Structural Analysis of an Alkali-Extractable Polysaccharide from the Seeds of *Retama raetam* ssp. *gussonei*

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An alkali-extractable polysaccharide (**1**) was isolated from the seeds of *Retama raetam* ssp. *gussonei*. Its composition and linkage determination have been investigated using component analysis, methylation analysis, hydrolysis studies, and NMR spectroscopic analysis. This was shown to contain D-xylose, D-glucose, and 4-*O*-methyl-D-glucuronic acid in a molar ratio of 7:2:1 and consisted of a backbone of β-(1→4)-linked D-xylopyranosyl residues, having branches of α-D-glucopyranosyl residues that contain (1→2)-linkages and 4-*O*-methyl-α-D-glucopyranosyluronic acid at O-2. This is the first report on the isolation of an alkali-extractable polysaccharide from this plant.

Retama raetam Webb & Berthel. ssp. *gussonei* (Webb). Greuter, locally named "R'tm" in Libya, is a plant belonging to the Fabaceae. It is common in the Mediterranean region and in the Sinai Peninsula.[1,2] The molecular and biochemical mechanisms associated with dormancy and drought tolerance in this desert plant have been elucidated previously.[2,3] According to a recent ethnobotanical survey in the northeastern region of Libya, 10 traditional herbal healers among 25 prescribe *R. raetam* ssp. *gussonei* for diabetes control and treatment, and it is also considered as a remedy for hypertension.[4−6] Some constituents from this plant have been reported, including daidzein, vicenin-2, naringenin, apigenin, kaempferol, quercetin, and kaempferol 7-*O*-glucoside in the seeds;[7] daidzein, daidzein 7,4′-dimethyl ether, chrysoeriol 7-*O*-glucoside, and orientin in the leaves;[8] and two flavone glycosides from its aerial parts.[9] However, despite this extensive utilization of *R. raetam* ssp. *gussonei*, no information has been published about the alkali-extractable polysaccharides present. In a previous paper, we have reported two water-soluble galactomannans from this plant.[10] In the present study, the results of investigation conducted on an alkali-extractable polysaccharide (**1**) from the seeds of dry *R. raetam* ssp. *gussonei* are presented.

Prior to aqueous extraction, lipid extraction of the dried seeds was carried out and resulted in 8.7% yield of total lipids. The extractive-free fibers from the outer integuments of the seeds contained 9.5% lignin, which was removed by three successive treatments with sodium chlorite. After defatting and delignifying, aqueous extraction was carried out to yield 9.4% of the extract at 100 °C, which afforded a highly viscous material. The aqueous extracts mainly consisted of mannose, galactose, and arabinose, but only 85% of them redissolved in water and the insoluble residue probably originated from the hemicellulosic fraction(s) of the seed coat. The residual materials of the aqueous extractions were sequentially extracted with 3, 6, and 15% aqueous NaOH at 80 °C to afford fractions I−III, which were obtained in yields of 8.2, 3.4, and 5.3% dry weight, respectively. The alkaline extractions gave a higher total yield (16.9%) than the aqueous extractions and suggested that the alkali disrupted the cell wall ultrastructure after water weakened it. The yield from fraction II to fraction III increased substantially, indicating that the increase of alkali concentration favored the release of hemicelluloses from the cell walls, as was described from data obtained by Sun and co-workers.[11] Fraction II was decolorized, which influenced the detection of isolation to some extent, using a weakly basic anion-exchange styrene macroreticular resin, in 61.6% yield. Then, the resulting sample was submitted to purification procedures such as freeze−thawing, Fehling precipitation, dissociation, and precipitation with 95% ethanol and afforded a purified polysaccharide (**1**, 0.68 g), giving a single peak on gel filtration through Sephadex G-100 and that was homogeneous by gel permeation chromatography (GPC) (Figure S1, Supporting Information). The weight-average molecular mass of **1** was 3.86×10^4 Da. The analytical results of these fractions are shown in Table S1 (Supporting Information), which showed some differences in terms of the fractions from sequential alkaline extractions. The glucose content decreased with each extraction, whereas the xylose content increased significantly. These data suggested that those polymers binding to cell walls to a lesser degree were released under mild alkaline extractions, while the less branched xylans, which were highly associated with the cell wall structure, were preferably released under stronger alkaline conditions. The presence of uronic acid was also observed in all crude fractions from the alkaline extracts.

