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Carbohydrate Research 333 (2001) 87–93

CARBOHYDRATE RESEARCH

Note

Structural analysis of a new glycosphingolipid from the lipopolysaccharide-lacking bacterium *Sphingomonas adhaesiva*

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Received 15 January 2001; accepted 19 April 2001

Abstract

A new glycosphingolipid, GSL-4B, was isolated from *Sphingomonas adhaesiva* and found to share the ceramide moiety with GSL-1 and GSL-3 from *Sphingomonas capsulata* studied earlier [Kawahara, K.; Moll, H.; Knirel, Y. A.; Seydel, U.; Zähringer, U. *Eur. J. Biochem.* **2000**, *267*, 1837–1846]. It is heterogeneous with respect to the long-chain bases *erythro*-2-amino-1,3-octadecanediol (sphinganine), (13*Z*)-*erythro*-2-amino-13-eicosene-1,3-diol, and (13*Z*)-*erythro*-2-amino-13,14-methylene-1,3-eicosanediol which in GSL-4B are present in the ratios of 1.1:1.0:1.1, and all bearing amide-linked (*S*)-2-hydroxymyristic acid. Methylation analysis and MALDI-TOF–MS along with ^{1}H and ^{13}C NMR spectroscopy showed that the carbohydrate part of GSL-4B has the structure of

α-D-Glc*p*-(1→4)-α-D-Gal*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*A-(1→1)-Cer

Keywords: *Sphingomonas adhaesiva*; Glycosphingolipid; Ceramide

While in most Gram-negative bacteria the major glycolipid in the outer membrane is the lipopolysaccharide (LPS), in bacteria belonging to family *Sphingomonadaceae*[1] this is replaced with glycosphingolipids (GSL).[2–4] Being amphiphilic molecules, GSL was found to possess functional and physicochemical similarities[5] as well as, in the case of GSL-4A, biological properties[6] with LPS, and is a useful chemotaxonomic marker.[1] Previously, we have determined structures of GSL from *Sphingomonas paucimobilis*[3] and *S. capsulata*,[4] which are characterised by heterogeneity in the ceramide moiety due to the presence of saturated, unsaturated, and cyclopropane-containing long-chain bases. In the present paper, we report on the structure of GSL from another species of the genus *Sphingomonas*, *Sphingomonas adhaesiva*.

A glycosphingolipid with a tetrasaccharide carbohydrate chain (GSL-4B) was isolated

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0008-6215/01/$ - see front matter © 2001 Published by Elsevier Science Ltd.
PII: S0008-6215(01)00111-2

from *S. adhaesiva* by silica gel-column chromatography. Chemical and GLC–MS studies, performed as described previously,[4] demonstrated that the ceramide moiety of GSL-4B has essentially the same composition and structure as that of GSL-1 and GSL-3 from *S. capsulata*[4] (Fig. 1). It includes the major long-chain bases *erythro*-2-amino-1,3-octadecanediol (sphinganine), (13*Z*)-*erythro*-2-amino-13-eicosene-1,3-diol (eicosasphing-13-enine), and (13*Z*)-*erythro*-2-amino-13,14-methylene-1,3-eicosanediol (13,14-methyleneeicosasphinganine) in the ratios 1.1:1.0:1.1, which are quantitatively substituted by amide-linked (*S*)-2-hydroxymyristic acid.

Sugar analysis of GSL-4B revealed D-Glc and D-Gal in the ratio 2:1, as well as D-GlcA. Methylation analysis of GSL-4B resulted in identification of terminal and 6-substituted glucose and 4-substituted galactose. Positive-ion mode MALDI-TOF mass spectrum of GSL-4B (Fig. 2) showed three major peaks for pseudomolecular ions $[M+Na]^+$ and $[M+K]^+$ derived from compounds with the molecular masses m/z 1189 [M_1], 1215 [M_2], and 1229 [M_3] Da. A similar result was obtained from the methyl esters of GSL-4B (GSL-4B$_{Me}$) prepared for NMR analysis, which again showed three major pseudomolecular peaks $[M+Na]^+$ derived from the molecular masses m/z 1203 [M_{1Me}], 1229 [M_{2Me}] and 1243 [M_{3Me}] Da all being shifted by 14 Da higher as compared to GSL-4B (data not shown). This pattern reflected the heterogeneity in the ceramide moiety (see above) and indicated the presence of a tetrasaccharide chain consisting of three hexose residues and one hexuronic acid in GSL-4B and one hexuronic acid methyl ester in GSL-4B$_{Me}$, respectively.

