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A 500-MHz ^{1}H-NMR Study on the *N*-Linked Carbohydrate Chain of Bromelain

^{1}H-NMR Structural-reporter-groups of Fucose α(1-3)-Linked to Asparagine-bound *N*-Acetylglucosamine

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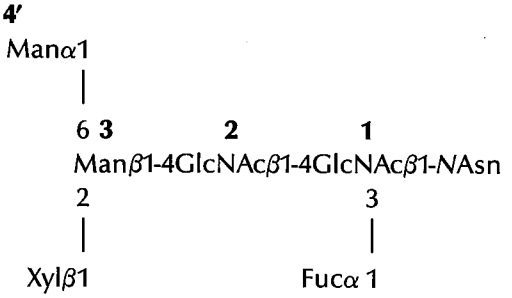
The 500-MHz ^{1}H-NMR characteristics of the *N*-linked carbohydrate chain Manα1-6[Xylβ1-2]Manβ1-4GlcNAcβ1-4[Fucα1-3]GlcNAcβ1-*N*Asn of the proteolytic enzyme bromelain (EC 3.4.22.4) from pineapple stem were determined for the oligosaccharide-alditol and the glycopeptide, obtained by hydrazinolysis and Pronase digestion, respectively. The ^{1}H-NMR structural-reporter-groups of the α(1-3)-linked fucose residue form unique sets of data for the alditol as well as for the glycopeptide.

The structural characterization of the *N*-linked carbohydrate chain(s) of the proteolytic enzyme bromelain (EC 3.4.22.4) from pineapple stem has been the subject of several investigations [1-11]. Finally, Ishihara *et al.* [11] reported the structure



in which all the sugars have the D-configuration except fucose. This structure can be extended with an additional mannose, α(1-6)-linked to Man-**4'**. In view of the unusual

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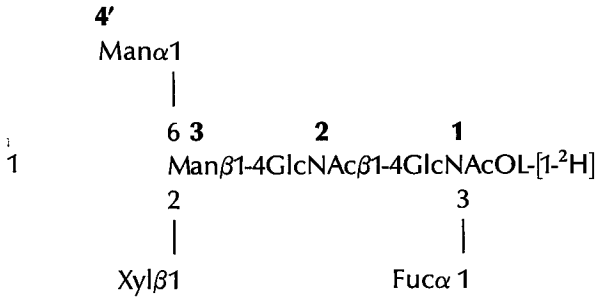
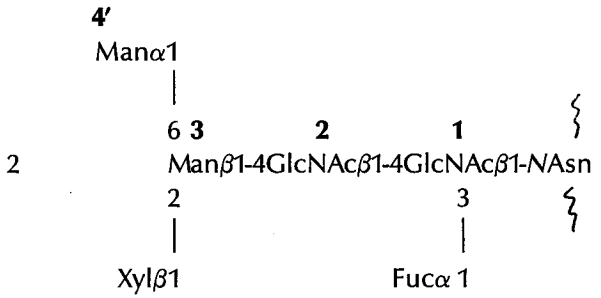
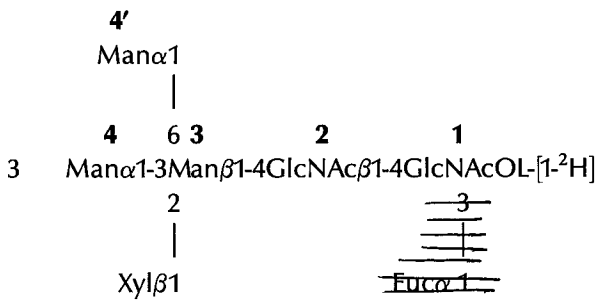

