

Characterization by Gas-Liquid Chromatography-Mass Spectrometry and Proton-Magnetic-Resonance Spectroscopy of Pertrimethylsilyl Methyl Glycosides obtained in the Methanolysis of Glycoproteins and Glycopeptides

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(Received 5 June 1975)

The quantitative analysis by gas chromatography of monosaccharides present in glycoproteins and glycopeptides using methanolysis, followed by re-*N*-acetylation and trimethylsilylation, gives rise to several peaks for each monosaccharide. The identity of these peaks for xylose, fucose, mannose, galactose, glucose, *N*-acetylglucosamine, *N*-acetylgalactosamine and *N*-acetylneuraminic acid was established for α - and β -methyl pyranosides and furanosides by combined g.l.c.-mass spectrometry and proton-magnetic-resonance spectroscopy. These data provide for the unambiguous interpretation of the gas chromatograms obtained in the application of this g.l.c. method, and supply basic information for the further application of mass spectrometry in this field.

For the quantitative analysis of monosaccharides present in glycoproteins and glycopeptides, a number of methods have been described. Methods have been based on measurement of reducing power, colorimetric and enzymic assays (Marshall & Neuberger, 1972), ion-exchange chromatography (Kennedy, 1974) and g.l.c. (Clamp, 1974). To obtain suitable starting materials for analysis, solvolysis of the glycoconjugates is necessary. The stabilities of the free monosaccharides to aqueous acid vary greatly from one type of sugar to another, necessitating different optimum hydrolysis conditions for each type of monosaccharide, each of which has to be determined individually.

G.l.c. makes it possible to analyse all monosaccharides in one step provided that a strictly controlled solvolytic procedure is used. It has been demonstrated that methanolysis is as efficient as hydrolysis at cleaving glycosidic linkages and causes less destruction of carbohydrate than does aqueous acid (Chambers & Clamp, 1971). By using these data, a g.l.c. analytical procedure was developed consisting of methanolysis followed by re-*N*-acetylation and trimethylsilylation (Clamp *et al.*, 1971, 1972). Most of the peaks in the gas chromatogram were characterized by co-chromatography with methyl pyranosides.

To obtain more conclusive evidence for the identity of the various peaks originating from saccharides that frequently occur in glycoproteins and glycopeptides and to provide basic information for further developments in this field, the methyl glycoside pat-

tern was investigated in detail by combined g.l.c.-mass spectrometry and $^1\text{H-n.m.r.}$ (nuclear-magnetic-resonance) spectroscopy.

Materials and Methods

Carbohydrates

D-Xylose, L-fucose, D-mannose, D-galactose, D-glucose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine were purchased from J. T. Baker Chemicals N.V., Deventer, The Netherlands and *N*-acetylneuraminic acid was obtained from Calbiochem, Los Angeles, Calif., U.S.A.

Methanolysis, re-N-acetylation and trimethylsilylation (Clamp *et al.*, 1971, 1972).

Dried sugar (1mg) was dissolved in 0.5ml of methanolic 1M-HCl. N_2 was bubbled through the solution for 30s and then the ampoule was sealed. After methanolysis for 24h at 85°C, the acid solution was neutralized by the addition of silver carbonate. Re-*N*-acetylation was carried out by the addition of 0.1ml of acetic anhydride. This mixture was kept at room temperature for 24h. The precipitate was triturated thoroughly, centrifuged (2000g for 5min) and the supernatant collected. The residue was washed twice with 0.5ml of dry methanol. The pooled supernatants were evaporated under reduced pressure at 35°C. The residue was dried for 12h in a vacuum

desiccator over P_2O_5 . Finally, the sample was trimethylsilylated with 0.1 ml of silylating agent (pyridine – hexamethyldisilazane – trimethylchlorosilane, 5:1:1, by vol.) for 30 min at room temperature.

