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STRUCTURAL STUDIES OF 4-*O*-ACETYL-α-*N*-ACETYLNEURAMINYL-(2→3)-LACTOSE, THE MAIN OLIGOSACCHARIDE IN ECHIDNA MILK

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ABSTRACT

The main oligosaccharide (50%) in the milk of the Australian echidna (*Tachyglossus aculeatus*) has been identified unequivocally as 4-*O*-acetyl-α-*N*-acetylneuraminyl-(2→3)-lactose. The 4-*O*-acetyl substituent of the sialic acid residue was characterised by g.l.c.–m.s. of the isolated (after mild, acid hydrolysis) and trimethylsilylated/esterified sialic acid, and by m.s. (after derivatisation) and 500-MHz, ^{1}H-n.m.r. spectroscopy of the intact oligosaccharide. Information about the glycosidic bonds was obtained by methylation analysis and 500-MHz, ^{1}H-n.m.r. spectroscopy. This animal species is the third one known to produce 4-*O*-acetylated sialic acid.

INTRODUCTION

The carbohydrate composition of the milk of monotremes (egg-laying mammals) differs greatly from that of the milk of placental mammals[1,2]. For the Australian echidna (*Tachyglossus aculeatus*), the milk oligosaccharides are mainly composed of sialyl-lactose (50%) and fucosyl-lactose (26%), whereas difucosyl-lactose (13%) and lactose (8%) are present in smaller proportions.

The structure of the sialyl-lactose was investigated by chemical and enzymic methods, and suggested to be 4-*O*-acetyl-α-*N*-acetylneuraminyl-(2→3)-lactose[2]. Conclusive evidence obtained by g.l.c., m.s., and 500-MHz, ^{1}H-n.m.r. spectroscopy is now presented for the structure of this oligosaccharide.

RESULTS AND DISCUSSION

Isolation of sialyl-lactose from echidna milk. — The milk oligosaccharide was isolated by gel filtration on Sephadex G-15 after removal of milk fat and protein, as described earlier[1,2].

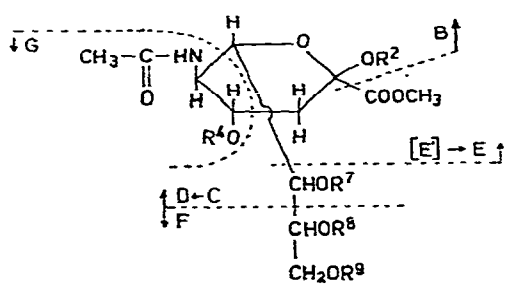
TABLE I

SPECIFIC FRAGMENT-IONS A-G IN THE MASS SPECTRA OF THE TRIMETHYLSILYLATED METHYL ESTER DERIVATIVES OF *N*-ACETYLNEURAMINIC ACID (Neu5Ac) AND 4-*O*-ACETYL-*N*-ACETYLNEURAMINIC ACID (Neu4,5Ac$_2$)

Neu5Ac[a] (R_{Neu5Ac} 1.00)	*Neu4,5Ac$_2$*[a] (R_{Neu5Ac} 1.18)	*Explanation*[b]
668	638	A: M − CH_3 (from Me_3Si-group)
624	594	B: M − $COOCH_3$
478	448	C: M − $CHOR^8CH_2OR^9$
298	298	D: M − $CHOR^8CH_2OR^9$ − R^2OH − R^4OH
317	—[c]	E: M − $CHOR^7CHOR^8CH_2OR^9$ − CH_3CONH_2
205	205	F: $CHOR^8CH_2OR^9$
173	143	G: $CH_3CONHCHCHOR^4$

[a]The g.l.c. retention times on 3.8% SE-30 at 215° are given relative to the trimethylsilylated derivative of the methyl ester of Neu5Ac (R_{Neu5Ac}). [b]For a discussion of the mass spectrometric method, see refs. 3–5. [c]If C-4 bears an *O*-acyl group, this fragment ion is not formed[3–5].

