*Biochem. J.* (1977) **165**, 173–176
*Printed in Great Britain*



# 3-*O*-Methyl Sugars as Constituents of Glycoproteins

## IDENTIFICATION OF 3-*O*-METHYLGALACTOSE AND 3-*O*-METHYLMANNOSE IN PULMONATE GASTROPOD HAEMOCYANINS

By RODERICK L. HALL* and EDWARD J. WOOD
*Department of Biochemistry, University of Leeds, 9 Hyde Terrace, Leeds LS2 9LS, U.K.*

and JOHANNIS P. KAMBERLING, GERRIT J. GERWIG and F. G. VLIEGENTHART
*Laboratory of Organic Chemistry, University of Utrecht, Croesestraat 79, Utrecht 2503, The Netherlands*

(*Received* 14 *April* 1977)

In addition to the already known monosaccharides fucose, xylose, mannose, galactose, glucose, *N*-acetylgalactosamine and *N*-acetylglucosamine, the carbohydrate part of the haemocyanin from *Helix pomatia* (Roman snail) contains 3-*O*-methylgalactose, and that from *Lymnaea stagnalis* (a freshwater snail) 3-*O*-methylgalactose and 3-*O*-methylmannose. The 3-*O*-methyl sugars were identified by g.l.c.–mass spectrometry of the corresponding trimethylsilyl methyl glycosides and the alditol acetates, and by co-chromatography with the synthetic reference substances.

Haemocyanins, the copper-containing respiratory proteins of many invertebrates, have been found to be glycoproteins. Dijk *et al.* (1970) reported that the haemocyanin from *Helix pomatia* (Roman snail) contains, in addition to fucose, xylose, mannose, galactose, glucose, *N*-acetylgalactosamine and *N*-acetylglucosamine, an unidentified sugar residue. Analyses of the haemocyanins of gastropods from marine, freshwater and terrestrial habitats (Hall & Wood, 1976) confirmed the findings for *H. pomatia*. Further, the haemocyanin from another pulmonate species, *Lymnaea stagnalis* (a freshwater snail), contains in addition a second unknown carbohydrate. These two unknown carbohydrates have now been identified as 3-*O*-methylgalactose and 3-*O*-methylmannose respectively by means of g.l.c.–mass spectrometry of the pertrimethylsilyl derivatives of the methyl glycosides, and of the alditol acetates.

## Materials and Methods

### *Preparation of haemocyanins*

Specimens of *H. pomatia* were obtained from T. Gerrard and Co., East Preston, Sussex, U.K., and the haemolymph was taken by cardiac puncture. The pooled haemolymph from ten snails was diluted with an equal volume of 0.1 M-sodium acetate buffer, pH 5.7, containing 0.01% $NaN_3$ and centrifuged at $26000g_{av.}$ for 20 min in an MSE Superspeed 50 centrifuge to remove tissue, shell debris and bacteria. The blue supernatant solution was then centrifuged for a further 2 h at 4°C and $133500g_{av.}$ in a Beckman L2-65 B ultracentrifuge. The resultant blue pellet of haemocyanin was resuspended in the sodium acetate buffer, sedimented again and finally resuspended in buffer and stored at 4°C. *L. stagnalis* haemocyanin was prepared in a similar way (see Hall *et al.*, 1975). Haemocyanin purified in this manner sedimented as a single boundary in the analytical ultracentrifuge, with a sedimentation coefficient of approximately 100 S.

* Present address: Department of Physiology, St. George's Hospital Medical School, Blackshaw Road, Tooting, London SW17 0QT, U.K.

### *Monosaccharide analysis*

Methyl glycosides derived from haemocyanins were prepared in the presence of mannitol as internal standard essentially by the method described by Clamp *et al.* (1971), except that methanolysis was carried out for 8 h at 80°C, and re-*N*-acetylation for 4 h at room temperature (20°C). The samples were analysed by g.l.c. and g.l.c.–mass spectrometry in two ways: (*a*) directly after trimethylsilylation or [$^2H_9$]trimethylsilylation and (*b*) after hydrolysis with 0.25 M-$H_2SO_4$ for 18 h at 100°C and subsequent reduction with $NaB^2H_4$ and acetylation (alditol [1-$^2H_1$]acetates) (Björndal *et al.*, 1967).