The ^1H and ^{13}C NMR spectra of GSL-4B$_{Me}$ (Figs. 3 and 4) contained signals for the sphingolipid moiety, which were in close agreement with the data for GSL-1 and GSL-3.[4] In particular, the spectra confirmed the presence of a cyclopropane ring (δ_H −0.42, 0.48, and 0.57; δ_C 10.6 and 15.5 for the methylene and me-

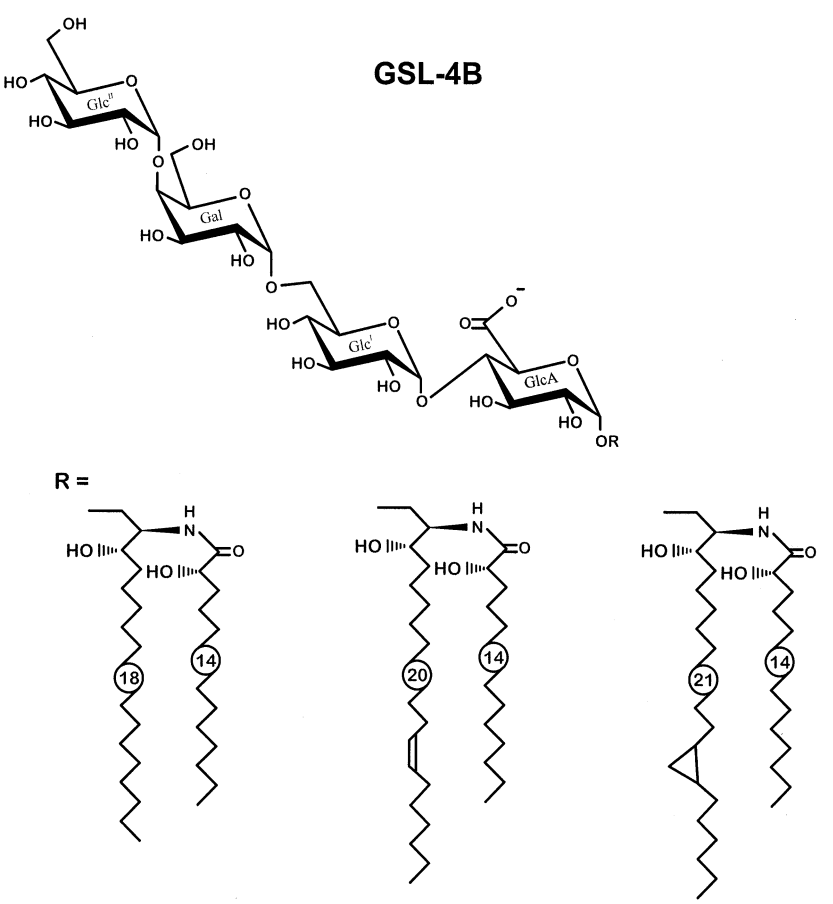

Fig. 1. Chemical structure of GSL-4B from *S. adhaesiva*.

