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A 360-MHz ^{1}H-NMR STUDY OF THREE OLIGOSACCHARIDES ISOLATED FROM COW κ-CASEIN *

H. VAN HALBEEK [a], L. DORLAND [a], J.F.G. VLIEGENTHART [a], A.-M. FIAT [b] and P. JOLLES [b]

[a] *Department of Bio-Organic Chemistry, State University of Utrecht, Croesestraat 79, 3522 AD Utrecht (The Netherlands) and* [b] *Laboratoire des Protéines, Université de Paris V, 45 rue des Saints-Pères, 75270 Paris Cedex 06 (France)*

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Summary

360-MHz ^{1}H-NMR spectra were recorded of NeuAcα(2 → 3)Galβ (1 → 3)GalNAc-ol (I), Galβ(1 → 3)[NeuAcα(2 → 6)]GalNAc-ol (II) and NeuAcα(2 → 3)-Galβ(1 → 3) [NeuAcα(2 → 6)]GalNAc-ol (III). The chemical shifts and coupling constants of the anomeric protons, the H-3ax and H-3eq of NeuAc, the GalNAc-ol skeleton protons, the H-3 of Gal and the *N*-acetyl protons of GalNAc-ol and NeuAc provide conclusive evidence for the identification of the primary structures. Compound II represents a novel carbohydrate chain of κ-casein.

Introduction

360-MHz ^{1}H-NMR spectroscopy has proved to be a highly powerful technique for the structure determination of the carbohydrate chains of glycoproteins. So far we investigated mainly oligosaccharides and glycopeptides related to glycan structures of the N-glycosidic type [1—8]. In the field of O-glycosidically linked chains the preparation of glycopeptides containing only a few amino acids is rather difficult. Very often structure determination of the carbohydrate portion(s) of this type of glycoprotein occurs at the level of reduced oligosaccharides released from these by means of alkaline borohydride reductive cleavage. This paper deals with the structure elucidation of three oligosaccharide alditols, (I), (II) and (III), isolated from cow κ-caseinoglycopeptide.

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Abbreviations: GalNAc-ol, 2-acetamido-2-deoxy-D-galactitol; Gal, D-galactose; NeuAc, *N*-acetylneuraminic acid.

For this series of compounds the NMR-method provides independent evidence for the primary structures, thereby confirming the structures of compounds I and III, obtained via methylation analysis [9].

Materials and Methods

Preparation of bovine κ-caseinoglycopeptide

Cow κ-casein was prepared according to McKenzie and Wake [10]. The κ-caseinoglycopeptide soluble in 12% trichloroacetic acid was obtained after chymosin digestion of cow κ-casein according to Jollès et al. [11].

Preparation of the sugar portions

The caseinoglycopeptide was treated with alkaline borohydride (0.05 M NaOH and 1.0 M $NaBH_4$) at 50°C under nitrogen in the dark according to Carlson [12]. After desalting on Dowex 50-X4 (H^+) and washing with methanol, the sugar moieties were isolated by filtration on Biogel P4 (250 × 1.2 cm) with water as eluent. For the purification of the sugar moieties preparative descending paper chromatograms (Whatman No. 1) were run for 24 h in the solvent ethyl acetate/pyridine/water/acetic acid (5 : 5 : 3 : 1, v/v). The sugars were detected with the periodate-benzidine reagent [13]. Three oligosaccharide-alditols were isolated: (I), R_{Gal} 0.43; (II), R_{Gal} 0.38; and (III), R_{Gal} 0.30; they were analyzed as previously described [9]. Compounds I and II were trisaccharide-alditols containing GalNAc-ol, Gal and NeuAc; compound III was a reduced tetrasaccharide containing GalNAc-ol, Gal and two residues of NeuAc.