A: (M − Me)
(from Me_3Si group)



Structure determination of the echidna sialic acid. — After cleavage from the sialyl-lactose, the identity of the sialic acid was established by a g.l.c.–m.s. microtechnique, using a series of characteristic fragment-ions which indicate the number, type, and position of the *O*-acyl substituents and the type of the *N*-acyl group in *N*,*O*-acylneuraminic acids[3–5]. The sialyl-lactose was treated with dilute acid, and the resulting sialic acid fraction was isolated by ion-exchange chromatography and derivatised with diazomethane and hexamethyldisilazane–chlorotrimethylsilane. In g.l.c.–m.s., a main component (70%) having the same retention time (R_{Neu5Ac} 1.18) and the same mass spectrum as the trimethylsilylated methyl ester derivative of 4-*O*-acetyl-*N*-acetylneuraminic acid (Neu4,5Ac$_2$) (Fig. 1), and a minor one (30%) corresponding to the derivative of *N*-acetylneuraminic acid (Neu5Ac)[3] were detected. Table I summarises the characteristic fragment ions A–G. Neu5Ac may be considered mainly as a decomposition product of Neu4,5Ac$_2$ (*O*-deacetylation during hydrolysis and isolation), since the isolated oligosaccharide was almost homogeneous (see Fig. 3).

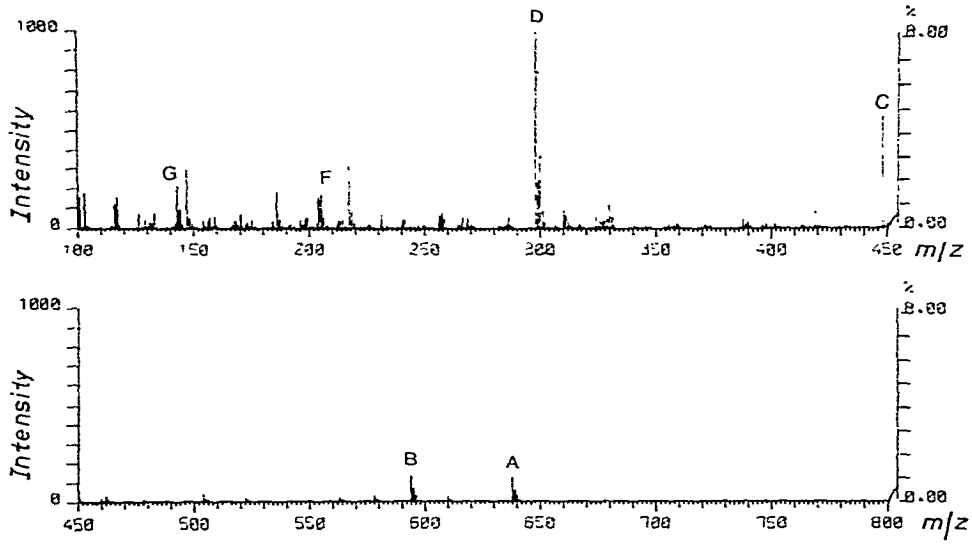


Fig. 1. Mass spectrum of the trimethylsilylated methyl ester of 4-*O*-acetyl-*N*-acetylneuraminic acid; only values $m/z > 100$ are given. For an explanation of the fragment ions A–G, see Table I.

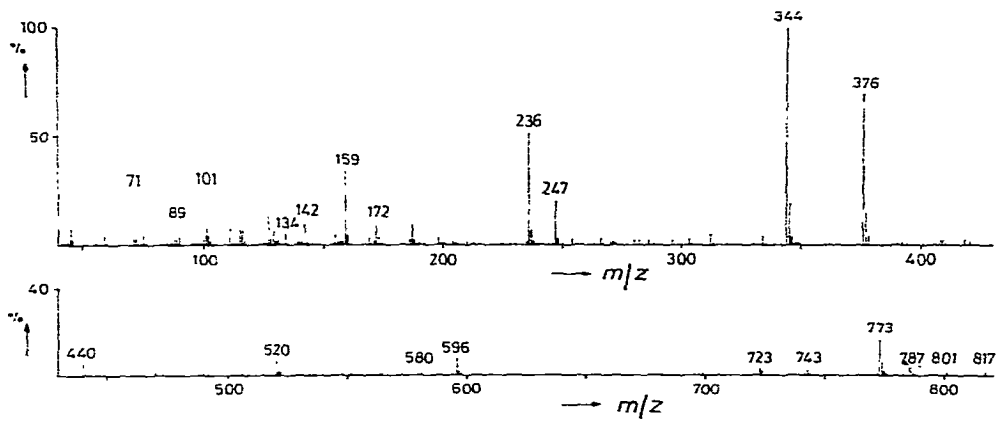


Fig. 2. Mass spectrum of permethylated α-*N*-acetylneuraminyl-(2→3)-lactitol-*1-d*; only values $m/z > 40$ are given. For an explanation of characteristic peaks, see text.