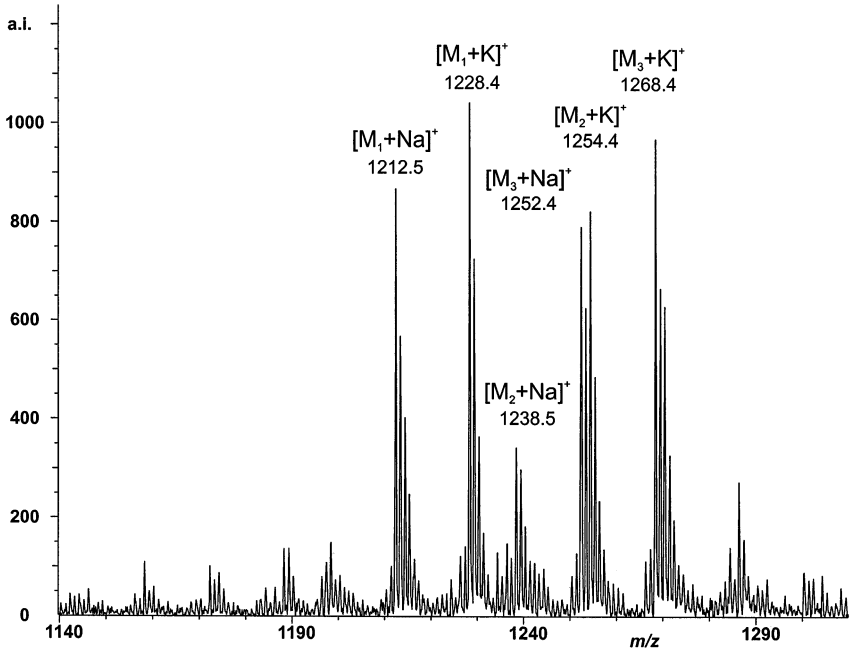

Fig. 2. Positive ion MALDI-REF-TOF mass spectrum of GSL-4B from *S. adhaesiva*. a.i., absolute intensity. M_1, M_2, and M_3 represent the intrinsic heterogeneity in the sphinganine part of GSL-4B. For details see text.

thine atoms, respectively) and a double bond (δ_H 5.26 and 1.93; δ_C 129.6 and 26.9 for the olefinic and allylic atoms, respectively). There were present also signals for four anomeric atoms of the carbohydrate moiety ($\delta_{H\text{-}1}$ 4.79, 4.85, 4.87, and 5.11; $\delta_{C\text{-}1}$ 98.7, 99.4, 100.3, and 101.0).

The ^1H and ^{13}C NMR spectra were assigned using 2D COSY, TOCSY, and H-detected $^1H,^{13}C$ HMQC experiments (Tables 1 and 2). The assignment was straightforward due to sufficient polarisation transfer in the TOCSY experiment within the whole spin system in Glc and $GlcA_{Me}$ and within the systems H-1,2,3,4; H-4,5; and H-5,6 in Gal. Characteristic $^3J_{H,H}$ coupling constant values showed that the four monosaccharides are in the pyranose form, and the $J_{1,2}$ values of < 4 Hz (Glc^{II}, 3.7 Hz; Gal, 3.6 Hz; Glc^{I}, 3.7 Hz; GlcA, 3.7 Hz) demonstrated that they all are α-linked.

Downfield displacements of the ^{13}C NMR signals for C-4 of Gal and $GlcA_{Me}$ and C-6 of one of the glucose residues (Glc^{I}) to δ 80.6, 80.0, 65.9, respectively, as compared with their positions in the spectra of the corresponding nonsubstituted monosaccharides,[7] revealed the substitution pattern in the trisaccharide chain. The chemical shifts for C-2,3,4,5,6 of the second glucose residue (Glc^{II}) were similar to those in nonsubstituted α-glucopyranose and, hence, this sugar occupies the terminal position. These data were in consistence with methylation analysis data (see above).

The ROESY spectrum revealed the following interresidue correlations between the anomeric protons and the protons at the linkage carbons: Glc^{II} H-1, Gal H-4; Gal H-1, Glc^{I} H-6a; Glc^{I} H-1, GlcA H-4; and $GlcA_{Me}$ H-1, sphingoid H-1a at δ 4.87/3.98; 4.85/3.52; 5.11/3.66; and 4.79/3.69, respectively. These data confirmed the modes of glycosylation of the monosaccharide and defined their sequence and the attachment of the carbohydrate moiety to the sphingoid at position 1.

Therefore, the carbohydrate chain of GSL-4B has the structure of a α-D-Glc*p*^{II}-(1→4)-α-D-Gal*p*-(1→6)-α-D-Glc*p*^{I}-(1→4)-α-D-Glc*p*A tetrasaccharide, and the structure of the whole native glycosphingolipid is established as shown in Fig. 1. As mentioned above, the ceramide moiety in GSL-4B from *S. adhaesiva* is identical to that in GSL-1 and GSL-3 from *S. capsulata*[4] whereas GSL-1 and GSL-4A from *S. paucimobilis*[3] have a similar sphin-