4'
Manα1
6 3
2
1
Manβ1-4GlcNAcβ1-4GlcNAcOL-[1-2H]
2
3
Xylβ1
Fucα 1

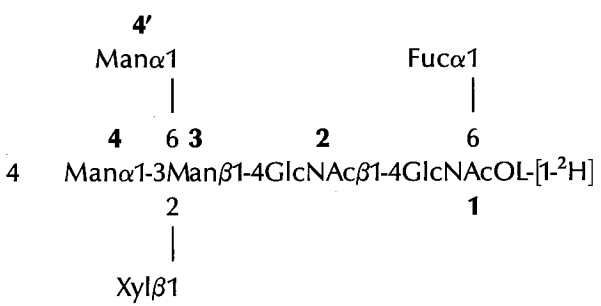
1


4'
Manα1
6 3
2
1
Manβ1-4GlcNAcβ1-4GlcNAcβ1-NAsn
2
3
Xylβ1
Fucα 1

2


4'
Manα1
4
6 3
2
1
Manα1-3Manβ1-4GlcNAcβ1-4GlcNAcOL-[1-2H]
2
3
Xylβ1
Fucα 1

3 [14]


4'
Manα1
Fucα1
4
6 3
2
6
Manα1-3Manβ1-4GlcNAcβ1-4GlcNAcOL-[1-2H]
2
1
Xylβ1

4 [14]

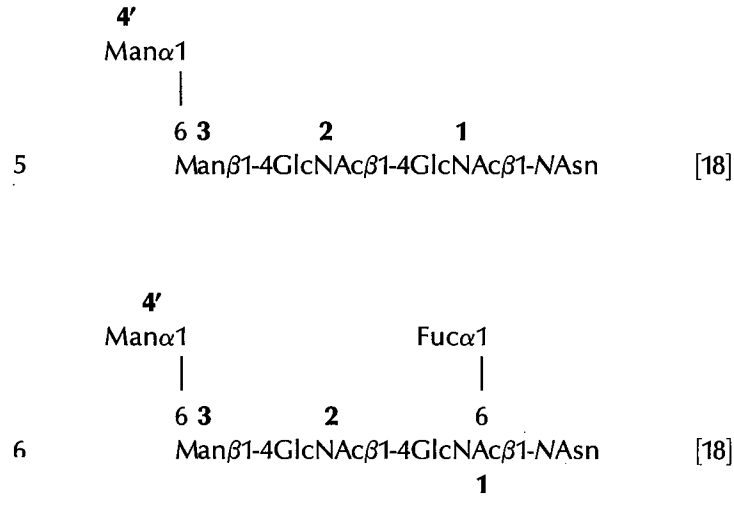


Figure 1. Carbohydrate structures discussed in this study.

features of the structure, namely, the occurrence of xylose β(1-2)-linked to the β-mannose, and in particular, the presence of fucose α(1-3)-linked to the asparagine-bound *N*-acetylglucosamine, the isolation of substantial amounts of this carbohydrate chain as an oligosaccharide-alditol and as a glycopeptide was carried out in order to establish the ^{1}H-NMR chemical shift values of structural-reporter-groups typical for these compounds.

Materials and Methods

Commercially available bromelain preparations (Boehringer, Mannheim, W. Germany; suspension in ammonium sulfate solution; Sigma Chemical Co., St Louis, MO, USA; approx. 50% protein) were purified using Sephadex G-100 gel filtration and SE- or SP-Sephadex C-50 cation-exchange chromatography [5].

For the preparation of neutral hexasaccharide-alditol-[1-^{2}H], compound 1 in Fig. 1, the thoroughly dried, purified (Boehringer) bromelain (200 mg) was subjected to the hydrazinolysis procedure, including high-voltage paper electrophoresis and Bio-Gel P-4 fractionation [12-14]. Sugar analysis [15] of compound 1 indicated Fuc:Xyl:Man:GlcNAc:GlcNAcOL in the molar proportions 0.7 : 0.9 : 2.2 : 1.0 : 0.5.