Compound **1** showed absorption bands at 3385, 2958, 2927, 1726, 1637 (br), 891, and 761 cm^{-1} in the IR spectrum. The band at 891 cm^{-1} was ascribed to β-type glycosidic linkages in the polysaccharide,[12] while the broad band at 1637 cm^{-1} was due to bound water.[13] In turn, the band at 1726 cm^{-1} was ascribed to carboxyl groups in the uronic acid.

On complete acid hydrolysis, **1** yielded xylose and glucose as the neutral sugars and 4-*O*-methylglucuronic acid and aldobiouronic acid as the acidic sugars. The uronic acid in **1** was reduced completely and hydrolyzed. The resulting product yielded xylose, glucose, and 4-*O*-methylglucose in a molar ratio of 7:2:1. The absolute configurations of the sugars presented in **1** were determined essentially as described by Leontein et al.,[14] by GLC of their acetylated glycosides, using (+)-2-butanol,[15] which indicated that they all had the D-configuration.

Graded acid hydrolysis of **1** was conducted with 50% aqueous formic acid for 3 h at 100 °C and gave xylose, glucose, and several oligomers. Of these, an acidic oligomer, $[\alpha]_D^{25}$ (in H_2O) +115 and equivalent mass 332 Da, was hydrolyzed with 1.5 M TFA at 100 °C for 2.5 h in a sealed tube and gave three products on paper chromatography that had the same mobility as those of the unreacted oligomer, 4-*O*-methylglucuronic acid, and xylose. The equivalent weight value and specific rotation indicated that the oligomer might be 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose. To prove this, aldobiouronic acid was converted into its methyl ester, methyl glycoside, and this derivative was reduced with lithium aluminum hydride in tetrahydrofuran. This carboxyl-reduced al-

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Table 1. Methylation Analysis of Products from (1) the Hydrolysate of Reduced, Methylated Aldobiouronic Acid and (2) the Hydrolysate of Methylated, Reduced Aldobiouronic Acid

			molar ratio of products	
sugar[a]	$[\alpha]_D^{25}$ (in H_2O)	t_R[b]	(1)	(2)
2,3,4,6-Glc	+78	1.00	1.00	
2,3,4-Glc	+66	2.27		0.99
3,4-Xyl	+20	1.36	0.93	0.94

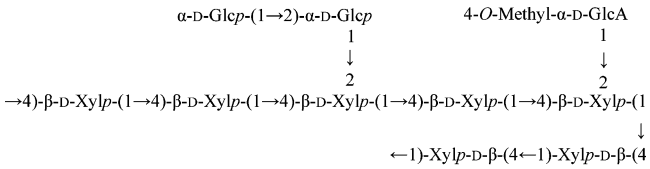
[a] 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose; 2,3,4-Glc = 2,3,4-tri-*O*-methyl-D-glucose; 3,4-Xyl = 3,4-di-*O*-methyl-D-xylose. [b] Retention times of the corresponding alditol acetates, relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucose as 1.00.

dobiouronic acid and the original aldobiouronic acid were then methylated by a modified Hakomori procedure.[16] The methylated aldobiouronic acid was reduced with lithium aluminum hydride. These two products were then separately hydrolyzed, and the hydrolyzates were both studied by GLC and by isolating the products on paper chromatography. The results, shown in Table 1, together with those discussed earlier, proved that the oligomer was indeed 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose.