Isolation of anomeric forms of methyl glycosides

For the isolation of the different anomeric pyranose and furanose forms of the methyl glycosides of the neutral and acetamido sugars, 400 mg of each monosaccharide was methanolysed and re-*N*-acetylated as described above, but with proportionally greater quantities of reagents. The separation of the various forms of each methyl glycoside was performed on 0.5 mm plates (20 cm × 20 cm) of silica gel 60F254 (E. Merck, Darmstadt, Germany) in the solvent system benzene–propan-2-ol–water (9:10:1, by vol.) (Nozawa *et al.*, 1969). As spray reagent a mixture of 0.2% naphthoresorcinol in methanol and 20% (v/v) H_2SO_4 in methanol (1:1, v/v) was used. The different zones were eluted with pyridine and trimethylsilylated as described by Kamerling *et al.* (1971). Pure fractions of the various anomeric pyranose and furanose forms could not be obtained in all cases. However, comparison of the enriched fractions with the original mixture greatly facilitated the interpretation of the mass spectra and the 1H -n.m.r. spectra.

G.l.c.

A Varian Aerograph 2740-30-01 gas chromatograph equipped with a dual flame ionization detector and coiled glass columns (2.00 m × 4.0 mm internal diam.) packed with 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. The injection port temperature and the detector temperature were 200° and 220°C respectively.

For the analysis of mixtures of monosaccharides, the oven temperature was programmed from 135° to

200°C at 0.5°C/min., but the isolated methyl glycoside fractions were run isothermally at temperatures dependent on the type of saccharide.

G.l.c.–mass spectrometry

The 75 eV mass spectra were recorded on a Jeol JGC-1100/JMS-07 combination at an ion-source temperature of 250°C, an accelerating voltage of 3 kV or 1.5 kV and an ionizing current of 300 μA . As column material 3% SE-30 on Chromosorb W (AW-DMCS), 80–100 mesh, was used. The oven temperature was dependent on the type of sample.

1H -n.m.r. spectroscopy

The 1H -n.m.r. spectra were recorded at 100 MHz with a Varian HA-100 spectrometer, locked on tetramethylsilane, or at 220 MHz with a Varian HR-220 spectrometer, both operating in the field sweep mode at room temperature. As solvents [2H_6]acetone ($\delta_{tetramethylsilane} = 2.05$ p.p.m.) and [2H_6]benzene were used (both from E. Merck).

Results and Discussion

In Fig. 1 a typical gas chromatogram of a mixture of the trimethylsilyl derivatives of the various anomeric methyl glycosides of D-xylose, L-fucose, D-mannose, D-galactose, D-glucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine and *N*-acetylneuraminic acid is presented. For the characterization of each gas chromatographic peak, the eight monosaccharides were studied separately.

Two types of experiment were carried out. (a) The mixture of anomeric pyranose and furanose forms obtained after methanolysis and derivatization of a monosaccharide was investigated directly by g.l.c., g.l.c.–mass spectrometry and p.m.r. (proton magnetic resonance). (b) The mixture of anomeric pyranose and furanose forms obtained after methanolysis and

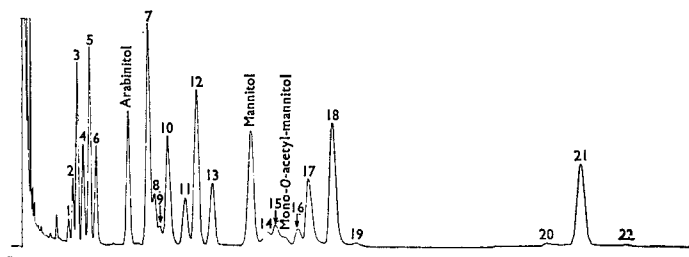


Fig. 1. Gas chromatogram of pertrimethylsilyl methyl glycosides

The peaks are numbered in their order of elution and are identified in Table 1. The internal standards are arabinitol and mannitol. Further details are given in the text.

Table 1. Gas chromatography, mass spectrometry and n.m.r. spectroscopy of pertrimethylsilyl methyl glycosides

For details of the peaks see Fig. 1. For ^1H -n.m.r. spectroscopy the chemical shifts were obtained in $[\text{D}_6]\text{acetone}$. For mass spectrometry of peaks 20, 21 and 22 see the data of Kamerling *et al.* (1974, 1975). The mass spectrum of peak 22 shows that besides 9-*O*-acetyl-*N*-acetylneuraminic acid a very small amount of another mono-*O*-acetyl compound is also present. The α - and β -pyranose used in co-chromatography of peaks 20 and 21 were synthesized as described by Yu & Ledeen (1969). The 9-*O*-acetyl- β -pyranose used for co-chromatography of peak 22 was synthesized as described by Kamerling *et al.* (1975)