For the preparation of glycopeptide 2, 500 mg of the denatured purified glycoprotein material (Sigma) were subjected to exhaustive Pronase digestion [14]. After fractionation on Bio-Gel P-6 [14], the main glycopeptide fraction was lyophilized. Sugar analysis indicated Fuc:Xyl:Man:GlcNAc in the molar proportions 1.1 : 1.1 : 2.4 : 2.0 (the *N*-acetylglucosamine value has been corrected for non-cleaved GlcNAc-Asn [15]). Methylation analysis [16] gave rise to the partially methylated alditol acetates indicative for terminal

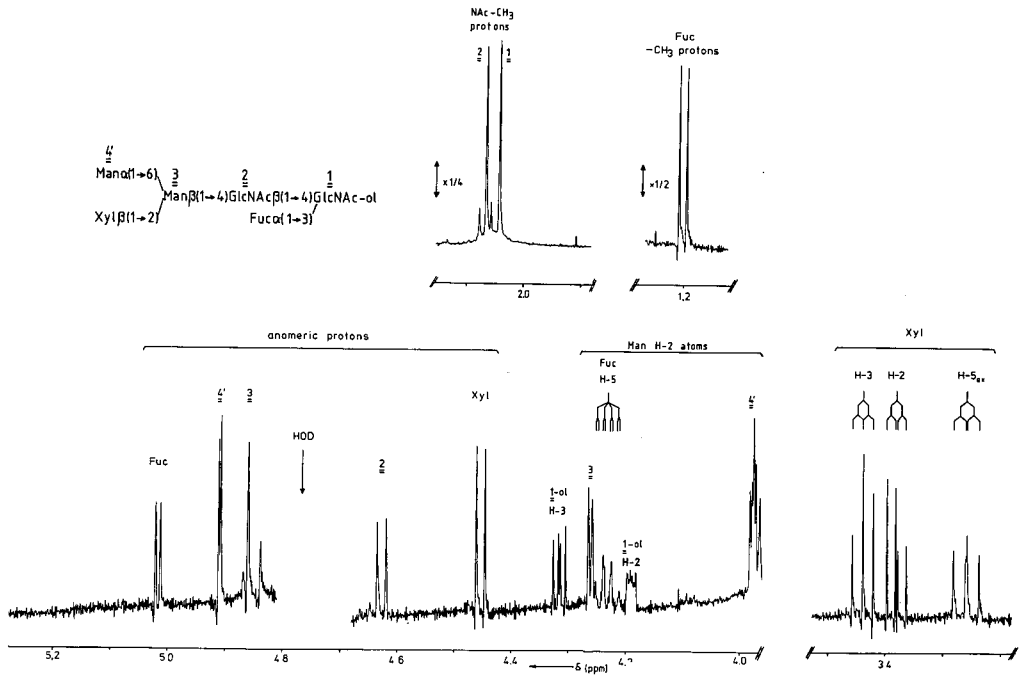


Figure 2. Structural-reporter-group regions of the 500-MHz ^{1}H-NMR spectrum (^2H_2O; p^{2}H 7; 27°C) of oligosaccharide-alditol-[1-^{2}H] obtained from bromelain. The numbers in the spectrum refer to the corresponding residues in the structure. The relative-intensity scale of the *N*-acetyl and Fuc-CH_3 proton regions differs from that for other parts of the spectrum, as indicated.

mannose, xylose and fucose; 2,6-substituted mannose; 4-substituted *N*-acetylglucosamine; and 3,4-substituted *N*-acetylglucosamine residues, in the molar ratios 1.1 : 1.1 : 1.2 : 1.0 : 1.0 : 0.5. Amino acid analysis showed Asp:Glu:Ser:Pro:Gly:GlcNAc in the molar proportions 2.0 : 1.9 : 0.9 : 1.1 : 0.7 : 2.0, which can accommodate the presence of the reported peptide sequence Asn-Asn(carbohydrate)-Glu-Ser [17] in the glycopeptide fraction. Additional amino acids were detected in molar ratios of less than 0.4. Further purifications were not carried out.