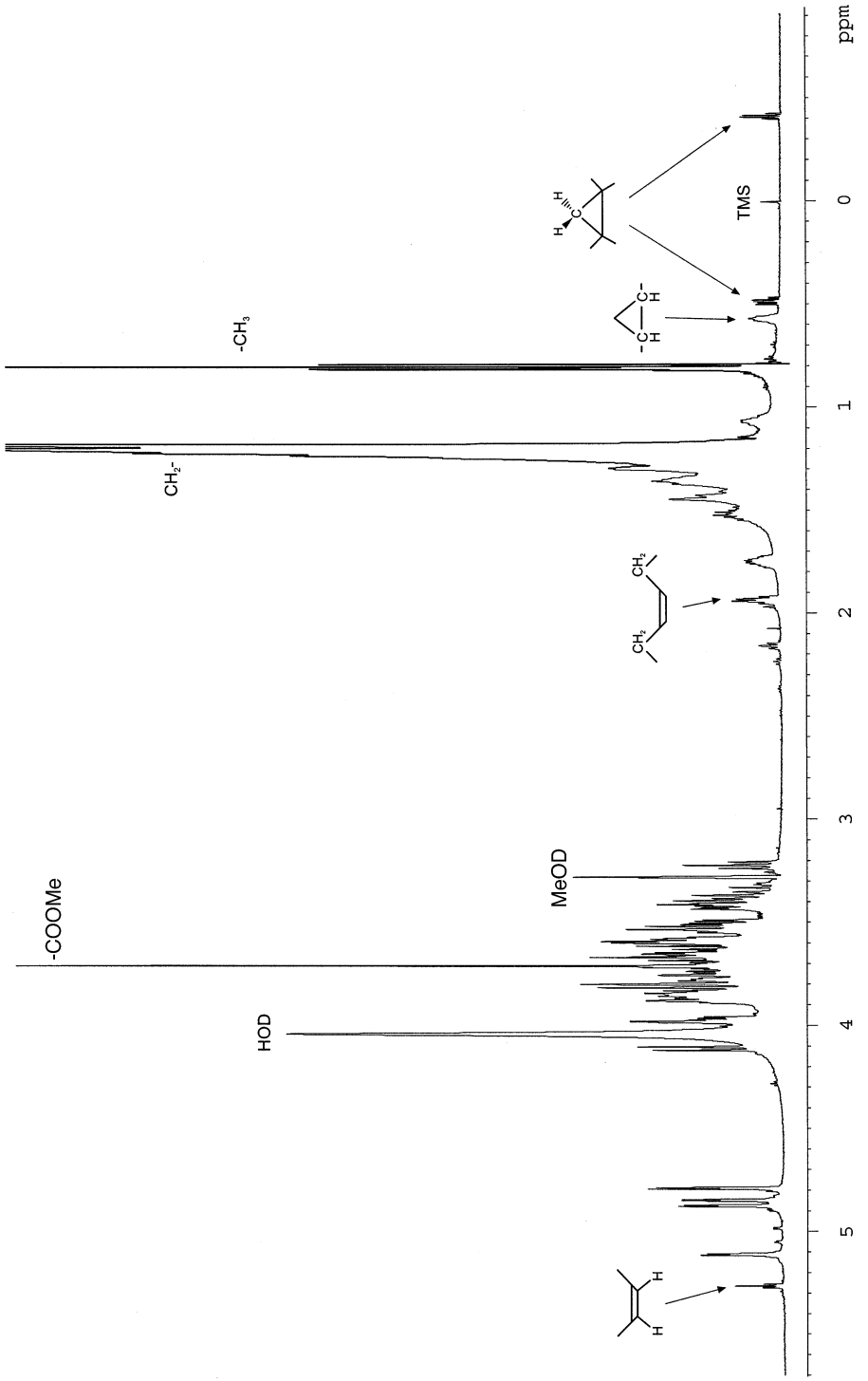

Fig. 3. 600-MHz ^{1}H NMR spectrum of GSL-4B$_{Me}$ from *S. adhaesiva*.

golipid, differing only in a significantly lower content of the unsaturated sphingobase. Another common feature of the *Sphingomonas* glycosphingolipids, with one exception reported recently,[8] is the attachment to ceramide of the same monosaccharide, α-D-glucuronic acid, whereas, when present, the further sugar chains are different (Fig. 1).

1. Experimental

Growth of bacteria.—*S. adhaesiva* GIFU 11458 was cultivated in the liquid medium described previously,[4] using a 30-L jar fermenter at 30 °C for 24 h. The bacteria were killed by heating (100 °C, 30 min), washed with distilled water, and lyophilised.