Peak	Methyl-glycoside	R_{mannitol}	%	Mass spectrometry	^1H -n.m.r. spectroscopy	Co-chromatography	Conclusion
1	Xyl	0.21	6	$m/e\ 204 \ll m/e\ 217$	$\delta = 4.63\ \text{p.p.m. (2.5 Hz)}$	—	β -f
5	Xyl	0.30	62	$m/e\ 204 > m/e\ 217$	$\delta = 4.54\ \text{p.p.m. (3.2 Hz)}$	α -p	α -p
6	Xyl	0.33	32	$m/e\ 204 > m/e\ 217$	$\delta = 4.05\ \text{p.p.m. (7.0 Hz)}$	β -p	β -p
2	Fuc	0.23	16	$m/e\ 204 \ll m/e\ 217$	$\delta = 4.64\ \text{p.p.m. (2.6 Hz)*}$	—	β -f
3	Fuc	0.25	50	$m/e\ 204 > m/e\ 217$	$\delta = 4.50\ \text{p.p.m. } (\approx 3\ \text{Hz})\dagger$	α -p	α -p
4‡	Fuc	0.27	34	$m/e\ 204 > m/e\ 217$	$\delta = 4.04\ \text{p.p.m. (7.0 Hz)}$	β -p	β -p
	Fuc	0.27		$m/e\ 204 \ll m/e\ 217$	$\delta = 4.66\ \text{p.p.m. (4.2 Hz)}$	—	α -f
7	Man	0.55	90	$m/e\ 204 > m/e\ 217$	$\delta = 4.49\ \text{p.p.m. (1.6 Hz)}\S$	α -p	α -p
9	Man	0.61	8	$m/e\ 204 > m/e\ 217$	$\delta = 4.38\ \text{p.p.m. (0.8 Hz)}\S$	β -p	β -p
10	Man	0.64	2	$m/e\ 204 \ll m/e\ 217$; $m/e\ 205$	Not determined	—	f
8	Gal	0.58	22	$m/e\ 204 \ll m/e\ 217$; $m/e\ 205$	$\delta = 4.64\ \text{p.p.m. (2.6 Hz)}$	—	β -f
10‡	Gal	0.64	54	$m/e\ 204 > m/e\ 217$	$\delta = 4.55\ \text{p.p.m. (2.2 Hz)}$	α -p	α -p
	Gal	0.64		$m/e\ 204 \ll m/e\ 217$; $m/e\ 205$	$\delta = 4.67\ \text{p.p.m. (4.3 Hz)}$	—	α -f
11	Gal	0.72	24	$m/e\ 204 > m/e\ 217$	$\delta = 4.09\ \text{p.p.m. (7.3 Hz)}$	β -p	β -p
8	Glc	0.58	2	$m/e\ 204 \ll m/e\ 217$; $m/e\ 205$	Not determined	—	f
12	Glc	0.77	72	$m/e\ 204 > m/e\ 217$	$\delta = 4.63\ \text{p.p.m. (3.2 Hz)}$	α -p	α -p
13	Glc	0.83	26	$m/e\ 204 > m/e\ 217$	$\delta = 4.14\ \text{p.p.m. (7.1 Hz)}$	β -p	β -p
14	GlcNAc	1.09	6	$m/e\ 173 \ll m/e\ 186$; $m/e\ 205$	$\delta = 4.97\ \text{p.p.m. (5.0 Hz)}$	—	α -f
16	GlcNAc	1.23	13	$m/e\ 173 > m/e\ 186$	$\delta = 4.42\ \text{p.p.m. (8.0 Hz)}$	β -p	β -p
18	GlcNAc	1.37	77	$m/e\ 173 > m/e\ 186$	$\delta = 4.50\ \text{p.p.m. (3.5 Hz)}$	α -p	α -p
19¶	GlcNAc	1.47	4	$m/e\ 173 > m/e\ 186$	Not determined	Free α, β -p	Free p
15**	GalNAc	1.12	25	$m/e\ 173 \ll m/e\ 186$; $m/e\ 205$	$\delta = 4.62\ \text{p.p.m. (4.5 Hz)}$; $4.66\ \text{p.p.m. (1.5 Hz)}$	—	α, β -f
17	GalNAc	1.28	72	$m/e\ 173 > m/e\ 186$	$\delta = 4.50\ \text{p.p.m. (3.5 Hz)}$	α -p	α -p
	GalNAc	1.28		$m/e\ 173 > m/e\ 186$	$\delta = 4.50\ \text{p.p.m. (7.8 Hz)}$	β -p	β -p
18††	GalNAc	1.37	3	$m/e\ 173 > m/e\ 186$	Not determined	Free α, β -p	Free p
20	AcNeu	2.34	4	—	—	α -p	α -p
21	AcNeu	2.46	92	—	—	β -p	β -p
22	AcNeu	2.68	4	—	—	9- <i>O</i> Ac- β -p	9- <i>O</i> Ac- β -p

