Eur. J. Biochem. *160*, 621 – 625 (1986)
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# Primary structure of a low-molecular-mass N-linked oligosaccharide from hemocyanin of *Lymnaea stagnalis*

## 3-*O*-methyl-D-mannose as a constituent of the xylose-containing core structure in an animal glycoprotein

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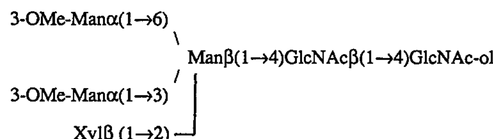
(Received June 30, 1986) – EJB 86 0681

Hemocyanin from the freshwater snail *Lymnaea stagnalis* is a high-molecular-mass copper-containing oxygen-transport protein, which occurs freely dissolved in the hemolymph. It is a glycoprotein containing fucose, xylose, 3-*O*-methylmannose, 3-*O*-methylgalactose, mannose, galactose, *N*-acetylgalactosamine and *N*-acetylglucosamine residues as sugar constituents. The *N*-glycosidic carbohydrate chains of this glycoprotein were released by hydrazinolysis of a pronase digest and subsequently fractionated as oligosaccharide-alditols on Bio-Gel P-4 followed by Lichrosorb-$NH_2$. Investigation with 500-MHz $^1$H-NMR spectroscopy, in conjunction with sugar and methylation analysis revealed the lowest-molecular-mass glycan chain to have the structure:




The hemocyanins of the gastropods *Helix pomatia* and *Lymnaea stagnalis* are the only animal glycoproteins, reported so far, having partially *O*-methylated carbohydrate chains [1]. Sugar analysis of these glycoproteins revealed the presence of Fuc, Xyl, 3-OMe-Gal, Man, Gal, GalNAc and GlcNAc for *H. pomatia* hemocyanin and Fuc, Xyl, 3-OMe-Man, 3-OMe-Gal, Man, Gal, GalNAc and GlcNAc for *L. stagnalis* hemocyanin. Recently, the structures of the low-molecular-mass N-linked oligosaccharides of *H. pomatia* hemocyanin have shown to be Manα(1→6)[Manα(1→3)][Xylβ(1→2)]-Manβ(1→4)GlcNAcβ(1→4)[Fucα(1→6)]$_{0-1}$GlcNAc-ol [2]. The detailed characterization of the very complex high-molecular-mass structures, built up from these core structures and the additional monosaccharides 3-OMe-Gal, Gal, GalNAc and GlcNAc, is still in progress.

In the course of a series of studies on the structures of the carbohydrate chains of hemocyanins from different species the analysis of the primary structure of the lowest-molecular-mass N-linked oligosaccharide from the hemocyanin of the freshwater snail *L. stagnalis* is reported.

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*Abbreviations.* Fuc, L-fucose; Xyl, D-xylose; 3-OMe-Man, 3-*O*-methyl-D-mannose; 3-OMe-Gal, 3-*O*-methyl-D-galactose; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc-ol, *N*-acetyl-D-glucosaminitol; HPLC, high-performance liquid chromatography; GLC-MS, gas-liquid chromatography mass spectrometry; SDS, sodium dodecyl sulfate.

## MATERIALS AND METHODS

### *Isolation of hemocyanin from* L. stagnalis

Specimens of *L. stagnalis* were collected from the canal in Leeds and were immediately bled via the hemal pore. The hemolymph was collected in 0.1 M acetate buffer, pH 5.7, at 4°C containing the following to prevent proteolysis: 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM pepstatin, 0.1 M antipain, 0.1 M chymostatin, 0.1 M leupeptin, 0.2 units/ml aprotinin, as well as 0.005% (w/v) thiomersalate as preservative. The pooled hemolymph from several hundred snails was centrifuged at low speed to remove debris and bacteria and the hemocyanin sedimented by ultracentrifugation for 2 h and 4°C at 130000 × *g*. The resulting blue pellet was resuspended in 0.1 M acetate buffer, pH 5.7, and resedimented twice. The purity of the final material was checked by SDS/polyacrylamide gel electrophoresis on a 4–16% gel slab.

### *Preparation of a copper-free pronase digest*

A solution of 550 mg hemocyanin in 10 mM Pipes buffer, pH 7.0, containing 0.2 M NaCl and a trace of thiomersalate as preservative, was stripped of copper, denatured with urea and digested by pronase, as described [2], yielding 55 mg glycopeptide mixture.