Methylation analysis of the echidna sialyl-lactitol. — For the methylation studies, the sialyl-lactose was converted into sialyl-lactitol-*1-d* with sodium borodeuteride. During the reduction and/or the methylation, the native *O*-acetyl group is split off. The mass spectrum of the permethylated sialyl-lactitol-*1-d* is shown in Fig. 2. The peaks in the high-mass range at m/z 817 (M — CH_3), 801 (M — OCH_3), 787 (M — CH_2OCH_3), 773 (M — $COOCH_3$), 743 (M — $CHOCH_3CH_2OCH_3$), and 723 (M — CH_2OCH_3 — CH_3OH — CH_3OH) are in accordance with a completely methylated Neu5Ac-lactitol-*1-d*. The sequence of the monomeric units (Neu5Ac→

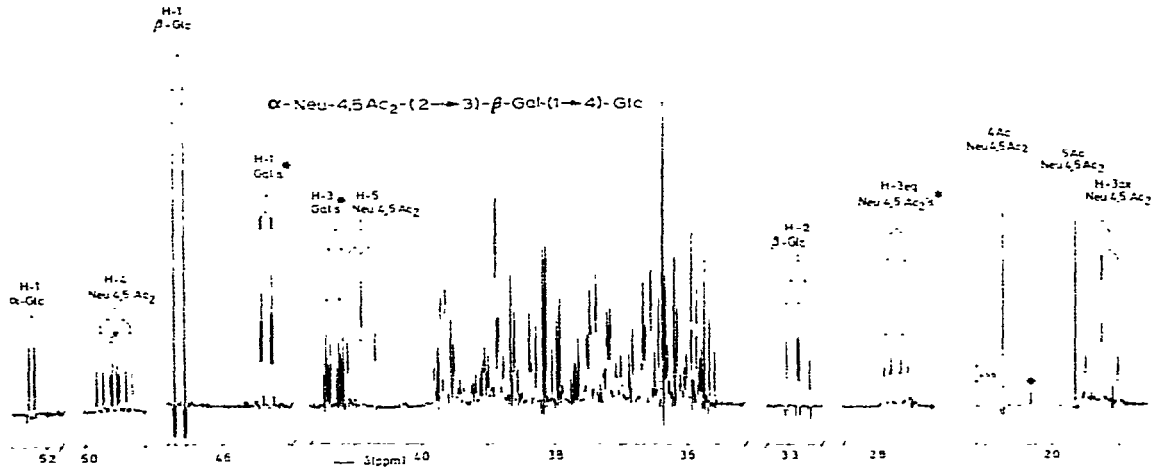


Fig. 3. 500-MHz, ^{1}H-n.m.r. spectrum of 4-*O*-acetyl-α-*N*-acetylneuraminyl-(2→3)-lactose, recorded in D_2O at 300 K and pD 7; for further details, see Table II; *signals resolved due to the presence of α and β anomers of the oligosaccharide (see text); •methyl resonance due to the presence of 2% of α-*N*-acetylneuraminyl-(2→3)-lactose.

Gal→Glc-ol-*1-d*) can be deduced directly from the mass spectrum: *m/z* 376-344-312 (aA_1-aA_2-aA_3), 580–548 (baA_1-baA_2), 596-564, 440-408 (bcA_1-bcA_2), 236 (cA_1) [for the explanation of the symbols, see refs. 6–8]. It has been demonstrated earlier[8,9] that a relatively intense peak at *m/z* 159 is indicative of a (1→3) linkage between the hexopyranosyl units in Hex*p*→Hex*p*→Hex-ol. Hence, the intense peak at *m/z* 159 in the mass spectrum of the permethylated Neu5Ac-lactitol-*1-d* is characteristic of a (2→3) linkage between Neu5Ac and Gal*p*. This conclusion is supported by the absence of the bcJ_1 fragment (*m/z* 296 is ~2%) and the H_1^2 or H_1^3 fragment in the Gal*p* residue ($CHOCH_3$ — $CHOCH_3$; *m/z* 88 ~3%). The presence of the alditol fragments at *m/z* 90 and 134, in combination with the absence of a peak at *m/z* 178, are in agreement[8,10] with a (1→4) linkage between Gal*p* and Glc-ol-*1-d*. The occurrence of both linkages was further proved by g.l.c.–m.s. of the partially methylated alditol acetates[11,12] 4-*O*-acetyl-1,2,3,5,6-penta-*O*-methylglucitol-*1-d* and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol-*1-d*. These data also prove the pyranoid ring form for Gal.