* The doublet appears as a higher-order pattern.

† The doublet appears as a higher-order pattern; in $[\text{D}_6]\text{benzene}$ a normal doublet with $J_{1,2} = 3.0\ \text{Hz}$.

‡ Mass spectrometry of the mixture is $m/e\ 204 > m/e\ 217$.

§ See for reference the $J_{1,2}$ values of Streefkerk *et al.* (1973) and of Haverkamp *et al.* (1975).

|| Because of the very small amounts, ^1H -n.m.r. spectroscopy could not be applied. Mostly these peaks do not interfere with the quantitative determination of galactose.

¶ Owing to the small amount no ^1H -n.m.r. spectroscopy could be performed. On 3.8% SE-30 the α - as well as the β -pyranose forms have the same retention time. However, the two anomers can be separated on 3% OV-17.

** The furanose forms could not be separated by t.l.c. in the applied solvent system.

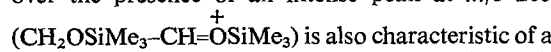
†† Owing to the small amount, ^1H -n.m.r. spectroscopy could not be applied. On 3.8% SE-30 the α - as well as the β -pyranose forms have the same retention time. However, the two anomers can be separated on 3% OV-17. Mostly this peak will not interfere with the quantitative analysis of *N*-acetylglucosamine. It has to be noted, that in the case of the large-scale preparations sometimes this peak is present in a somewhat higher concentration (up to 8% of the total amount of the methyl glycosides of *N*-acetylgalactosamine).

subsequent re-*N*-acetylation was fractionated by t.l.c. Each fraction was analysed by g.l.c., g.l.c.-mass spectroscopy and p.m.r. The combined results are summarized in Table 1.

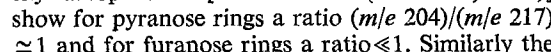
For the interpretation of the mass spectra and ^1H -n.m.r. spectra of the neutral and acetamido sugars, the following basic data were used.

(a) Mass spectrometry

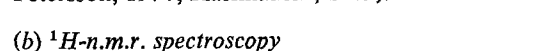
By mass spectroscopy it is possible to discriminate between pyranose and furanose ring forms. For pertrimethylsilyl aldohexoses it has been found that the ratio of the intensities of the peaks at m/e 204



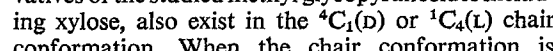
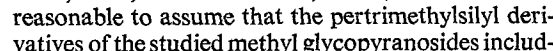
and at m/e 217



is useful (DeJongh *et al.*, 1969; Petersson, 1974; Vink *et al.*, 1972). Pyranose rings give rise to a ratio $(m/e\ 204)/(m/e\ 217) > 1$, whereas furanose rings show a ratio ≤ 1 . Moreover the presence of an intense peak at m/e 205



is also characteristic of a furanose ring. The mass spectra of the pertrimethylsilyl aldopentoses [i.e. arabinose (Petersson, 1974)] show for pyranose rings a ratio $(m/e\ 204)/(m/e\ 217) \approx 1$ and for furanose rings a ratio ≤ 1 . Similarly the ratio of the intensities of the peaks at m/e 173



can be used for the characterization of the ring forms of the pertrimethylsilyl 2-acetamido-2-deoxyaldohexoses (Kärkkäinen, 1969; Vink *et al.*, 1974). A ratio $(m/e\ 173)/(m/e\ 186) > 1$ points to a pyranose ring and a ratio ≤ 1 to a furanose ring. In the last case an intense peak at m/e 205 is also present. From the literature it can be concluded that the same reasoning holds for pertrimethylsilyl methyl glycosides (DeJongh *et al.*, 1969; Petersson, 1974; Kärkkäinen, 1969).