### *Hydrazinolysis procedure and fractionation*

The thoroughly dried glycopeptide mixture was suspended in 1 ml of anhydrous hydrazine and heated for 8 h at 100°C.

After evaporation of hydrazine, the material was re-*N*-acetylated and reduced as described [3]. A small amount of the sample (0.6 mg) was reduced with $NaB^3H_4$ and the remaining part (23 mg) with $NaB^2H_4$ [2]; whereafter 50 µCi (1%) of the $^3$H-labelled oligosaccharide-alditols were mixed with the oligosaccharide-[1-$^2$H]-alditols. Paper electrophoresis (Whatman 3MM paper, 70 V/cm, 90 min) was carried out using a pyridine/acetic acid/water buffer (3:1:387; v/v), pH 5.4. The oligosaccharide-alditols were recovered from the paper by elution with water.

The neutral oligosaccharide-alditols were fractionated on two connected Bio-Gel P-4 columns (2 × 100 cm each; − 400 mesh; Bio-Rad) eluted with water (20 ml/h, 1.7 ml fractions) at 55 °C [4]. Oligosaccharide-alditols were monitored by refractive index detection and scintillation counting. The lowest-molecular-mass fraction was further fractionated by HPLC using a Perkin-Elmer series 3 liquid chromatograph, equipped with a Rheodyne injection valve. A column (4 × 250 mm) of Lichrosorb-$NH_2$ (5 µm, Merck) was used. The column was run isocratically with a mixture of acetonitrile/water (75:25, v/v) for 55 min at room temperature at a flow rate of 1 ml/min. Fractions of 1 ml were collected and assayed at 205 nm and by scintillation counting [5].

### *500-MHz $^1$H-NMR spectroscopy*

Carbohydrates were repeatedly exchanged in $^2H_2O$ (99.96 atom % $^2$H, Aldrich) with intermediate lyophilization. $^1$H-NMR spectra were recorded on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating at 500 MHz in the Fourier-transform mode at a probe temperature of 27 °C. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [6]. Chemical shifts ($\delta$) are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly to acetone ($\delta$ = 2.225 ppm) [7].

### *Sugar analysis*

Samples containing 50 nmol carbohydrate were subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85 °C) followed by GLC of the trimethylsilylated (re-*N*-acetylated) methyl glycosides on a capillary CPsil 5 CB WCOT fused silica column (0.34 mm × 25 m, Chrompack) [1, 8].

### *Methylation analysis*

Methylation analysis was carried out essentially as described by Waeghe et al. [9]. After permethylation of the oligosaccharide-alditol (100 µg), using $C^2H_3I$, the permethylated material was hydrolysed with 4 M trifluoroacetic acid and the obtained mixture of partially methylated monosaccharides reduced with $NaB^2H_4$ in water (10 µg/µl). Combined GLC-MS was performed on a Carlo Erba GC/Kratos MS 80/Kratos DS55 system; electron energy, 70 eV; accelerating voltage, 2.7 kV; ionizing current, 100 µA; ion-source temperature, 225 °C; CPsil 5 CB WCOT fused silica column (0.34 mm × 25 m; Chrompack); oven temperature program, 120 °C during 2 min, 120−240 °C at 4 °C/min.

## RESULTS AND DISCUSSION

The purity of *L. stagnalis* hemocyanin was checked by SDS/polyacrylamide gelelectrophoresis (Fig. 1). The material showed a major band at 450 kDa, which corresponds to the subunit (1/20th molecule) of the hemocyanin. The small amounts of lower-molecular-mass contaminants are believed to be digestion products of the hemocyanin notwithstanding the precautions taken. No 'low'-molecular-mass (i.e. $M_r$ < 100000) material was detected.

The sugar analysis of the native hemocyanin is presented in Table 1. The quantitative data indicated a carbohydrate content of 3% (w/w). To facilitate the structural analysis of the N-linked carbohydrate chains, they were released from a pronase digest of the glycoprotein (sugar analysis, Table 1) by hydrazinolysis. After re-*N*-acetylation and reduction (sugar analysis, Table 1) high-voltage paper electrophoresis showed only a small amount of acidic material. The neutral fraction (97%) was separated on Bio-Gel P-4 in four fractions (Fig. 2)