For ^{1}H-NMR analysis, the carbohydrate samples were repeatedly treated with ^2H_2O at room temperature, with intermediate lyophilization, finally using 99.96% ^2H_2O (Aldrich, Milwaukee, WI, USA). 500-MHz ^{1}H-NMR spectra were obtained using a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating in the Fourier transform mode at a probe temperature of 27°C [18]. Resolution-enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [19]. Chemical shifts (δ) are expressed in ppm downfield from the signal for internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), and measured by reference to internal acetone (δ 2.225).

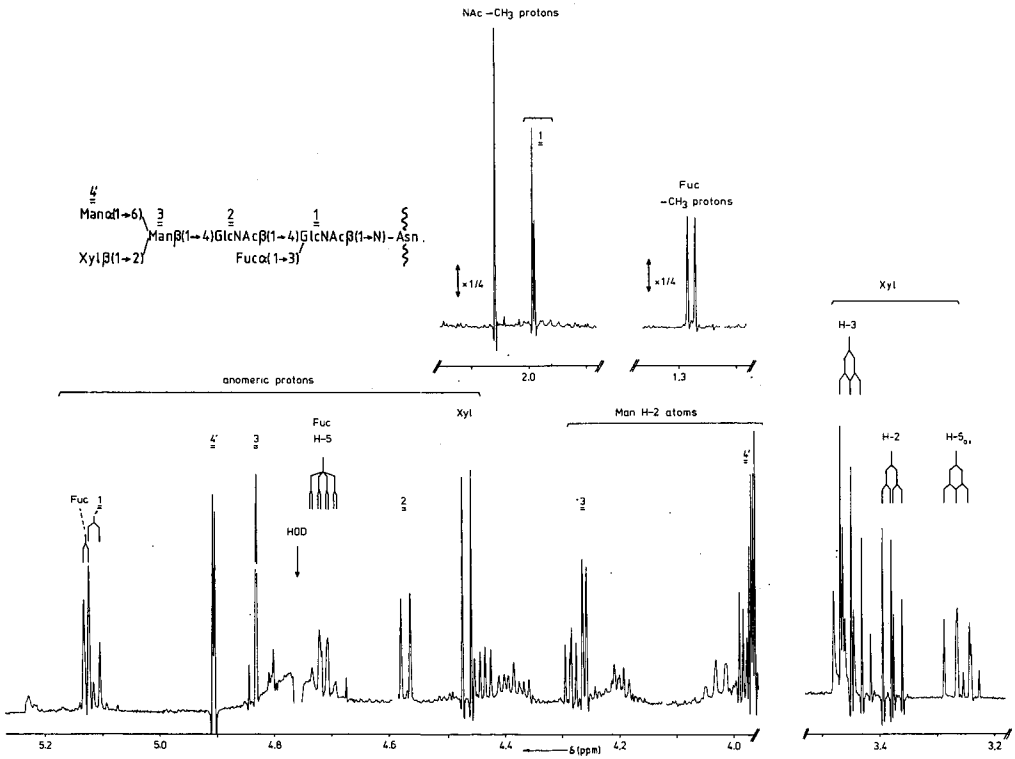


Figure 3. Structural-reporter-group regions of the 500-MHz ^{1}H-NMR spectrum (^2H_2O; p^{2}H 7; 27°C) of glycopeptide 2 obtained from bromelain. The numbers in the spectrum refer to the corresponding residues in the structure. The relative-intensity scale of the *N*-acetyl and Fuc-CH_3 proton regions differs from that for other parts of the spectrum, as indicated. For GlcNAc-**1** H-1 only the main signal has been indicated.

Results and Discussion

The sugar analysis and methylation analysis data of oligosaccharide-alditol 1 and glycopeptide 2 (see Fig. 1) indicated that only the reported hexasaccharide was present [11]. In none of the bromelain preparations was there any indication of the occurrence of a carbohydrate chain extended at Man-**4'** with an α(1-6)-linked mannose residue. Although not discussed here, we have evidence that the hydrazinolysis procedure as used for the preparation of compound 1 results in the removal of the (1-3)-linked fucose and degradation of the reducing terminal *N*-acetylglucosamine residue (see also [9]).