The carboxyl-reduced **1** was methylated three times by a modified Hakomori procedure.[16] After hydrolysis, reduction, and acetylation, GLC of the alditol acetates from the partially methylated **1** showed five peaks corresponding to 2,3,4-tri-*O*-methyl-Xyl, 3-*O*-methyl-Xyl, 2,3-di-*O*-methyl-Xyl, 2,3,4,6-tetra-*O*-methyl-Glc, and 3,4,6-tri-*O*-methyl-Glc derivatives in an approximate molar ratio of 1.00:2.17:5.11:2.09:1.06 (Table 2). The carboxyl-reduced **1**, different from the native polysaccharide, revealed the presence of 4-*O*-methylglucuronic acid as nonreducing end-units of Glc*p* (approximate 50%) from the 2,3,4,6-tetra-*O*-methyl-Glc derivative, which indicated that 4-*O*-methylglucuronic acid and D-glucosyl groups, respectively, are attached to position 2 of different D-xylopyranosyl residues. Compound **1** was hydrolyzed by xylanase from *Trichoderma virida*. The elution profile of the digest on Sephadex G-25 showed xylose, xylobiose, and several oligosaccharides. Thus, the results indicated that the backbone of **1** contained (1→4)-linkages. The proportion of oligosaccharides decreased with the increase in incubation time, indicating the presence of a majority of β-(1→4)-linkages in the polysaccharide. Finally, only two oligosaccharides (OGSa and OGSb) remained to inhibit the enzyme to degrade the molecule completely into small fragments. OGSa and carboxyl-reduced OGSb (CR-OGSb), respectively, were converted into their alditol acetates.[16] GLC of alditol acetates from the partially methylated OGSa showed three peaks corresponding to 3,4-di-*O*-methylxylitol, 2,3,4,6-tetra-*O*-methylglucitol, and 3,4,6-tri-*O*-methylglucitol in a molar ratio of 1.00:0.99:1.03, which indicated that the structure of the OGSa fragment is Glc(1→2)-Glc(1→2)-Xyl. However, GLC of alditol acetates from partially methylated CR-OGSb showed only two peaks, corresponding to 3,4-di-*O*-methylxylitol and 2,3,4,6-tetra-*O*-methylglucitol in a molar ratio of 1.00:1.06, which confirmed that the structure of the CR-OGSb fragment is 2-*O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-D-xylose. Accordingly, **1** contains a backbone mainly composed of (1→4)-linked D-xylopyranosyl residues. The side chains attached to positions 2 of the D-xylopyranosyl residues contain (1→2)-linked D-glucosyl groups and 4-*O*-methyl-α-D-glucopyranosyluronic acid in a molar ration of 2:1 at O-2, respectively.

The 500 MHz ^{1}H NMR spectrum of **1** showed the resonances of three anomeric protons to be well separated at δ 4.67, 5.33, and 5.21, which were assigned as (1→4)-β-D-Xyl*p* (residue A), (1→2)-α-D-Glc*p* (residue B), and 2-*O*-(α-D-4-*O*-methyl-Glc*p*A) (residue C), respectively. The relevant signals occurred in two regions, namely, the anomeric region (δ 5.6−4.9 for α-anomers and δ 4.9−4.3 for β-anomers) and the ring proton region (δ 4.5−3.0).[17] This confirmed that residue A is linked β-glycosidically, which agreed with the presence of an IR band at 891 cm^{-1}. Residue B and residue C are linked α-glycosidically. The chemical shifts from 3.2 to 4.1 ppm, which showed overlapping peaks, were assigned to protons of carbons C-2 to C-5 (or C-6) of the glycosidic ring. The broad-band decoupled ^{13}C NMR spectrum (Figure 1) confirmed the substitution pattern of xylose revealed by the methylation studies. All of the resonances were resolved,[18−21] and their chemical shifts are recorded in Table 3, which showed **1** to be a complex polysaccharide with three signals in the anomeric region; those signals at δ 101.3, 99.3, and 98.2 were from the anomeric carbons of residue A,[22] residue B,[23] and residue C.[22] The signal at δ 75.6, compared with C-4 of the corresponding monosaccharide at δ 68.9, characteristic of C-4-substituted xylopyranosyl residues, and the signals at δ 71.8 and 78.6, characteristic of C-2-unsubstituted and -substituted xylopyranosyl residues, indicated that **1** contains both (1→4)- and 2-linkages present in the xylopyranosyl residues. The signals at δ 77.3 and 71.7, corresponding to C-2-substituted and -unsubstituted glucosyl residues, compared with the glucosyl ring carbon signals of α-D-Glc*p*,[24] showed (1→2)-linked glucopranosyl branches in **1**. The signals at δ 168.6, 79.7, and 53.8 corresponded to a carboxyl group, C-4, and −CH_3 from the 4-*O*-methylglucuronic acid units. The xylosyl ring carbon atoms at δ 73.6 and 65.3 corresponded to C-3 and C-5 of residue A, respectively. The glucosyl ring carbon atom signals at δ 74.1, 69.8, 72.0, and 61.6 corresponded to C-3, C-4, C-5, and C-6 of residue B. The other signals for residue C were C-3 (73.2 ppm) and C-5 (70.6 ppm), respectively.