(b) ^1H -n.m.r. spectroscopy

In many cases it is possible to differentiate between α and β forms of pyranose and of furanose rings by p.m.r. For the pertrimethylsilyl derivatives of mannose, galactose, glucose, *N*-acetylglucosamine and *N*-acetylgalactosamine (Streefkerk *et al.*, 1973, 1974a) and the permethyl derivatives of mannose, galactose and glucose (Haverkamp *et al.*, 1975), it has been demonstrated that the pyranose rings exist in the $^4\text{C}_1(\text{D})$ chair conformation. The derivatives of fucose occur in the $^1\text{C}_4(\text{L})$ chair conformation (Haverkamp *et al.*, 1975; Streefkerk *et al.*, 1974b). Therefore it is reasonable to assume that the pertrimethylsilyl derivatives of the studied methyl glycopyranosides including xylose, also exist in the $^4\text{C}_1(\text{D})$ or $^1\text{C}_4(\text{L})$ chair conformation. When the chair conformation is known, the values of the coupling constants $J_{1,2}$ can be interpreted in terms of α and β configurations. Equatorial-axial, axial-equatorial and di-equatorial

couplings between H_1 and H_2 give rise to $J_{1,2}$ values of 0–4 Hz, whereas di-axial couplings have $J_{1,2}$ values of 6–8 Hz. In furanose rings, $J_{1,2}$ can theoretically vary between 3.5 and 8 Hz for *cis* H_1 – H_2 couplings (dihedral angle 0° – 45°) and between 0 and 8 Hz for *trans* H_1 – H_2 couplings (dihedral angle 75° – 165°) (Lemieux & Lineback, 1963). However, in our studies on pertrimethylsilyl and permethyl derivatives of galactose and glucose it has been shown that *trans*-couplings give rise to $J_{1,2}$ values of 0–3.5 Hz and *cis*-couplings to $J_{1,2}$ values of 4.0–5.5 Hz (J. Haverkamp & D. G. Streefkerk, unpublished work; Vilkas *et al.*, 1973). Therefore, for furanose rings the $J_{1,2}$ values are also characteristic for the α and β configurations.

The signals of the anomeric protons of the pertrimethylsilyl methyl glycosides have a higher chemical shift (δ -value) than the signals of the non-anomeric protons, although sometimes solvent effects disturb this pattern. It has to be noted that for any interpretation of the $J_{1,2}$ values the ring forms (pyranose or furanose) have to be known (the latter information can be obtained by mass spectroscopy).

The gas chromatographic peaks of *N*-acetylneuraminic acid were investigated by g.l.c. and g.l.c.-mass spectroscopy. The mass spectral data of these peaks have been compared with those published by Kamerling *et al.* (1975). Obviously the anomeric configuration cannot be determined by ^1H -n.m.r. spectroscopy. In ^{13}C -n.m.r. spectroscopy the methyl esters of the α - and β -methyl glycosides of *N*-acetylneuraminic acid showed nearly identical chemical shifts for the anomeric carbon atoms (J. P. Kamerling, unpublished work). However, the identity of the peaks could be deduced from co-chromatography with synthetic products.

Sometimes we observed that the internal standard mannitol shows, apart from the main peak, a very small additional peak with a higher retention time; its mass spectrum is in accordance with the presence of one *O*-acetyl group attached to one of the primary hydroxyl functions. A similar phenomenon has been found in the case of *N*-acetylneuraminic acid. These *O*-acetylated derivatives are formed during the re-*N*-acetylation step. For the qualitative and quantitative determination of the carbohydrate composition of glycoproteins and glycopeptides, the g.l.c. method is frequently applied. In the method described in the present paper, the monosaccharide constituents of glycoproteins are released and converted into methyl glycosides by methanolysis and made suitable for g.l.c. by re-*N*-acetylation and trimethylsilylation. The pertrimethylsilyl methyl glycosides give rise to multiple peaks in the gas chromatogram owing to the various anomeric pyranose and furanose forms. So far, the assignment of the peaks has been based solely on co-chromatography. This study describes for each of the commonly occurring monosaccharides

in glycoproteins the precise anomeric form that contributes to the intensity of a peak in the gas chromatogram. This identification by means of instrumental analysis furnishes a valuable addition to the g.l.c. method, since chromatography is in principle a separation method.

