to TMS, and the coupling constants were in Hz. HRESIMS spectra were recorded with a Q-trap LC-MS-MS system using a Turbo ionspray source. Column chromatography was performed on Si gel G (200−300 mesh, Qingdao Haiyang Chemical Factory) and Sephadex LH-20 (Pharmacia Co.) columns. TLC was carried out using Si gel GF_{254} (Qingdao Haiyang Chemical Factory) plates. HPLC was performed using a YMC Chromatorex C18 10 μm preparative column (22 × 250 mm). The identification of fatty acid methyl esters was based on the GC-MS retention times and GC-MS spectra using a DB-1MS column (0.25 × 0.25 × 30 mm). After 3 min, the column temperature was increased from 80 °C to 250 °C at a gradient of 10 °C/min. Quantitative determination was based on the area of the GC peaks.

Collection. The red alga *Gracilaria asiatica* was collected in May 2004 in Indonesia and identified by Prof. Xiao Fan (Institute of Oceanology, Chinese Academy of Sciences, Qingdao, 266071, China). A voucher specimen (No. 200405) is deposited at the Herbarium of Shenyang Pharmaceutical University.

Extraction and Isolation. The collected red alga (dry weight 4.5 kg) was soaked in MeOH at rt for 3 × 72 h and filtered. After the extracts were concentrated in vacuo at 40 °C, the residue was suspended in H_2O and then successively partitioned between EtOAc and n-BuOH. The EtOAc-soluble fraction (8.6 g) was subjected to Si gel (200−300 mesh) CC using gradients of increasing acetone (0−100%) in $CHCl_3$ and separated into 10 fractions (I−X) on the basis of TLC analyses. Fraction VII was separated by CC over Sephadex LH-20 using $CHCl_3$/MeOH (1:1) as the eluent to give three corresponding subfractions. The last two subfractions of VII were then purified by reversed-phase preparative HPLC (RP-C18, MeOH/H_2O, 97:3) to give **1** (3 mg) and **2** (2.5 mg). Fraction VI was purified by Sephadex LH20 (MeOH) to afford **3** (4 mg).

Gracilarioside (1): white, amorphous powder; $[\alpha]^{20}_D$ +12.1 (*c* 0.005, MeOH); CD (α-hydroxy acid methyl esters, MeOH) λ_{max} 211.3 nm ($\Delta\epsilon$ −0.88); UV (MeOH) λ_{max} (log ϵ) 205.0 (0.94); ^1H and ^{13}C NMR, see Table 1; ESIMS (pos) *m/z* 856 $[M + H^+]$; HRESIMS (pos) *m/z* 856.6898 $[M + H]^+$ (calcd for $C_{49}H_{93}NO_{10}$, 856.6878).

Gracilamide A (2): white, amorphous powder; $[\alpha]^{20}_D$ +8.5 (*c* 0.002, MeOH); CD (α-hydroxy acid methyl esters, MeOH) λ_{max} 213.6 nm ($\Delta\epsilon$ −0.73); UV (MeOH) λ_{max} (log ϵ) 196.6 (0.31); ^1H and ^{13}C NMR, see Table 1; ESIMS (pos) *m/z* 694 $[M + H^+]$; HRESIMS (pos) *m/z* 694.6361 $[M + H]^+$ (calcd for $C_{43}H_{83}NO_5$, 694.6350).

Gracilamide B (3): white, amorphous powder; $[\alpha]^{20}_D$ +16.7 (*c* 0.008, MeOH); CD (α-hydroxy acid methyl esters, MeOH) λ_{max} 210.5 nm ($\Delta\epsilon$ −0.96); UV (MeOH) λ_{max} (log ϵ) 203.6 (0.70); ^1H and ^{13}C NMR, see Table 1; ESIMS (pos) *m/z* 696 $[M + H^+]$; HRESIMS (pos) *m/z* 696.6490 $[M + H]^+$ (calcd for $C_{43}H_{85}NO_5$, 696.6506).

Mixture (5) of long-chain bases after methanolysis of 1: $[\alpha]^{20}_D$ +10.4 (*c* 0.004, MeOH); ^1H NMR (300 MHz, $CDCl_3$) δ 4.10 (m), 3.85 (m), 3.50 (m), 3.45 (m), 2.35 (m), 1.63 (m), 1.26 (m), 0.98 (d, $J = 6.7$ Hz), 0.92 (d, $J = 6.7$ Hz), 0.85 (t).