From the results of component, methylation, and hydrolysis analysis and IR and NMR spectra, **1** was suggested for the structure of the alkali-extractable polysaccharide from the seeds of *R. raetam* ssp. *gussonei*. Compound **1** is a polymer of β-(1→4)-linked D-xylopranosyl residues having branches at *O*-2 and is composed of α-D-glucopyranosyl residues containing (1→2)-linkages and 4-*O*-methyl-α-D-glucopyranosyluronic acid. For every seven D-xylopyranosyl residues in the main chain, there is one uronic acid group, and for approximately 15 such D-xylopyranosyl residues, there is one (1→2)-linked D-glucopyranosyl branch.



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Experimental Section

General Experimental Procedures. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. IR spectra (KBr) were recorded with a Beckman Acculab 10 instrument. NMR spectra were recorded on a Bruker 500 instrument. For ^{1}H NMR spectroscopy at 70 °C, the sample (10 mg) was repeatedly dissolved in D_2O (5 × 5 mL), and the solution was lyophilized. The final freeze-dried sample was dissolved in 1 mL of 99.99% D_2O. For ^{13}C NMR spectroscopy at 50 °C, the sample was dissolved in D_2O. The molecular weight of **1** was determined by a GPC technique.[25] GLC was conducted on a Hewlett-Packard model 419 and a Hewlett-Packard model 5713 gas chromatograph each equipped with a flame-ionization detector. Resolutions were performed on glass columns (1.83 m × 6 mm) containing (a) 1.3% of ECNSS-M on Gas Chrom Q (100−200 mesh) and (b) 2.3% of OV-225 on GC-Q (100−200 mesh) at 210 °C for acetate sugars and 1% of OV-225 on Gas Chrom Q (80−100 mesh) at 170 °C for acetates of partially methylated sugars. Uronic acids were determined colorimetrically using a method described in the literature.[26] A styrene macroreticular resin (D303) was purchased from Shanghai Huazheng Science & Technology, Co, Ltd., Ecust, People's Republic of China.

Plant Material. Dried seeds obtained from the whole plant (*R. raetam* ssp. *gussonei*) were provided by the Biotechnology Research Center (Tripoli, Libya). The identity of the plant was confirmed by Dr. Hongxiang Sun (College of Animal Science, Zhejiang University, People's Republic of China), and a sample was deposited in the

Table 2. GLC-MS Results of Partially *O*-Methylalditol Acetates Formed on Methylation Analysis of Carboxy-Reduced **1** Derived from the Seeds of *R. raetam* ssp. *gussonei*

alditol acetate	approximate molar ratio[a]	fragments (diagnostic ions, *m*/*z*)	proposed structure
2,3,4-tri-*O*-methylxylitol	1.00	88, 101, 102, 118, 129, 161	Xyl-(1→
3-*O*-methylxylitol	2.17	87, 88, 129, 130, 189, 190	→4)-Xyl-(1→ 2 ↑
2,3-di-*O*-methylxylitol	5.11	87, 102, 118, 129, 189	→4)-Xyl-(1→
2,3,4,6-tetra-*O*-methylglucitol	2.09	71, 87, 101, 117, 129, 145, 161, 205	Glc-(1→
3,4,6-tri-*O*-methylglucitol	1.06	87, 88, 101, 129, 130, 145, 161, 190	→2)-Glc-(1→

[a] Relative to 2,3,4-tri-*O*-methylxylitol.