### *G.l.c.*

G.l.c. of [$^2H_9$]trimethylsilyl methyl glycosides was performed on a Varian Aerograph 2740-30-01 gas chromatograph equipped with a flame-ionization detector and a WCOT glass capillary column (25 m × 0.35 mm internal diam.) coated with SE-30 as stationary phase (LKB-Producter A.B., Stockholm, Sweden). The carrier-gas $N_2$ flow rate was

1 ml/min and the make-up gas $N_2$ flow rate 30 ml/min. The injection-port temperature and the detector temperature were 200 and 220°C respectively. The oven temperature was programmed from 135 to 220°C at 1°C/min.

G.l.c. of alditol [1-$^2H_1$]acetates was carried out on the same type of gas chromatograph equipped with a dual flame-ionization detector, and coiled glass columns (2.00m×4.0mm internal diam.) packed with 3.8% SE-30 on Chromosorb W (AW-DMCS) (HP, 80–100 mesh). The carrier-gas $N_2$ flow rate was 40 ml/min. The injection port, detector and oven temperatures were 200, 220 and 190°C respectively.

*G.l.c.–mass spectrometry*

The 75eV mass spectra were recorded on a Jeol JGC-1100/JMS-07 combination at an ion-source temperature of 250°C, an accelerating voltage of 3kV and an ionizing current of 300$\mu$A. As column material, 3.8% SE-30 on Chromosorb W (AW-DMCS) (HP, 80–100 mesh) was used. The carrier-gas $N_2$ flow rate was 40 ml/min, and the oven-temperature programme conditions were the same as described above for methyl glycosides and alditol acetates respectively.

## Results

In Fig. 1 the capillary gas chromatogram of the mixture of trimethylsilyl methyl glycosides derived from the *H. pomatia* haemocyanin is presented. To characterize the three 'unknown' peaks 7, 8 and 9, the mixture of trimethylsilyl methyl glycosides and the corresponding [$^2H_9$]trimethylsilyl derivatives were investigated by g.l.c.–mass spectrometry. On a packed column of SE-30 the three peaks are not as well separated as on the capillary SE-30 column, but g.l.c.–mass spectrometry could only be performed with packed columns. Comparison of the mass spectra of the labelled and unlabelled derivatives