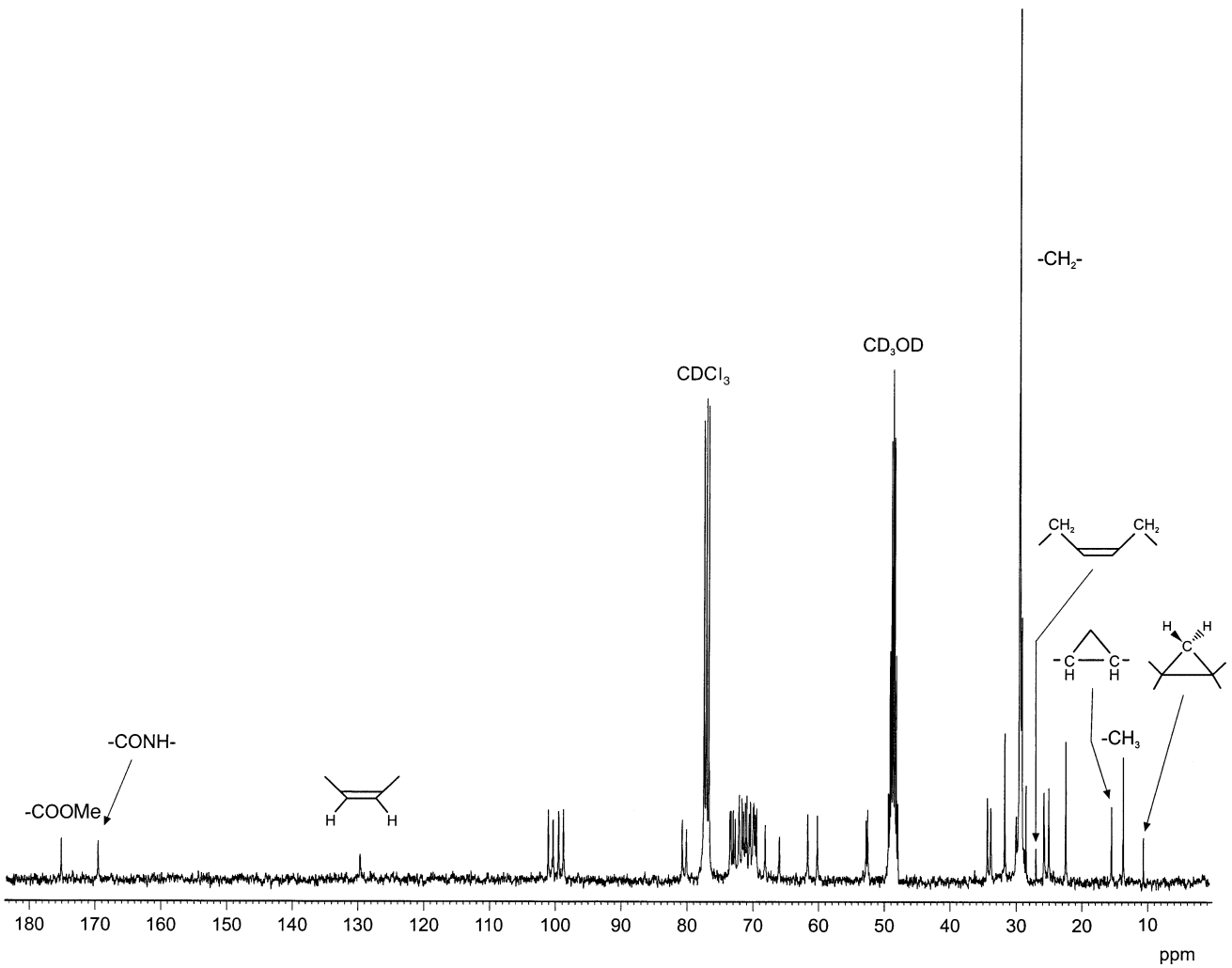

Fig. 4. 90-MHz ^{13}C NMR spectrum of GSL-4B$_{Me}$ from *S. adhaesiva*.

Table 1
^1H NMR chemical shifts for the carbohydrate moiety of GSL-4B$_{Me}$ (δ, ppm) [a]

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
α-D-Glc*p*^{II}-(1→	4.87	3.42	3.61	3.22	3.83	3.58	3.80
→4)-α-D-Gal*p*-(1→	4.85	3.65	3.76	3.98	3.87	3.63	3.73
→6)-α-D-Glc*p*^{I}-(1→	5.11	3.40	3.53	3.59	3.36	3.52	3.87
→4)-α-D-Glc*p*A$_{Me}$-(1→	4.79	3.49	3.81	3.66	4.11		

[a] Additional chemical shifts for ceramide protons at N- and O-linked carbons: δ 3.69, 3.78, 3.84, and 3.58 for H-1a,1b,2,3 of sphingoids, respectively, and δ 3.97 for H-2 of (*S*)-2-hydroxymyristic acid.