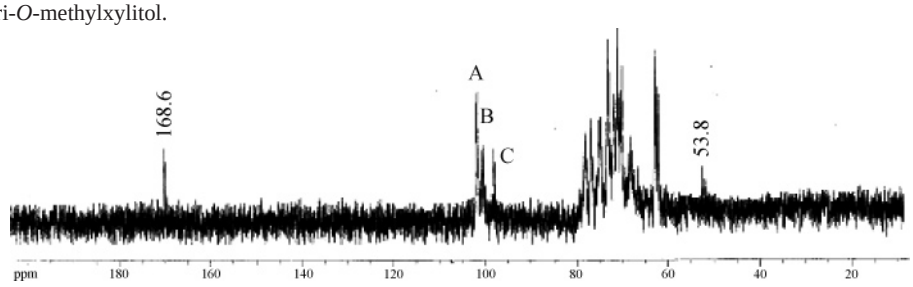

Figure 1. ^{13}C NMR spectrum of **1** from the seeds of *R. raetam* ssp. *gussonei* in D_2O at 50 °C; numerical values are in δ (ppm); A, residue A; B, residue B; C, residue C.

Table 3. ^{13}C NMR Chemical Shifts of **1** Isolated from the Seeds of *R. raetam* ssp. *gussonei*

	chemical shifts (ppm)[a]							
sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	carboxyl group	methyl group
1[b]								
(1→4)-β-D-Xyl*p* (residue A)	101.3	71.8, 78.6[c]	73.6	75.6, 68.9[d]	65.3			
(1→2)-α-D-Glc*p* (residue B)	99.3	77.3, 71.7[e]	74.1	69.8	72.0	61.6		
2-*O*-(α-D-4-*O*-methyl-Glc*p*A) (residue C)	98.2	72.2	73.2	79.7	70.6		168.6	53.8

[a] In ppm downfield relative to the signal for Me_4Si. [b] **1** obtained by freeze−thawing, Fehling precipitation, dissociation, and precipitation with ethanol. [c] *O*-Substituted C-2 of xylopyranosyl residues. [d] *O*-Unsubstituted C-4 of xylopyranosyl unit from the corresponding monosaccharide. [e] *O*-Unsubstituted C-2 of glucopyranosyl residues.

Herbarium of the Department of Chemistry (Zhejiang University, People's Republic of China), as voucher no. DFC 04613.

Extraction and Isolation. The dried seeds (800 g) from *R. raetam* ssp. *gussonei* were fed through a mill, which was not fitted with a screen. After several repetitions of this procedure, practically all the seeds were broken into several pieces. The split seeds (300 g) were extracted successively with refluxing EtOH (95%, 2 h, twice), $CHCl_3$−MeOH (3:1 v/v, 2 h, twice), and acetone (1 h, once). The extractive-free material (65 g) was delignified by treatment with aqueous sodium chlorite (0.85%) in 0.1 M sodium acetate−acetic acid buffer (pH 4.2) for 8 h at 70 °C, and the residue was recovered by filtration and freeze-dried. The above whole process was repeated once. The resulting material (58.8 g) was successively extracted with hot water for 5 h at 100 °C, then with 3% sodium hydroxide (fraction I), 6% sodium hydroxide (fraction II), and 15% sodium hydroxide (fraction III), in each case for 8 h at 80 °C under refluxing conditions. The alkaline solutions were made neutral with acetic acid, and each fraction was isolated by precipitation with ethanol and centrifugation. These alkali-soluble fractions were submitted to a column of a weakly basic anion-exchange styrene macroreticular resin (D303), which was used as decolorizer. The decolorized fraction II (1.23 g) was used in the subsequent studies. This fraction was purified through a freeze−thawing procedure,[27] and the supernatant was then submitted to a precipitation process, using Fehling solution, dissociation of the complex with acid, and isolation by repeated precipitation with ethanol.

Purity Determination of 1. *(a) Gel Filtration.* A solution of **1** (5.3 mg) in 0.15 M sodium chloride (1 mL) buffer was applied to a column (1.6 × 60 cm) of Sephadex G-100 and eluted with the same buffer. Fractions (1 mL) were analyzed by the phenol−sulfuric acid method.[28] (b) *Gel Permeation Chromatography (GPC).* The GPC system was incorporated in a Waters 515 instrument (Milford, MA). Two columns in series (Ultrahydrogel 250 and Ultrahydrogel 2000, Waters) were used. The eluent was distilled water, and the column temperature was maintained at 35 °C. The eluent was monitored with a Waters 2410 refractive index detector. The sample concentration was 4.5 mg/mL. The standards used to calibrate the column were purified dextran fractions having definite molecular masses ranging from 2000 Da to 119 kDa. All data provided by the GPC system were collected and analyzed using the Waters Millenium 32 software package.