360 MHz ^{1}H-NMR spectroscopy

For ^{1}H-NMR spectroscopic analysis the neutralized oligosaccharide-alditols were repeatedly treated with ^2H_2O (100.0% ^{2}H, Aldrich, Milwaukee, U.S.A.) at room temperature and intermediate lyophilization. The 360-MHz ^{1}H-NMR spectra were recorded on a Bruker HX-360 spectrometer, operating in the Fourier Transform mode at a probe temperature of 25°C. Chemical shifts are given relative to sodium-2,2-dimethyl-2-silapentane-5-sulphonate (indirectly to acetone in ^2H_2O: $\delta = 2.225$ ppm) with an accuracy of 0.003 ppm. GalNAc-ol (A) was prepared by reduction of GalNAc (Baker) by $NaBH_4$. The model compound Galβ(1 → 3)GalNAc-ol (B) was isolated from hog submaxillary gland mucin glycoproteins by Dr. D. Aminoff (Ann Arbor, U.S.A.) [14]. The reference substance NeuAcα(2 → 6)GalNAc-ol (C) was isolated from gangliosides by Dr. R. Veh (Bochum, F.R.G.).

Results

360-MHz ^{1}H-NMR spectra were recorded of the three major oligosaccharide-alditols I, II and III, obtained from cow κ-caseinoglycopeptide after alkaline borohydride reductive cleavage. The interpretation of these spectra in terms of structural assignments was carried out based on spectra of appropriate reference compounds A, B and C. The chemical shifts of the anomeric protons, the protons of GalNAc-ol, the H-3 protons of NeuAc residues and the N-acetyl protons are compiled in Table I. The assignment of all signals in the spectrum of

TABLE I

^{1}H-NMR CHEMICAL SHIFTS OF CHARACTERISTIC PROTONS OF CONSTITUENT MONOSACCHARIDES FOR OLIGOSACCHARIDE ALDITOLS I, II AND III AND REFERENCE COMPOUNDS A, B AND C

Residue	Proton	Chemical shift (ppm) in					
		GalNAc-ol (A)	Galβ(1 → 3)-GalNAc-ol (B)	NeuAcα(2 → 6)-GalNAc-ol (C)	NeuAcα(2 → 3)-Galβ(1 → 3)-GalNAc-ol (I)	Galβ(1 → 3)-[NeuAcα(2 → 6)]-GalNAc-ol (II)	NeuAcα(2 → 3)-Galβ(1 → 3)-[NeuAcα(2 → 6)]-GalNAc-ol (III)
GalNAc-ol	H_1	3.740	3.81	3.73	3.7—3.8	3.7—3.8	3.75—3.80
	H_1	3.683	3.73	3.67	3.7—3.8	3.7—3.8	3.75—3.80
	H_2	4.246	4.396	4.248	4.392	4.384	4.382
	H_3	3.850	4.062	3.839	4.077	4.059	4.070
	H_4	3.389	3.506	3.410	3.494	3.531	3.517
	H_5	3.930	4.198	4.020	4.191	4.245	4.246
	H_6	3.671	3.69	3.839	3.65—3.70	3.85	3.84
	H_6'	3.651	3.65	3.528	3.65—3.70	3.481	3.470
	NAc	2.055	2.050	2.055	2.047	2.048	2.041
Gal	H_1	—	4.475	—	4.546	4.474	4.542
	H_2	—	3.564	—	3.57—3.62	3.57	3.634
	H_3	—	3.754	—	4.123	3.76	4.120
NeuAcα(2 → 3)	H_{3ax}	—	—	—	1.802	—	1.805
	H_{3eq}	—	—	—	2.770	—	2.772
	NAc	—	—	—	2.032	—	2.033
NeuAcα(2 → 6)	H_{3ax}	—	—	1.702	—	1.695	1.696
	H_{3eq}	—	—	2.727	—	2.728	2.723
	NAc	—	—	2.036	—	2.034	2.033