Table 2
^{13}C NMR chemical shifts for the carbohydrate moiety of GSL-4B$_{Me}$ (δ, ppm) [a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
α-D-Glc*p*^{II}-(1→	101.0	72.0	73.3	70.3	72.9	61.5
→4)-α-D-Gal*p*-(1→	98.7	69.4	69.8	80.6	69.9	60.1
→6)-α-D-Glc*p*^{I}-(1→	100.3	72.1	73.4	71.1[b]	69.6	65.9
→4)-α-D-Glc*p*A$_{Me}$-(1→	99.4	70.9	72.6	80.0	70.5	175.0

[a] Additional chemical shifts for N- and O-linked ceramide carbons: δ 68.0, 52.7, and 71.4[b] for C-1,2,3 of sphingoids, respectively, and δ 71.6 for C-2 of (*S*)-2-hydroxymyristic acid.
[b] Assignment could be interchanged.

Isolation of glycosphingolipid.—Dried cells were homogenised with 2:1 (v/v) $CHCl_3$–MeOH to remove phospholipids, and GSL was extracted twice from the residual cells with 1:3 (v/v) $CHCl_3$–MeOH at 80 °C for 1 h. The crude extract was applied to a column of Silica Gel 60 (E. Merck, Germany) for purification. Lipids were eluted using a stepwise gradient of 4:1 to 1:3 (v/v) $CHCl_3$–MeOH. The elution was monitored by TLC of Silica Gel 60 (E. Merck) with a solvent system of 25:15:4:2 (v/v/v/v) $CHCl_3$–MeOH–AcOH–water. The yield of pure GSL-4B obtained by this way from 5 g of dry cells (25 g wet cells) was 20 mg (0.4%, w/w).

Composition and methylation analysis.—Fatty acids and long-chain bases in ceramide were analysed as described previously.[4] Neutral sugars were determined as the alditol acetates by GLC on a CBP-10 column using a GC-14 A instrument (Shimadzu, Japan) after acid hydrolysis (0.1 M HCl, 100 °C, 48 h). Uronic acid was determined by the carbazole–H_2SO_4 method[9] after hydrolysis with 2 M H_2SO_4 (100 °C, 5 h). Methylation was performed by the method of Hakomori[10] with some modifications,[11] the product was hydrolysed with 1 M CF_3COOH at 120 °C for 2 h, and liberated sugars were conventionally reduced with $NaBH_4$, peracetylated (Ac_2O–pyridine), and analysed by GLC–MS on a Hewlett–Packard HP 5985 instrument equipped with a SE-54 column (Weeke, Germany).

NMR spectroscopy.—^{13}C NMR spectra were recorded on a Bruker AM-360 spectrometer at 90.5-MHz. ^1H NMR and 2D ^1H,^1H and H-detected ^1H,^{13}C HMQC experiments were run on a Bruker DRX-600 instrument. The purified GSL-4B (free acid form) was esterified with diazomethane in 1:1 (v/v) $CHCl_3$–MeOH at 20 °C for 5 min to give the corresponding methyl ester of GSL-4B (GSL-4B$_{Me}$). GSL-4B$_{Me}$ showed a significantly improved signal resolution as compared with the native compound. The sample was dissolved in 4:1 (v/v) $CDCl_3$–CD_3OD, and the spectra were measured at 27 °C. Chemical shifts are referenced to internal tetramethylsilane (δ_H 0 ppm) or internal $CHCl_3$ (δ_C 77.0 ppm). A mixing time of 100 and 200 ms was used in TOCSY and ROESY experiments, respectively.

MALDI-TOF–MS.—Positive-ion mode MALDI-REF-TOF–MS was performed with a Bruker-Reflex II mass spectrometer (Bruker–Franzen Analytik, Germany) in reflector configurations at an acceleration voltage of 20 kV. The sample was prepared in 0.5 M matrix solution of 2,5-dihydroxybenzoic acid (Aldrich) in MeOH. The instrument was mass calibrated externally with similar compounds of known chemical structure.

Acknowledgements

The authors are grateful to H. Moll, I. Mizuta, and Y. Suzuki for excellent technical assistance. Financial support from Kibun Foods Inc., and Kibun Food Chemifa Co. is also acknowledged.

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