Total Acid Hydrolysis and Monosaccharide Composition Analysis. Compound **1** (5.5 mg) and inositol (2.5 mg) were taken in a round-bottom flask, and 1.5 M TFA was added, followed by boiling in a water bath for 8 h. After hydrolysis was complete, excess acid was removed by co-distillation with distilled water. These hydrolyzates were concentrated and freeze-dried, followed by successive reduction with $NaBH_4$ and acetylation with Ac_2O−pyridine (1:1, v/v, 3 mL) at 85 °C for 6 h. The resulting alditol acetates obtained were analyzed by GLC as indicated above and identified by their typical retention times and electron impact profiles.

Paper Chromatographic Studies. Paper partition chromatography was performed on Whatman No. 1 for qualitative separations and 3 mm papers for large amounts. The solvents used were as follows: *A*, 9:3:2 EtOAc−pyridine−water; *B*, 10:3:3 EtOAc−acetic acid−water. Sugars were detected by alkaline silver nitrate and aniline oxalate.

Methylation Linkage Analysis of 1. Compound **1**, dissolved in dimethyl sulfoxide, was then methylated with methyl iodide and sodium hydroxide as a catalyst according to a modified Hakomori procedure,[16] with the whole process repeated twice. The fully methylated product showed no OH absorption bands in the region 3600−3300 cm^{-1} of the IR spectrum. The methylated **1** was first hydrolyzed with 90% formic acid for 6 h at 100 °C and then with 2 M TFA for 3 h at the same temperature. The hydrolyzate was then reduced with $NaBH_4$, and the alditol acetate was prepared as usual. The alditol acetate of the methylated sugar was analyzed by GLC using columns a and b and by GLC-MS using a fused silica capillary column (30 × 25 mm) coated with a 0.2 μm film of OV-1.

Graded Acid Hydrolysis of 1. Under refluxing conditions, a solution of **1** (80 mg) in 50% aqueous formic acid (40 mL) was heated for 3 h at 100 °C. The formic acid was removed under reduced pressure by

co-distillation with distilled water. Paper chromatography (Whatman paper, solvent *A* and *B*) of the hydrolysate was used to isolate the hydrolysate.

Preparation of Carboxyl-Reduced 1. Compound **1** (50 mg) was solubilized in 20 mL of 0.01 M HCl (pH 4.75) by stirring for 2 h. Then, 250 mg of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (250 mg) was added to a stirred dispersion of **1**, and the pH value of the solution was maintained at 4.75 by dropwise addition of 0.01 M HCl. After 75 min, the solution was stirred and 550 mg of $NaBH_4$ was added over 30 min, with the pH being kept at pH 7.0 by concurrent addition of 4 M HCl. After stirring for 2 h, the solution was dialyzed against distilled water for 36 h and then lyophilized. The whole process was repeated once.

Enzymatic Degradation. Xylanase, purchased from Megazyme (Bray, Ireland), was prepared from *Trichoderma viride*. A solution (5 mL) containing 6 units of xylanase of **1** (10.1 mg) in 0.05 M sodium acetate buffer (pH 4.8), after adding a few drops of toluene, was stored at 37 °C for 16, 24, and 50 h. Then, the mixture was stirred and heated at 100 °C for 10 min to inactivate the enzyme, cooled, passed through a column of Dowex 50W-X8 cation-exchange resin (Bio-Rad) to remove Na^+, and then centrifuged. The supernatant solution was concentrated, and the digest (4 mL) was applied to a column (1.6 × 60 cm) of Sephadex G-25 and eluted with distilled water. Fractions (3 mL) were collected, and the sugar content was determined by the phenol−sulfuric acid method.[28]

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Supporting Information Available: Elution profile of compound **1** with GPC (Figure S1) as well as data of preliminary analysis of alkaline extracts and **1** (Table S1) are available free of charge via the Internet at http://pubs.acs.org.

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