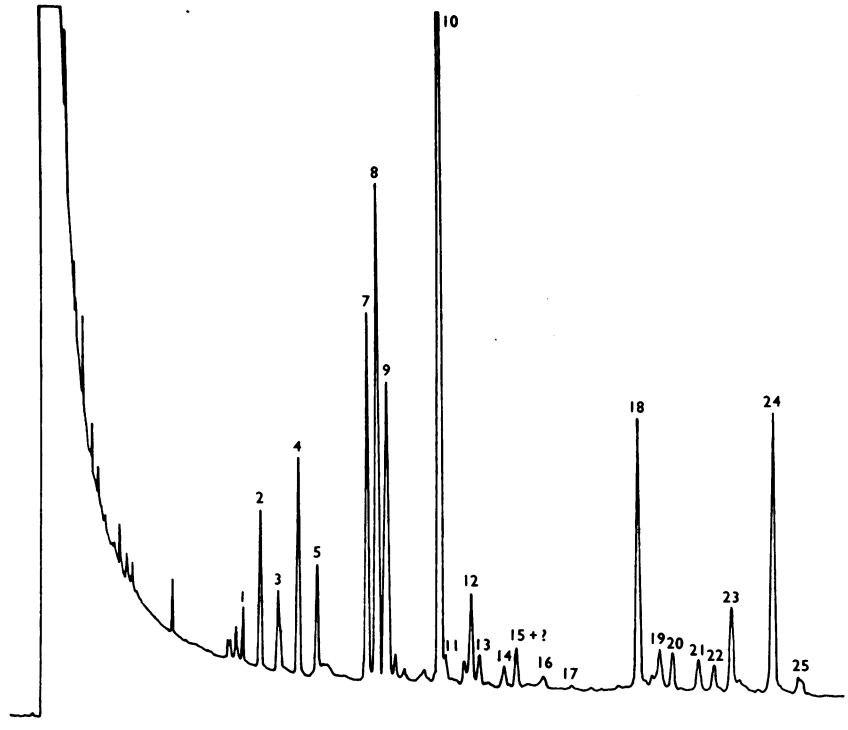


Fig. 1. *SE-30 capillary gas chromatogram of the trimethylsilyl derivatives of the methyl glycosides derived from the haemocyanin of Helix pomatia*

The peaks are identified as follows: 1, 2 and 3, fucose; 4 and 5, xylose; 6, 3-*O*-methylmannose; 7, 8 and 9, 3-*O*-methylgalactose; 10, 12 and 14, mannose; 11, 13 and 15, galactose; 16 and 17, glucose; 18, mannitol (internal standard); 19, 22, 24 and 25, *N*-acetylglucosamine; 20 and 23, *N*-acetylgalactosamine; 21, mono-*O*-acetylmannitol (by-product of the internal standard).

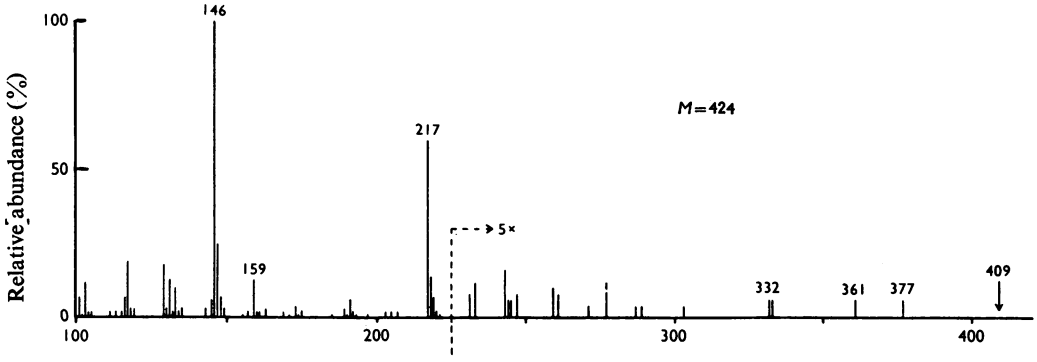


Fig. 2. *Mass spectrum of the trimethylsilyl derivative of methyl 3-O-methylmannopyranoside*
Only values >*m/e*100 are given.