The 500-MHz ^{1}H-NMR spectra of compounds 1 and 2, recorded in ^2H_2O, are depicted in Figs. 2 and 3, respectively. Relevant NMR parameters of these compounds together with NMR data of the reference compounds 3-6 (see Fig. 1) [14, 18] are compiled in Table 1.

Table 1. Relevant ^{1}H-NMR characteristics of constituent monosaccharides for the oligosaccharide-alditol-[1-^{2}H] 1 and the glycopeptide 2 derived from bromelain, together with those of reference substances 3-6.

Residue[a]	Reporter group	Chemical shift (ppm)[b]					
							
		3	4	1	5	6	2
GlcNAc-1-(OL)	H-1				5.071	5.076	5.121[d]
	H-2	4.239	4.219	4.189	n.d.[c]	n.d.	n.d.
	H-3	n.d.	n.d.	4.315	n.d.	n.d.	n.d.
	NAc	2.057	2.058	2.043	2.014	2.013	2.000[d]
GlcNAc-**2**	H-1	4.634	4.718	4.625	4.618	4.690	4.579
	NAc	2.073	2.081	2.066	2.076	2.095	2.066
Man-**3**	H-1	4.883	4.884	4.859	4.767	4.770	4.839
	H-2	4.270	4.270	4.262	4.080	4.083	4.268
Man-**4**	H-1	5.122	5.124				
	H-2	4.039	4.040				
Man-**4'**	H-1	4.913	4.914	4.909	4.915	4.916	4.913
	H-2	3.983	3.982	3.976	3.968	3.967	3.988
Fuc^6	H-1		4.898			4.877	
	H-5		4.077			4.125	
	CH_3		1.225			1.209	
Fuc^3	H-1			5.017			5.136
	H-5			4.232			4.722
	CH_3			1.202			1.285
Xyl	H-1	4.449	4.449	4.453			4.474
	H-2	3.377	3.379	3.379			3.385
	H-3	3.437	3.453	3.439			3.456
	H-5ax	3.250	3.253	3.258			3.273

[a] For numbering of monosaccharide residues and complete structures, see Fig. 1. A superscript at the Fuc residue indicates to which position of the adjacent monosaccharide it is linked.

[b] Chemical shifts are given in ppm downfield from internal sodium 4, 4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O (27°C). Compounds are represented by shorthand symbolic notation [14,18]: ●, GlcNAc; ♦, Man; □, Fuc; ⊠, Xyl.

[c] n.d., not detected.

[d] Chemical shift values of the main glycopeptide(s).

Comparison of the structural-reporter-groups of oligosaccharide-alditol 1 (Fig. 1) with those of the reference alditols 3 and 4 leads to the following comments. The set of structural-reporter-group signals of xylose in compound 1, namely, H-1 (δ 4.453), H-2 (δ 3.379), H-3 (δ 3.439) and H-5ax (δ 3.258), show essentially the same chemical shift values

as those observed for compounds 3 and 4 (Table 1). Apparently, the presence of the mannose residue (Man-**4**) α(1-3)-linked to Man-**3** essentially does not influence the NMR parameters of the xylose. As was demonstrated earlier [14], the attachment of a xylose residue in the presence of both Man-**4'** and Man-**4** has a distinct influence on the position of the Man-**3** H-1 signal (Manα1-6[Manα1-3]Manβ1-4GlcNAcβ1-4GlcNAcOL, δ 4.78 [14]; compared to the values δ 4.883 and 4.884 for compounds 3 and 4, respectively). Compared with 3 and 4, the absence of Man-**4** in compound 1 causes an upfield chemical shift effect on Man-**3** H-1 of Δδ -0.024 ppm relative to the positions in 3 and 4 (compound 1, δ 4.859). Finally, the position of the Man-**4'** H-1 and H-2 signals, being typical for a terminal Manα(1-6) residue [18], are not influenced by the presence of Man-**4** (compare compound 1 with 3 and 4).