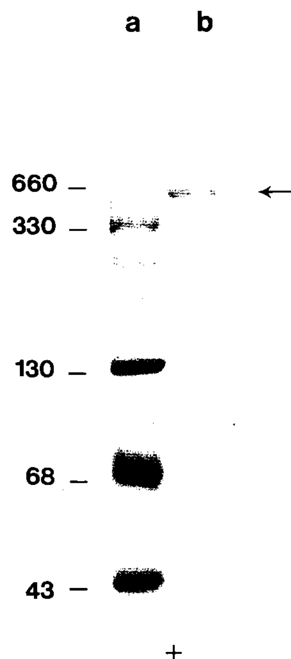



Fig. 1. *SDS/polyacrylamide gel electrophoresis with purified* L. stagnalis *hemocyanin, gradient gel 4−16%.* Lane a, molecular mass markers, values in kDa, from bottom: ovalbumin, bovine serum albumin, β-galactosidase, thyroglobulin and its dimer; lane b, *L. stagnalis* hemocyanin, approx. 450−500 kDa

Table 1. *Molar carbohydrate composition of hemocyanin from* Lymnaea stagnalis *and related fractions*

| Monosaccharide | Glycoprotein | Pronase digest | Hydrazinolysate | IVa | IVb |
|---|---|---|---|---|---|
| Fuc | 1.1 | 0.9 | 0.9 | | |
| Xyl | 0.7 | 0.7 | 0.7 | 0.9 | 0.4 |
| 3-OMe-Man | 1.1 | 1.1 | 1.1 | 1.9 | 1.0 |
| 3-OMe-Gal | 0.3 | 0.3 | 0.3 | | |
| Man | 3.0[a] | 3.0[a] | 3.0[a] | 1.0[b] | 1.0[b] |
| Gal | 1.3 | 0.9 | 1.2 | | |
| GalNAc | 1.2 | 1.0 | 0.8 | | |
| GlcNAc | 2.4 | 1.9 | 1.3 | 0.6 | 0.4 |
| GlcNAc-ol | | | 0.6 | 0.5 | 0.7 |

[a] Man taken as 3.
[b] Man taken as 1.

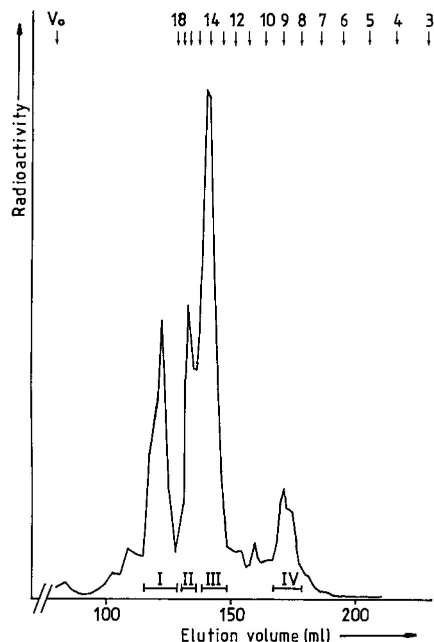



Fig. 2. *Elution profile on Bio-Gel P-4 (−400 mesh) of $^3$H-labelled oligosaccharide-[1-$^2$H]-alditols derived from* L. stagnalis *hemocyanin.* The column was eluted with bidistilled water a 55°C. Fractions of 1.7 ml were collected at a flow rate of 20 ml/h and assayed for $^3$H radioactivity. Fractions I−IV were pooled. The arrows at the top indicate the elution positions of glucose oligomers generated by a dextran hydrolysis. The numbers at the top indicate the glucose units

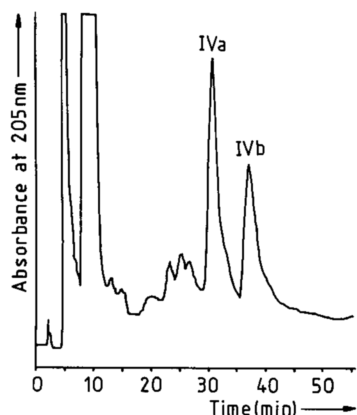



Fig. 3. *Elution profile on Lichrosorb-NH$_2$ (HPLC) of oligosaccharide-[1-$^2$H]-alditols derived from Bio-Gel P-4 fraction IV.* The column was run isocratically with a mixture of acetonitrile/water (75:25, v/v) at a flow rate of 1 ml/min. Fractions IVa and IVb were pooled

in relative amounts of: I, 24%; II, 18%; III, 42%; and IV, 16%. The low-molecular-mass carbohydrate fraction IV was further separated by HPLC, yielding two fractions denoted IVa and IVb (Fig. 3). Combination of the sugar analysis (Table 1) and the methylation analysis data (Table 2) of fraction IVa suggested the presence of an oligosaccharide structure comparable with the lowest-molecular-mass structure from *Helix pomatia* α-hemocyanin, namely Manα(1→6)-[Manα(1→3)][Xylβ(1→2)]Manβ(1→4)GlcNAcβ(1→4)GlcNAc-ol (reference compound R [2]), except that for *L. stagnalis* this