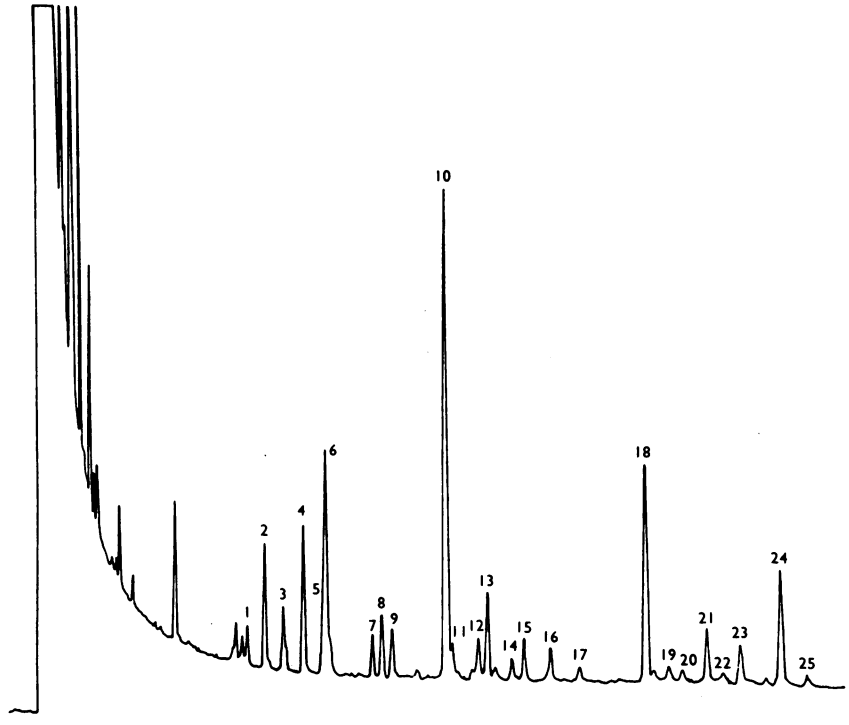


Fig. 3. *SE-30 capillary gas chromatogram of the trimethylsilyl derivatives of the methyl glycosides derived from the haemocyanin of Lymnaea stagnalis*
The peaks are identified as described in the legend to Fig. 1.

corresponding to the unknown peaks indicated the presence of a methyl 3-*O*-methylhexoside. The mass spectrum recorded on the shoulder in the ascending curve of the unresolved peaks demonstrated the presence of a furanose ring form, because the intensity of the peak at *m/e* 146 (predominantly $Me_3SiO$–$\overset{2}{C}H$–$\overset{3}{C}H$–$OCH_3$*) was smaller than that of the peak at *m/e*159 (predominantly $Me_3SiO$-$\overset{2}{C}H$-$\overset{3}{C}H(OCH_3)$-$\overset{4}{C}H$) (Kamerling *et al.*, 1975; J. P. Kamerling, unpublished

* Abbreviation: $Me_3Si$-, trimethylsilyl-.

work). The spectra taken on the top and the shoulder in the descending curve gave evidence for the presence of pyranose ring forms. Fig. 2 shows the mass spectrum of a methyl 3-*O*-methylhexopyranoside as its trimethylsilyl derivative. The intense peaks at *m/e* 146 (predominantly $Me_3SiO-\overset{2}{C}H-\overset{3}{C}H-OCH_3$) and at *m/e* 217 ($Me_3SiO-\overset{2}{C}H-\overset{3}{C}H=\overset{4}{C}H-OSiMe_3$) in the mass spectra of trimethylsilyl (methyl) mono-*O*-methylhexopyranos(id)es are typical for the presence of an *O*-methyl group at C-3 (Petersson & Samuelson, 1968; Petersson, 1974; De Jongh *et al.*, 1969). The observed peak pattern in the gas chromatogram suggested a *galacto*-configuration for the hexose. G.l.c. of the trimethylsilyl derivative of synthetic methyl 3-*O*-methylgalactoside gave rise indeed to the same pattern as the methyl 3-*O*-methylhexoside (co-chromatography). The mass spectra were also identical.

The identification of 3-*O*-methylgalactose in the haemocyanin from *H. pomatia* was confirmed by the analysis of the alditol acetates. The unknown alditol acetate was characterized by g.l.c. and g.l.c.–mass spectrometry as 3-*O*-methylgalactitol (co-chromatography; presence of the primary fragment ions at *m/e* 190 and *m/e* 261) (Jansson *et al.*, 1976; Lönngren & Svensson, 1974).

In Fig. 3 the capillary gas chromatogram of the mixture of trimethylsilyl methyl glycosides derived from the haemocyanin of *L. stagnalis* is given. Besides the monosaccharides identified previously, the three peaks 7, 8 and 9, and another unknown peak 6, which overlaps partly the second xylose peak, were observed (Hall & Wood, 1976). The unknown peaks were investigated in the same way as described for the haemocyanin of *H. pomatia*. The peaks 7, 8 and 9 were identified as anomeric forms of methyl 3-*O*-methylgalactoside and peak 6 as methyl 3-*O*-methylmannopyranoside. The *manno*-configuration was proven by co-chromatography with the trimethylsilyl derivative of synthetic methyl 3-*O*-methylmannoside.

## Discussion

In the last few years a vast number of partially *O*-methylated monosaccharides have been found in naturally occurring biopolymers, obtained from various plant, bacterial and fungal materials.