The structural-reporter-group signals of the fucose α(1-3)-linked to GlcNAcOL-**1** (H-1, δ 5.017; H-5, δ 4.232; CH_3, δ 1.202) differ drastically from those reported for fucose α(1-6)-linked to GlcNAcOL-**1** (H-1, δ 4.898; H-5, δ 4.077; CH_3, δ 1.225). The chemical shift effects on the chitobiitol unit caused by the α(1-3)- and α(1-6)-linked fucose residues are also different. This is most pronounced for the *N*-acetyl signals of GlcNAcOL-**1** and GlcNAc-**2**. The presence of α(1-3)-linked fucose in compound 1 leads to upfield shifts of both resonances, as compared with their positions in compound 3 (Δδ -0.014 and -0.007 ppm, respectively). In the case of the α(1-6)-linked fucose residue in compound 4 the *N*-acetyl signal of GlcNAcOL-**1** is hardly affected, whereas the *N*-acetyl signal of GLcNAc-**2** is shifted downfield 0.008 ppm, when compared to compound 3. The position of H-2 of GlcNAcOL-**1** is influenced much more in compound 1 than in 4. Compared with compound 3, in 1 an upfield shift of 0.050 ppm is observed. In the latter case also GlcNAcOL-**1** H-3 resonates away from the bulk of skeleton protons. The assignment of this signal was made by selective irradiation of GlcNAcOL-**1** H-2.

Comparison of the structural-reporter-groups of glycopeptide 2 (Fig. 3) with those of the glycopeptides 5 and 6 shows the following features. The set of structural-reporter-group signals of α(1-3)-linked fucose in 2, namely, H-1 (δ 5.136), H-5 (δ 4.722) and CH_3 (δ 1.285) differ enormously, when compared to the set observed for the α(1-6)-linked fucose in 6 (see Table 1). It has to be noted that due to the heterogeneity in the peptide backbone, the GlcNAc-**1** H-1 and NAc signals show heterogeneity. The rather downfield position of the GlcNAc-**1** H-1 signal for glycopeptide 2 at δ 5.121 cannot be attributed merely to the influence of the type of fucose linkage, because the peptide moiety can considerably influence this chemical shift value [18]. The attachment of fucose at C-3 instead of C-6 of GlcNAc-**1** has also a clear effect on the δ-values of GlcNAc-**2** H-1 and NAc. Compared to compound 5, these values are shifted upfield (Δδ -0.039 and -0.010 ppm, respectively) when fucose is (1-3)-linked to GlcNAc-**1**, while they are found at downfield positions for (1-6)-linked fucose (Δδ 0.072 and 0.019 ppm, respectively).

The structural-reporter-groups of xylose (H-1, H-2, H-3, H-5ax) are found at more downfield positions for glycopeptide 2, compared to oligosaccharide-alditol 1. The chemical shift value of Xyl H-3 was assigned by selective irradiation of H-2. The sensitivity of the Man-**3** H-1 and H-2 structural-reporter-groups to the attachment of xylose through a β(1-2)-linkage mentioned above was also found in glycopeptide 2. These signals (δ 4.839 and 4.268, respectively) are observed at rather downfield positions when compared with those in glycopeptides 5 and 6.

When comparing oligosaccharide-alditol 1 with glycopeptide 2, it appears that in 2 the chemical shift values of the structural-reporter-groups of fucose occur at well-

pronounced more downfield positions: H-1, $\Delta\delta$ 0.119 ppm; H-5, $\Delta\delta$ 0.490 ppm; and CH_3, $\Delta\delta$ 0.083 ppm. It is evident that the alditol chain (GlcNAcOL-**1**) and the ring structure (GlcNAc-**1**) influence the NMR parameters of fucose quite differently.

Acknowledgements

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