Mixture (8) of fatty acid methyl ester after methanolysis of 3: ^1H NMR (300 MHz, $CDCl_3$) δ 4.11 (m), 3.51 (s), 2.07 (m), 1.27 (m), 1.01 (d, $J = 7.2$ Hz), 0.93 (d, $J = 7.2$ Hz), 0.86 (t).

GC-MS Analysis of Methyl-Branched Fatty Acid Methyl Esters. Gracilarioside (**1**): Methyl-branched methyl myristates: $t_R = 16.73$ min; MS *m/z* (relative intensities) 256 (15), 199 (10), 185 (9), 173 (1), 172 (2), 171 (6), 167 (6), 159 (2), 158 (3), 157 (12), 153 (5), 149 (3), 135 (4), 71 (15), 57 (23).

Gracilamide A (**2**): Methyl-branched methyl myristates: $t_R = 16.67$ min; MS *m/z* (relative intensities) 256 (14), 199 (9), 185 (9), 173 (1), 172 (1), 171 (5), 167 (4), 159 (1), 158 (1), 157 (10), 153 (4), 149 (2), 135 (2), 71 (14), 57 (25).

Gracilamide B (**3**): Methyl-branched methyl pentadecanoate: $t_R = 17.74$ min; MS *m/z* (relative intensities) 270 (16), 213 (15), 199 (10), 187 (2), 186 (3), 185 (11), 173 (2), 172 (2), 171 (7), 167 (6), 163 (3), 149 (2), 135 (2), 71 (14), 57 (26).

General Procedure for Chemical Degradation. Commercially available reagents were used without further purification. Gracilarioside (**1**) or gracilamide A or B (**2**, **3**) (1.0 mg) was hydrogenated in acetic acid (1.0 mL) in the presence of PtO_2 (7.0 mg) under atmospheric H_2 for 14 h. The reaction mixture was filtered and evaporated to dryness. The residue was treated with 0.9 mol/L HCl in 82% aqueous MeOH (2.0 mL) at 80 °C for 18 h. It was concentrated and separated by flash chromatography (Si gel, 200−300 mesh) eluted with $CHCl_3$ to give the fatty acid methyl esters (**4**), and with MeOH to give the sphingosines (**5**) and a methyl glycoside. The mixture of **5** (gracilarioside) and the methyl glycoside were partitioned between $CHCl_3$ and H_2O for further separation. Then, **4** and **5** were separately subjected to Lemieux

oxidation. Hence, 0.023 mol/L aqueous $KMnO_4$ and 0.09 mol/L $NaIO_4$ (2.0 mL) were slowly added to a mixture containing **4** or **5**, *t*-BuOH (1.0 mL), and 0.04 mol/L aqueous K_2CO_3 (0.5 mL). Then, the mixtures were stirred for 18 h at 37 °C, quenched with 2.5 mol/L H_2SO_4 (0.1−0.3 mL) and saturated aqueous Na_2SO_3, and then extracted with Et_2O. The organic layer was dried over Na_2SO_4. Finally, the concentrated, dried residue was esterified with excess CH_2N_2 in Et_2O overnight. The resulting esters were used for GC-MS analysis.

Cytotoxicity Assay. The cytotoxic effect of gracilarioside (**1**) and two gracilamides (**2**, **3**) on A375-S2 cells was measured by MTT assay as described. All the cells were cultured at 5×10^4 cells/well in 96-well plates (NUNC, Roskilde, Denmark). Four hours before the end of incubation, 20 μL of MTT solutions (5.0 mg/L) was added to each well. Resulting crystals were dissolved in DMSO. The result was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay using a plate microreader (TECAN SPECTRA, Wetzlar, Germany). The percentage of cell growth inhibition was calculated as follows: cell death (%) = [A492 (control) − A492 (drug)]/A492 (control) × 100.

Absolute Configuration of Glucose. According to the reported method,[8] the absolute configuration of glucose was determined by comparison of the retention time of its derivative obtained from the methanolysis with that of standard samples using HPLC (column SiO_2; eluent *n*-hexane/ethanol (95:5); flow rate 1 mL/min; detection at 230 nm). The retention times of the derivatives of the sugars were as follows: D-glucose 42.56 min, L-glucose 40.10 min.

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Supporting Information Available: ^{1}H NMR, ^{13}C NMR, and HRESIMS spectra of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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