To verify the data, the reference compounds α-Neu5Ac-(2→3)-lactitol-*1-d* and α-Neu5Ac-(2→6)-lactitol-*1-d* were permethylated and analysed by m.s. The mass spectrum of the reference α-(2→3) isomer was identical to that depicted in Fig. 2.

Mass spectrometry of the pertrimethylsilylated methyl ester. — The mass spectrum of the pertrimethylsilylated methyl ester of the sialyl-lactose showed, in the high-mass range, peaks at *m/z* 1394 (M — CH_3), 1350 (M — $COOCH_3$), and 1204 (M — $CHOSiMe_3CH_2OSiMe_3$). Comparison of these *m/z* values with those[13] for the pertrimethylsilylated methyl esters of α-Neu5Ac-(2→3)-lactose and α-Neu5Ac-

(2→6)-lactose show a negative shift of 30 m.u., indicating the presence of one *O*-acetyl group in the echidna oligosaccharide. The location of the *O*-acetyl group in the Neu5Ac unit can be deduced from the sequence fragment-ions *m/z* 564–504 (aA_1-aA_2), 942 (baA_1), and 451-361-271 (cA_1-cA_2-cA_3). Compared with the reference Neu5Ac-lactoses, the fragment ions aA_1, aA_2, and baA_1 were shifted by 30 m.u. The latter information, in combination with the occurrence of the above-mentioned peak at *m/z* 1204, demonstrates the *O*-acetyl group to be located at position C-4 of the Neu5Ac residue. As discussed previously for *N*,*O*-acylneuraminic acid derivatives[3–5], the presence of the fragment M − $CHOSiMe_3CH_2OSiMe_3$ excludes the presence of an *O*-acyl group at C-7 of Neu5Ac. The shift of *m/z* 173 ($CH_3CONHCH$-$CHOSiMe_3$) to *m/z* 143 accords with the assignment of the *O*-acetyl group to position 4. The mass spectrum does not contain pertinent data that can be used for the determination of the type of glycosidic linkages.

500-MHz, ^{1}H-n.m.r. spectroscopy. — The ^{1}H-n.m.r. spectrum of the sialyl-lactose, recorded in D_2O at 300 K and pD 7, is depicted in Fig. 3. The chemical shifts and coupling constants of the structural reporter groups[14] are listed in Table II. For comparison, the data of the corresponding protons of the reference compounds α-Neu5Ac-(2→3)-lactose, α-Neu5Ac-(2→6)-lactose, and lactose are included[15,16]. The ^{1}H-n.m.r. spectrum is a superposition of the subspectra of the α and β anomers of the sialyl-lactose. The areas of the H-1 signals of the reducing glucopyranose unit lead to an α/β ratio of 7:10. The Gal H-1 resonance is observed as two well-resolved doublets corresponding to the α and β anomers. The same effects are detected for the Gal H-3 and sialic acid H-3*eq* resonances. These small differences in chemical shift of the last three resonances reflect differences in total conformation of the α and the β anomer of the sialyl-lactose.

The relatively downfield position of the Gal H-3 resonance (δ 4.13), compared to that of the corresponding proton in β-Gal*p*-(1→4)-Glc*p* (δ 3.62), accords with a 3-substituted Gal. This conclusion is supported by the chemical shift (δ 4.11) of the Gal H-3 resonance in the ^{1}H-n.m.r. spectrum of α-Neu5Ac-(2→3)-lactose (see Table II). In both sialyl-lactoses, the Gal H-1 resonances show similar chemical shifts. The (1→4)-linkage between Gal and Glc cannot be deduced from the ^{1}H-n.m.r. spectrum.