GalNAc-ol (A) was carried out straightforwardly; the chemical shifts and coupling constants were refined by computer simulation of the spectrum. The spectrum of the disaccharide-alditol Galβ(1 → 3)GalNAc-ol (B) shows, in comparison to that of GalNAc-ol, a relatively large shift increment for the H-3 of GalNAc-ol, which is useful for the characterization of the (1 → 3) linkage between Gal and GalNAc-ol. The coupling constant $J_{1,2}$ (7.3 Hz) of Gal is indicative of a β-glycosidic bond. The chemical shifts of the GalNAc-ol protons in the trisaccharide (I) are almost identical to those of the disaccharide (B), showing that the substitution of GalNAc-ol is identical in both compounds. The set of chemical shift values of the H-3 protons of NeuAc (δH-3ax = 1.802 ppm, δH-3eq = 2.770 ppm) is indicative of an α-type of linkage of NeuAc to C-3 of a Gal residue (see Ref. 1). Further, the chemical shift increments of H-1 and H-3 of Gal with regard to compound B are characteristic for the NeuAcα(2 → 3)Galβ-(1 → ···) sequence [1], while the coupling constant $J_{1,2}$ (7.9 Hz) of Gal shows the β-type of linkage between Gal and GalNAc-ol. From the foregoing it is clear that oligosaccharide (I) can be conceived as an extension of (B) with an α(2 → 3) linked NeuAc residue.

In the NMR-spectrum of NeuAcα(2 → 6)GalNAc-ol (C) the chemical shift value of the H-3eq proton of NeuAc (δ = 2.727 ppm) is indicative of an α-type of linkage [15]. The oppositely directed changes in the chemical shifts of H-6 and H-6′ of GalNAc-ol and the change in the geminal coupling constant, $J_{6,6'}$

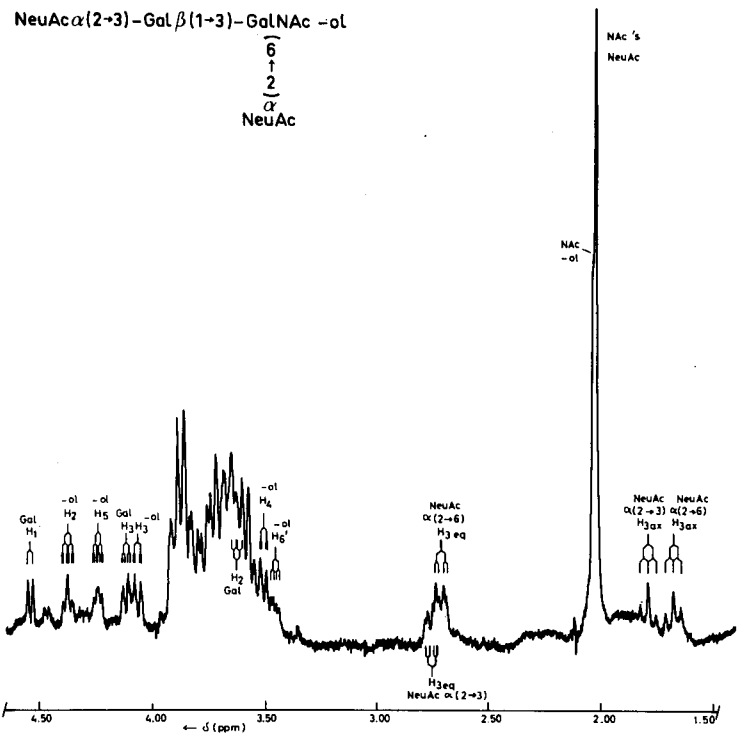

Fig. 1. 360-MHz ^{1}H-NMR spectrum of NeuAcα(2 → 3)Galβ(1 → 3)[NeuAcα(2 → 6)]GalNAc-ol (compound III) in ^2H_2O at 25° C and p^{2}H = 7.