Table 2. *Methylation analysis of the oligosaccharide-alditol fraction IVa of the hydrazinolysate*

| Partially methylated alditol acetate | Molar ratio |
|---|---|
| 2,3,4-Tri-*O*-[$^2$H]methyl-xylitol | 0.6 |
| 3-Mono-*O*-methyl-2,4,6-tri-*O*-[$^2$H]methyl-mannitol | 2.0[a] |
| 4-Mono-*O*-[$^2$H]methyl-mannitol | 0.8 |
| 1,3,5,6-Tetra-*O*-[$^2$H]methyl-2-*N*-[$^2$H]methyl-acetamido-2-deoxyglucitol | 0.9 |
| 3,6-Di-*O*-[$^2$H]methyl-2-*N*-[$^2$H]methyl-acetamido-2-deoxyglucitol | 0.9 |

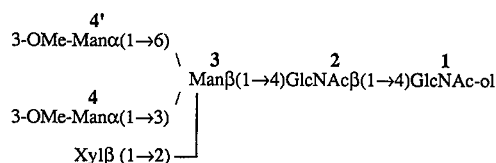
[a] 3-Mono-*O*-methyl-2,4,6-tri-*O*-[$^2$H]methyl-mannitol taken as 2.0.

Table 3. *Relevant $^1$H chemical shifts of structural-reporter groups of constituent monosaccharides for fraction IVa from* Lymnaea stagnalis *hemocyanin and those for reference compound R [2]*
Chemical shifts are in ppm downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate in $^2H_2O$ at 27°C acquired at 500 MHz (but were actually measured relative to internal acetone: δ = 2.225 ppm). For the numbering of the monosaccharide residues and complete structures, see Fig. 4. In the table heading, the structures are represented by short-hand symbolic notation (cf. [2, 7]): ●- , GlcNAc; ♦- , Man; (♦)- , 3-OMe-Man; and ⊠- , Xyl. n.d. = not detected

| Residue | Reporter group | Chemical shift in compound R | Chemical shift in compound IVa |
|---|---|---|---|
| | | ppm | |
| GlcNAc-**1**-ol | H-2 | 4.239 | 4.237 |
| | NAc | 2.057 | 2.056 |
| GlcNAc-**2** | H-1 | 4.634 | 4.620 |
| | NAc | 2.073 | 2.065 |
| Man-**3** | H-1 | 4.883 | 4.898 |
| | H-2 | 4.270 | 4.281 |
| Man-**4** | H-1 | 5.122 | 5.168 |
| | H-2 | 4.039 | 4.305 |
| | $OCH_3$ | | 3.443[a] |
| Man-**4′** | H-1 | 4.913 | 4.961 |
| | H-2 | 3.983 | 4.230 |
| | $OCH_3$ | | 3.415[a] |
| Xyl | H-1 | 4.449 | 4.453 |
| | H-2 | 3.377 | 3.378 |
| | H-3 | 3.437 | n.d. |
| | H-$5_{ax}$ | 3.250 | 3.264 |
| | H-$5_{eq}$ | n.d. | 4.015 |

[a] Assignments may have to be interchanged.

structure is extended with 3-*O*-methyl groups attached to both terminal α-Man residues:



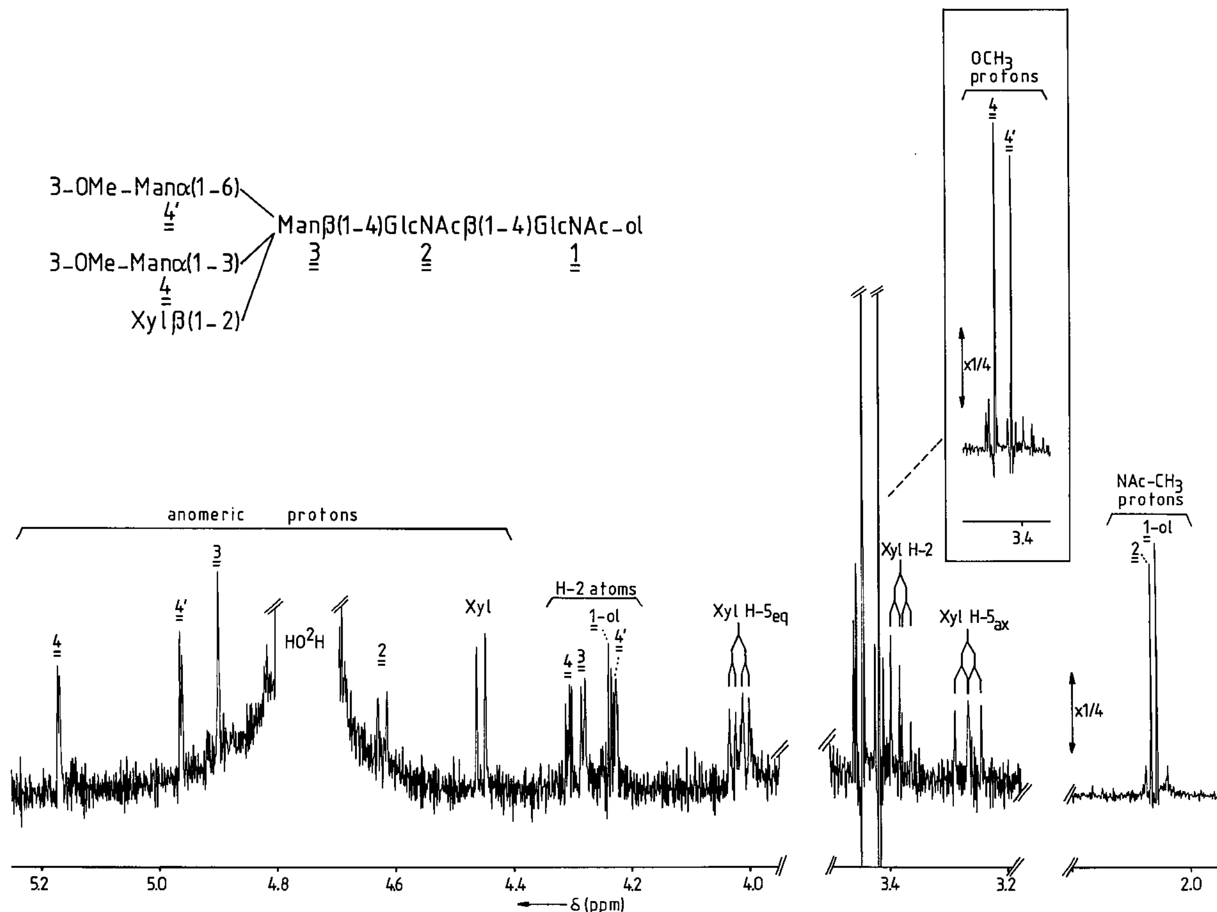



Fig. 4. *Structural-reporter-group regions of the resolution-enhanced 500-MHz $^1H$-NMR spectrum of oligosaccharide-[1-$^2H$]-alditol fraction IVa, derived from* L. stagnalis *hemocyanin recorded in $^2H_2O$ at 27°C.* The numbers in the spectrum refer to the corresponding residues in the structure. The relative intensity scale of the *N*-acetyl and *O*-methyl regions differs from that of the other parts of the spectrum as indicated

Conclusive evidence was obtained by using 500-MHz $^1H$-NMR spectroscopy. The structural-reporter-group regions of the $^1H$-NMR spectrum of fraction IVa, recorded in $^2H_2O$, are presented in Fig. 4. The equal intensity of the anomeric proton signals point to the presence of a single compound. Relevant NMR parameters are listed in Table 3 together with those of reference compound R from *H. pomatia* α-hemocyanin [2]. Comparing IVa with R shows that the chemical shift signals of the structural-reporter-groups of GlcNAc-**1**-ol, H-2 and NAc, are not affected by the *O*-methylation of C-3 of the terminal Man residues. However H-1 and NAc of GlcNAc-**2** have slightly shifted upfield in IVa ($\Delta\delta$ −0.014 ppm and −0.008 ppm, respectively). The structural-reporter-groups of Man-**3**, H-1 and H-2, are also slightly influenced by the *O*-methylation of Man-**4** and Man-**4′**; both protons resonate at lower values of $\delta$ ($\Delta\delta$ 0.015 and 0.011 ppm, respectively) in IVa.