The presence of two acetyl groups in the oligosaccharide is indicated by the occurrence of two methyl resonances at δ 1.965 and 2.072. The location of an *O*-acetyl group at position 4 of the sialyl residue can be deduced from the chemical shifts of H-3*ax*, H-4, and H-5. When compared with the chemical shifts of the corresponding protons in the spectrum of α-Neu5Ac-(2→3)-lactose (Table II), the H-4 signal has been shifted downfield by 1.27 p.p.m. (α-effect), the H-5 signal by 0.26 p.p.m. (β-effect), and the H-3*ax* signal by 0.13 p.p.m. (β-effect). The Ac-5 methyl resonance is also influenced (Δδ 0.07). The assignments of the Ac-4 and Ac-5 singlets accord with the data observed for β-Neu4,5Ac_2 (Ac-4, δ 2.065; Ac-5, δ 1.992). The Neu4,5Ac_2 H-4 and H-5 resonances for the sialyl-lactose were assigned on the basis of selective

TABLE II

^{1}H-CHEMICAL SHIFTS AND COUPLING CONSTANTS OF SOME RELEVANT PROTONS IN THE N.M.R. SPECTRA[a] OF α-Neu4,5Ac$_2$-(2→3)-LACTOSE (ECHIDNA SIALYLLACTOSE) (500 MHz), α-Neu5Ac-(2→3)-LACTOSE (360 MHz)[15], α-Neu5Ac-(2→6)-LACTOSE (360 MHz)[15], AND LACTOSE (360 MHz)[16].

	Glc anomer	*α-Neu4,5Ac$_2$-(2→3)-β-Gal-(1→4)-Glc*	*α-Neu5Ac-(2→3)-β-Gal-(1→4)-Glc*	*α-Neu5Ac-(2→6)-β-Gal-(1→4)-Glc*	*β-Gal-(1→4)-Glc*
Chemical shifts (δ)					
Glc					
H-1	α	5.220	5.220	5.225	5.224
H-1	β	4.662	4.661	4.667	4.665
H-2	β	3.284	3.281	3.308	3.286
Gal					
H-1	α	4.533	4.530	4.429	4.443
H-1	β	4.531	4.528	4.429	4.443
H-3	α	4.132	4.114	3.5–4.0[b]	3.62
H-3	β	4.128	4.110		
Neu5Ac					
H-3*ax*	α,β	1.925	1.799	1.739	
H-3*eq*	α	2.774	2.757	2.715	
H-3*eq*	β	2.772	2.757	2.715	
H-4	α,β	4.957	3.688	3.658	
H-5	α,β	4.088	3.825	3.836	
Ac-5	α,β	1.965	2.030	2.030	
Ac-4	α,β	2.072			

Coupling constants (J)					
Glc					
$J_{1,2}$	α	3.8	3.8	3.8	3.8
$J_{1,3}$	β	7.9	7.9	8.1	8.0
$J_{2,3}$	β	9.2	8.5	8.4	8.5
Gal					
$J_{1,2}$	α	7.9	7.8	7.8	7.8
$J_{1,2}$	β	7.8	7.8	7.8	7.8
$J_{2,3}$	α,β	3.2	3.3	N.d.[c]	N.d.
$J_{3,4}$	α,β	9.9	9.9	N.d.	N.d.
Neu5Ac					
$J_{3ax,4}$	α,β	11.9	12.1	12.0	
$J_{3eq,4}$	α,β	4.9	4.9	4.8	
$J_{3ax,3eq}$	α,β	−12.4	−12.4	−12.4	
$J_{4,5}$	α,β	10.1	N.d.	N.d.	
$J_{5,6}$	α,β	10.7	N.d.	N.d.	

[a]The spectra were recorded in D_2O at a probe temperature of 300 K and a pD of 7. Internal standard: acetone (δ 2.225 relative to DSS). [b]Signal is hidden in the bulk of the proton signals for the sugar skeleton. [c]Not determined.

decoupling experiments and on the various coupling constants which give the multiplets their characteristic shape.

The anomeric configuration of Neu5Ac can usually be inferred from the chemical shifts of the Neu5Ac H-3*eq* and H-4 resonances. Analyses of a series of model substances in our laboratory have shown that, for α anomers, H-3*eq* varies between 2.6 and 2.8 p.p.m., and H-4 between 3.6 and 3.8 p.p.m. For β anomers, these ranges are 2.1 to 2.5 p.p.m. and 3.9 to 4.2 p.p.m., respectively. For the echidna Neu4,5Ac_2-(2→3)-lactose, only the chemical shift of the H-3*eq* resonance (δ 2.77) can be used to assign the α configuration. Owing to the *O*-acetyl group at C-4 of Neu4,5Ac_2, the chemical shift of the H-4 resonance is no longer a good criterion. The β configuration of the (1→4)-linkage between Gal*p* and Glc*p* is demonstrated by the $J_{1,2}$ value of 7.8/7.9 Hz for the Gal H-1 resonance.