from −11.7 Hz in GalNAc-ol to −10.0 Hz in the disaccharide (C) are characteristic for the attachment of NeuAc to C-6 of GalNAc-ol (see Ref. 16). The aforementioned shift increments from (A) to (C) are also observed in the step from (B) to (II). Consequently, the trisaccharide-alditol (II) differs from the disaccharide (B) only in the attachment of NeuAc, linked $\alpha(2 \rightarrow 6)$ to GalNAc-ol. The structure of this novel compound is corroborated by comparison of the spectra of (C) and (II), which shows that the extension with Gal affects mainly the chemical shift of H-3 of GalNAc-ol, exactly as described for the step from (A) to (B). These data prove the structure of oligosaccharide (II) to be: Galβ-(1 → 3)[NeuAcα(2 → 6)]GalNAc-ol.

The 360-MHz ^{1}H-NMR spectrum of the bisialo-oligosaccharide (III) is given in Fig. 1. The typical NMR parameters of compounds I and II can be recognized in this spectrum. The set of chemical shift values of the H-3 protons of a NeuAc-residue (δH-3ax = 1.805 ppm; δH-3eq = 2.772 ppm), together with the chemical shifts of H-1 and H-3 of Gal show that, as in compound I, a NeuAc residue is $\alpha(2 \rightarrow 3)$ linked to Gal. The presence of the other NeuAc residue $\alpha(2 \rightarrow 6)$ linked to GalNAc-ol can be derived from the second set of H-3 protons of a NeuAc residue (δH-3ax = 1.696 ppm; δH-3eq = 2.723 ppm), in combination with the chemical shifts of H-6 and H-6′, and the value of $J_{6,6'}$ (−9.8 Hz) of GalNAc-ol. The latter data are in agreement with those of compound II. These results confirm the structure of the tetrasaccharide-alditol (III) to be as follows: NeuAc$\alpha(2 \rightarrow 3)$Gal$\beta(1 \rightarrow 3)$[NeuAc$\alpha(2 \rightarrow 6)$] GalNAc-ol.

Discussion

This study shows that high-resolution ^{1}H-NMR spectroscopy is also suited for the unambiguous determination of the primary structure of oligosaccharide alditols, related to O-glycosidically linked carbohydrate chains of glycoproteins of the mucin type. As for the N-glycosidically linked chains, the anomeric protons and some specific non-anomeric protons can be used as structural reporter groups to define the sequence as well as type and configuration of the glycosidic linkages of the constituting monomers, provided that at least the carbohydrate composition is known.

As could be expected on the basis of our earlier observations on the ^{1}H-NMR spectra of oligosaccharides containing sialic acid [1], we observed that the occurrence of NeuAc$\alpha(2 \rightarrow 6)$ linked to GalNAc-ol gives rise to a unique set of chemical shift values of the H-3 protons. The δH-3ax is sensitive for the substitution at position C-3 of the alditol. Comparison of the data of the compounds C and II shows a shift decrement of 0.007 ppm upon attachment of Gal in β-linkage. Similarly, the set of chemical shift values of the H-3 protons of NeuAc in the sequence NeuAc$\alpha(2 \rightarrow 3)$Gal$\beta(1 \rightarrow 3)$GalNAc-ol is also unique. The occurrence of GalNAc-ol influences specifically the chemical shift of H-3eq, since its value is different if GlcNAc or Glc are present instead of GalNAc-ol [1,7]. The resonances of the N-acetyl protons of amino sugars are, in general, highly informative with regard to the substitution pattern of these constituents [5]. This can be illustrated for the signal of the NAc group of GalNAc-ol, which starts with a chemical shift of δ = 2.055 ppm for GalNAc-ol (A) itself and ends at δ = 2.041 ppm for the tetrasaccharide-alditol (III). Intermedi-

ate values are found for the other compounds. It is interesting to note that compound II represents a novel carbohydrate chain of κ-casein. Apparently different partial structures of compound III occur in this glycoprotein. Their metabolic significance still remains to be established.

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