3-*O*-Methyl-D-galactose occurs as a component of polysaccharides in *Ulmus fulva* (slippery elm) mucilage (Hough *et al.*, 1950), in the lauraceous tree *Sassafras albidum* (sassafras) (Springer *et al.*, 1965), in the leaves of deciduous trees (Bacon & Cheshire, 1971), and in various actinomycetes (Lechevalier & Gerber, 1970). The bacteria *Streptomyces griseus* (Candy & Baddiley, 1966), *Klebsiella* (Nimmich, 1970) and *Rhodopseudomonas* spp. (Weckesser *et al.*, 1973) contain 3-*O*-methyl-D-mannose residues, as do polysaccharides isolated from the fungus *Coccidioides immitis* (Porter *et al.*, 1971) and the O-antigen of the blue–green alga *Anacystis nidulans* (A. Katz, unpublished work).

The finding of 3-*O*-methylgalactose in the haemocyanin from *H. pomatia*, and both 3-*O*-methylgalactose and 3-*O*-methylmannose in that from *L. stagnalis*, appears to constitute the first recognition of methylated sugars in glycoproteins.

We are indebted to Dr. G. J. F. Chittenden (University of Nijmegen, Nijmegen, The Netherlands) and Professor J. Montreuil (University of Lille, Lille, France) for the generous gifts of 3-*O*-methyl-D-galactose and 3-*O*-methyl-D-mannose respectively. We thank Mr. C. Versluis (Laboratory of Analytical Chemistry, University of Utrecht) for recording the mass spectra. This investigation was supported in part by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). R. L. H. is grateful to the S.R.C. for studentship.

## References

Bacon, J. S. D. & Cheshire, M. V. (1971) *Biochem. J.* **124**, 555–562

Björndal, H., Lindberg, B. & Svensson, S. (1967) *Acta Chem. Scand.* **21**, 1801–1804

Candy, D. J. & Baddiley, J. (1966) *Biochem. J.* **98**, 15–18

Clamp, J. R., Bhatti, T. & Chambers, R. E. (1971) *Methods Biochem. Anal.* **19**, 229–344

De Jongh, D. C., Radford, T., Hribar, J. D., Hanessian, S., Beiber, M., Dawson, G. & Sweeley, C. C. (1969) *J. Am. Chem. Soc.* **91**, 1728–1740

Dijk, J., Brouwer, M., Coert, A. & Gruber, M. (1970) *Biochim. Biophys. Acta* **221**, 467–479

Hall, R. L. & Wood, E. J. (1976) *Biochem. Soc. Trans.* **4**, 307–309

Hall, R. L., Pearson, J. S. & Wood, E. J. (1975) *Comp. Biochem. Physiol. B* **52**, 211–218

Hough, L., Jones, J. K. N. & Hirst, E. L. (1950) *Nature (London)* **165**, 34–35

Jansson, P.-E., Kenne, L., Lidegren, H., Lindberg, B. & Lönngren, J. (1976) *Chem. Commun. Univ. Stockholm* no. 8

Kamerling, J. P., Gerwig, G. J., Vliegenthart, J. F. G. & Clamp, J. R. (1975) *Biochem. J.* **151**, 491–495

Lechavalier, M. P. & Gerber, N. N. (1970) *Carbohydr. Res.* **13**, 451–454

Lönngren, J. & Svensson, S. (1974) *Adv. Carbohydr. Chem. Biochem.* **29**, 41–106

Nimmich, W. (1970) *Biochim. Biophys. Acta* **215**, 189–191

Petersson, G. (1974) Thesis, Chalmers Techniska Hogsköla, Göteborg

Petersson, G. & Samuelson, O. (1968) *Sven. Papperstidn.* **71**, 731–738

Porter, J. F., Scheer, E. R. & Wheat, R. W. (1971) *Infect. Immun.* **4**, 660–661

Springer, G. F., Takahashi, T., Desai, P. R. & Kolecki, B. J. (1965) *Biochemistry* **4**, 2099–2113

Weckesser, J., Mayer, H. & Fromme, I. (1973) *Biochem. J.* **135**, 293–297