We thank Mr. C. Versluis (Laboratory of Analytical Chemistry, University of Utrecht, The Netherlands), Miss T. Volp (Organic Chemical Institute T.N.O., Utrecht, The Netherlands) and Dr. E. Talman and Mr. J. L. Hoogendoorn (T.N.O. Central Laboratories, Delft, The Netherlands) for recording the mass spectra, the 100 and the 220 MHz spectra respectively. This investigation was supported 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.), and by the award of a N.A.T.O. grant (J.R.C.).

References

- Chambers, R. E. & Clamp, J. R. (1971) *Biochem. J.* **125**, 1009–1018
- Clamp, J. R. (1974) *Biochem. Soc. Symp.* **40**, 3–16
- Clamp, J. R., Bhatti, T. & Chambers, R. E. (1971) *Methods Biochem. Anal.* **19**, 229–344
- Clamp, J. R., Bhatti, T. & Chambers, R. E. (1972) in *Glycoproteins* (Gottschalk, A., ed.), 2nd edn., pp. 300–321, Elsevier, Amsterdam
- DeJongh, D. C., Radford, T., Hribar, J. D., Hanessian, S., Bieber, M., Dawson, G. & Sweeley, C. C. (1969) *J. Am. Chem. Soc.* **91**, 1728–1740
- Haverkamp, J., de Bie, M. J. A. & Vliegthart, J. F. G. (1975) *Carbohydr. Res.* **39**, 201–211
- Kamerling, J. P., Vliegthart, J. F. G., Vink, J. & de Ridder, J. J. (1971) *Tetrahedron* **27**, 4275–4288
- Kamerling, J. P., Vliegthart, J. F. G. & Vink, J. (1974) *Carbohydr. Res.* **33**, 297–306
- Kamerling, J. P., Vliegthart, J. F. G., Versluis, C. & Schauer, R. (1975) *Carbohydr. Res.* **41**, 7–17
- Kärkkäinen, J. (1969) *Carbohydr. Res.* **10**, 113–120
- Kennedy, J. F. (1974) *Biochem. Soc. Trans.* **2**, 56–64
- Lemieux, R. U. & Lineback, D. R. (1963) *Annu. Rev. Biochem.* **32**, 155–184
- Marshall, R. D. & Neuberger, A. (1972) in *Glycoproteins* (Gottschalk, A., ed.), 2nd edn., pp. 224–299, Elsevier, Amsterdam
- Nozawa, Y., Uesaka, H., Suzuki, H. & Ito, Y. (1969) *J. Chromatogr.* **43**, 528–530
- Petersson, G. (1974) Thesis Chalmers Tekniska Högskola, Göteborg
- Streefkerk, D. G., de Bie, M. J. A. & Vliegthart, J. F. G. (1973) *Tetrahedron* **29**, 833–844
- Streefkerk, D. G., de Bie, M. J. A. & Vliegthart, J. F. G. (1974a) *Carbohydr. Res.* **33**, 339–349
- Streefkerk, D. G., de Bie, M. J. A. & Vliegthart, J. F. G. (1974b) *Carbohydr. Res.* **38**, 47–59
- Vilkas, E., Amar, C., Markovits, J., Vliegthart, J. F. G. & Kamerling, J. P. (1973) *Biochim. Biophys. Acta* **297**, 423–435
- Vink, J., Bruins Slot, J. H. W., de Ridder, J. J., Kamerling, J. P. & Vliegthart, J. F. G. (1972) *J. Am. Chem. Soc.* **94**, 2542–2544
- Vink, J., Heerma, W., Kamerling, J. P. & Vliegthart, J. F. G. (1974) *Org. Mass. Spectrom.* **9**, 536–547
- Yu, R. K. & Ledeen, R. (1969) *J. Biol. Chem.* **244**, 1306–1313