For an evaluation of the NMR parameters of the 3-*O*-methylated Man-**4** and Man-**4′** residues, the 500-MHz $^1H$-NMR data of the methyl α-D-glycosides of Man and 3-OMe-Man are recorded. Comparison of the latter NMR data (Table 4) clearly indicates the influence of 3-*O*-methylation on the values of $\delta$ for the various protons of methyl α-D-mannopyranoside. Similar chemical-shift effects are observed for the structural-reporter-groups H-1 and H-2 of Man-**4** and Man-**4′** ($0.042 \leq \Delta\delta \leq 0.048$ ppm for H-1 and $0.249 \leq \Delta\delta \leq 0.266$ ppm for H-2) when going from R to IVa (Table 3). The assignments of the H-2 signals of the methylated α-Man residues in IVa was based on selective $^1H$-decoupling of the H-1 signals. The 3-*O*-methyl groups resonate at $\delta$ 3.443 ppm and 3.415 ppm.

The Xyl structural-reporter-groups H-1 and H-2 are not essentially influenced by 3-*O*-methylation of the terminal Man residues. The chemical-shift signals of the H-5 atoms are both found in downfield positions, when IVa is compared with R. For IVa, the chemical shift of H-$5_{ax}$ is observed at $\delta$ = 3.264 ppm and the signal of H-$5_{eq}$, which was hidden under the signals of the sugar skelet protons ($\delta$ < 4.0 ppm) for R, resonates outside the bulk at 4.015 ppm. The assignment of H-$5_{eq}$ was based on selective $^1H$-decoupling of H-$5_{ax}$, in combination with the characteristic coupling constants $J_{4,5eq}$ (5.4 Hz) and $J_{5ax,5eq}$ (−11.5 Hz) [10]. THe H-3 signal could not be indicated exactly.

The 500-MHz $^1H$-NMR spectrum of IVb showed the presence of at least two components of which the structure could not be unraveled, due to the low amount of material.

In conclusion it has been established that there is a striking relationship between the low-molecular-mass N-linked carbohydrate chains of the hemocyanins from the gastropods *H. pomatia* and *L. stagnalis*. Both species possess a core structure with Xyl in β(1→2)-linkage to Man-**3** which, so far, has not been found for animal glycoproteins. Furthermore, they both have 3-*O*-methylated sugars in their carbohydrate chains. It is remarkable that 3-OMe-Man occurs only in *L. stagnalis*.

Table 4. *$^1$H-NMR data for the methyl α-D-glycopyranosides of mannose and 3-O-methyl-mannose*
Chemical shifts are in ppm downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate in $^2H_2O$ at 27°C acquired at 500 MHz (but were actually measured relative to internal acetone: $\delta$ = 2.225 ppm)

| Protons | Chemical shift of | |
|---|---|---|
| | α-Man | 3-OMe-α-Man |
| | ppm | |
| H-1 | 4.761 | 4.803 |
| H-2 | 3.929 | 4.178 |
| H-3 | 3.751 | 3.449 |
| H-4 | 3.640 | 3.674 |
| H-5 | 3.604 | 3.624 |
| H-6a | 3.898 | 3.889 |
| H-6b | 3.755 | 3.749 |
| 1-$OCH_3$ | 3.407 | 3.414[a] |
| 3-$OCH_3$ | | 3.430[a] |
| Coupling | | |
| | Hz | |
| $J_{1,2}$ | 1.8 | 1.9 |
| $J_{2,3}$ | 3.5 | 3.3 |
| $J_{3,4}$ | 10.0 | 9.8 |
| $J_{4,5}$ | 10.0 | 9.9 |
| $J_{5,6a}$ | 2.6 | 2.3 |
| $J_{5,6b}$ | 5.8 | 6.0 |
| $J_{6a,6b}$ | −12.3 | −12.2 |

[a] Assignments were made by comparison with [$^2$H]methyl 3-*O*-methyl-α-D-mannopyranoside.

This methylated sugar is not of dietary origin, but is synthesized in the cell [11]. The elucidation of the high-molecular-mass carbohydrate chains of the hemocyanin of *L. stagnalis* is in progress.

We thank Prof. Dr A. Liptak (University of Debrecen, Hungary) for the kind gift of methyl 2-*O*-benzyl-4,6-*O*-benzylidene-3-*O*-methyl-α-D-mannopyranoside. This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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