CONCLUSIONS

In this study, the identity of the echidna sialyl-lactose has been firmly established to be α-Neu4,5Ac_2-(2→3)-β-Gal*p*-(1→4)-Glc*p*, thereby excluding a (2→2)- and (2→4)-linkage between sialic acid and galactose, possibilities which were still open. These investigations have shown clearly the advantages of g.l.c.–m.s. and high-resolution, ^{1}H-n.m.r. spectroscopy for the direct determination of the number, type, and position of *O*-acyl substituents in complex carbohydrates. It may be noted that, besides horse and donkey, echidna is only the third animal species known to produce 4-*O*-acetylated sialic acid[3,20].

EXPERIMENTAL

Identification of the sialyl unit in sialyl-lactose. — Sialic acid was released from 0.5 mg of sialyl-lactose by hydrolysis with formic acid or 0.1M HCl, as reported previously[17]. For the analysis by g.l.c.–m.s., the product was esterified with diazomethane and trimethylsilylated[3-5].

Reduction and permethylation of sialyl-lactose. — The oligosaccharide (0.8 mg) was treated with 20 mg of sodium borodeuteride in 5 ml of water for 2 h at room temperature. After decomposition of the excess of borodeuteride with Dowex 50-X8 (H^+) resin, boric acid was removed by co-evaporation with methanol. The residue was dried over P_2O_5 in a vacuum desiccator, dissolved in dry methyl sulphoxide (1 ml), and methylated with 2M methylsulphinylmethanide in methyl sulphoxide (1 ml)/methyl iodide (1 ml) according to the method of Hakomori[12,18]. The permethylated oligosaccharide-alditol-*1-d*, obtained *via* chloroform extraction, was purified by gel filtration on Sephadex LH-20 [column, 35 × 2 cm; solvent system, chloroform–ethanol (1:1)]. To detect carbohydrates, 0.2% orcinol in 20% methanolic H_2SO_4 was used.

Methylation analysis of the permethylated sialyl-lactitol-1-d. — The permethylated sialyl-lactitol-*1-d* (0.5 mg) was hydrolysed with 90% formic acid (1 h, 100°),

followed by 0.13M H_2SO_4 (16 h, 100°). The neutral, partially methylated alditol acetates, prepared from the hydrolysis mixture as described by Jansson *et al.*[12], were analysed by g.l.c.–m.s.

Esterification and trimethylsilylation of sialyl-lactose. — To 0.3 mg of sialyl-lactose in dry methanol (1 ml) was added ethereal diazomethane until a faint-yellow colour was obtained. Then, the solution was immediately evaporated followed by trimethylsilylation of the residue[3–5].

Instrumental analysis. — G.l.c. was carried out on a Varian Aerograph 2740, equipped with dual flame-ionisation detectors and glass columns packed with 3.8% of SE-30 (2.00 m × 4.0 mm i.d.; for sialic acid derivatives)[4,5] or 3% of OV-225 (2.00 m × 2.0 mm i.d.; for partially methylated alditol acetates)[12] on Chromosorb WHP (100–120 mesh).

G.l.c.–m.s. was performed with a combined Hewlett-Packard 5710A gas chromatograph/Jeol JMS-D300 mass spectrometer/Jeol JMA-2000 mass-data analysis system. Mass spectra (70 eV) were recorded with an ion-source temperature of 200°, an accelerating voltage of 3 kV, and an ionising current of 300 μA. The column materials for g.l.c. were as described above.

Direct insertion m.s. was carried out on an AEI MS-902 apparatus with an ion-source temperature of 170–180°, an accelerating voltage of 4 or 8 kV, an electron energy of 50 eV, and an ionising current of 100 μA.

500-MHz, ^{1}H-n.m.r. spectroscopy was performed with a Bruker WM-500 spectrometer. Resolution enhancement of the spectrum was achieved by Lorentzian to Gaussian transformation, according to Ernst[14,19]. Before analysis, the sample (3 mg) was treated five times with D_2O, with intermediate lyophilisation, finally using 99.96% deuterated H_2O (Aldrich). The probe temperature was 300 K and the pD was adjusted to 7. The chemical shifts (δ) are expressed in p.p.m. downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS), but were actually measured by reference to internal acetone (δ 2.225) with an accuracy of 0.001 p.p.m.

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