

# Sulfated *N*-linked carbohydrate chains in porcine thyroglobulin

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*N*-linked carbohydrate chains of porcine thyroglobulin were released by the hydrazinolysis procedure. The resulting mixture of oligosaccharide-alditols was fractionated by high-voltage paper electrophoresis, the acidic fractions were further separated by high-performance liquid chromatography on Lichrosorb-NH<sub>2</sub>, and analyzed by 500-MHz <sup>1</sup>H-NMR spectroscopy and, partially, by permethylation analysis. Of the acidic oligosaccharide-alditols, the following sulfated carbohydrate chains could be identified: NeuAc $\alpha$ 2 $\rightarrow$ 6Gal $\beta$ 1 $\rightarrow$ 4GlcNAc $\beta$ 1 $\rightarrow$ 2Man $\alpha$ 1 $\rightarrow$ 3[(SO<sub>3</sub>Na $\rightarrow$ 3)Gal $\beta$ 1 $\rightarrow$ 4GlcNAc $\beta$ 1 $\rightarrow$ 2-Man $\alpha$ 1 $\rightarrow$ 6]Man $\beta$ 1 $\rightarrow$ 4GlcNAc $\beta$ 1 $\rightarrow$ 4[Fuc $\alpha$ 1 $\rightarrow$ 6]GlcNAc-ol and NeuAc $\alpha$ 2 $\rightarrow$ 6Gal $\beta$ 1 $\rightarrow$ 4(SO<sub>3</sub>Na $\rightarrow$ 6)<sub>0-1</sub>GlcNAc $\beta$ 1 $\rightarrow$ 2-Man $\alpha$ 1 $\rightarrow$ 3[NeuAc $\alpha$ 2 $\rightarrow$ 6Gal $\beta$ 1 $\rightarrow$ 4(SO<sub>3</sub>Na $\rightarrow$ 6)<sub>1-6</sub>GlcNAc $\beta$ 1 $\rightarrow$ 2Man $\alpha$ 1 $\rightarrow$ 6]Man $\beta$ 1 $\rightarrow$ 4GlcNAc $\beta$ 1 $\rightarrow$ 4[Fuc $\alpha$ 1 $\rightarrow$ 6]GlcNAc-ol. The sulfated structural elements for porcine thyroglobulin form novel details of *N*-linked carbohydrate chains. They contribute to the fine structure of these oligosaccharides and are another type of expression of microheterogeneity.

Thyroglobulin; Sulfated *N*-linked carbohydrate; (Porcine)

## 1. INTRODUCTION

Thyroglobulin, the major iodinated glycoprotein synthesized in the thyroid gland, is the polypeptide precursor of the thyroid hormones. Analysis of the carbohydrate chains of thyroglobulin (molecular mass ~660 kDa) from several species has demonstrated that mainly two types of chains occur, generally called unit-A type (oligomannose type) and unit-B type (*N*-acetyllactosamine type) [1–10]. For human thyroglobulin also unit-C type (mucin type) [4] and unit-D type (proteoglycan type) [11] chains have been indicated. In many cases  $\alpha$ 1 $\rightarrow$ 3Gal residues can form part of the unit-B type chains [12–14].

Concerning the *N*-linked oligosaccharide chains of porcine thyroglobulin [5,6], the literature data indicate the presence of Man<sub>5-9</sub>GlcNAc<sub>2</sub> structures, whereby the trimming of Man residues

seems to take place randomly [7]. For the *N*-acetyllactosamine type a series of partially sialylated di- (75%) and tri- (25%) antennary structures with an  $\alpha$ 1 $\rightarrow$ 6-linked Fuc residue at the Asn-bound GlcNAc unit have been reported [8]. In addition, a terminal Gal $\alpha$ 1 $\rightarrow$ 3Gal $\beta$ 1 $\rightarrow$ 4 element in the Man $\alpha$ 1 $\rightarrow$ 6 branch of a monosialylated ( $\alpha$ 2 $\rightarrow$ 6-linked NeuAc; Man- $\alpha$ 1 $\rightarrow$ 3 branch) diantennary structure has been shown to occur [14].

The recent finding of phosphate [10,15–17] and, especially, sulfate [18] groups in thyroglobulins from different biological origins prompted us to report data on the structural identification of sulfated *N*-linked unit-B type carbohydrate chains in porcine thyroglobulin.

## 2. MATERIALS AND METHODS

### 2.1. Porcine thyroglobulin

Porcine thyroglobulin was obtained from Sigma. SDS-polyacrylamide gel electrophoresis gave the usual pattern of two main bands, together with three faint bands of lower molecular mass [19,20]. Its sugar composition (Fuc/Man/Gal/GlcNAc/

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NeuAc = 0.4:3.0:1.2:2.6:0.4) and carbohydrate content (7%) are in accordance with literature data [1,2,21].

### 2.2. Preparation of oligosaccharide-alditols

The hydrazinolysis procedure on porcine thyroglobulin (six 125-mg portions) followed by high-voltage paper electrophoresis was carried out essentially as described [22,23]. The oligosaccharide-alditols were recovered from the paper by elution with water, yielding one neutral and three acidic fractions. The acidic fractions were further subfractionated by HPLC on Lichrosorb-NH<sub>2</sub> using elution systems of acetonitrile/15–30 mM KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub>, pH 5.2 [24], and monitored at 205 nm. Fractions were desalted on Bio-Gel P-2 (200–400 mesh) using water as eluent and refractive index detection.

### 2.3. Monosaccharide analysis

Carbohydrate samples were subjected to the methanolysis procedure and analyzed by GLC on CP Sil 5, as described [25].

### 2.4. Methylation analysis

Methylation analysis was carried out essentially as described [26]. Permethylated material was hydrolyzed with 4 M trifluoroacetic acid, the obtained mixtures of partially methylated monosaccharides were reduced with NaB<sup>2</sup>H<sub>4</sub> in water, and the partially methylated alditol acetates analyzed by GLC-MS.

### 2.5. 500-MHz <sup>1</sup>H-NMR spectroscopy

Oligosaccharide-alditols were repeatedly exchanged in <sup>2</sup>H<sub>2</sub>O (99.96 atom% <sup>2</sup>H) with intermediate lyophilization. Resolution-enhanced 500 MHz <sup>1</sup>H-NMR spectra were recorded in <sup>2</sup>H<sub>2</sub>O at 27°C on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands). 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) [27].

## 3. RESULTS

In the framework of our NMR studies on sulfated mono- [28] and oligo- [29,30] saccharides, porcine thyroglobulin was investigated for the presence of sulfated *N*-linked carbohydrate chains. The hydrazinolysis procedure in combination with high-voltage paper electrophoresis yielded one neutral (N) and three acidic (A1, A2, A3) fractions in the molar ratio of 27:35:28:10, respectively. As A2 and A3 turned out to contain the searched material, attention will only be paid to these fractions in this paper.

Subfractionation of A2, isolated from the 'double-negatively-charged' region of the paper electropherogram, on Lichrosorb-NH<sub>2</sub> yielded a series of peaks, of which A2b is of interest. The <sup>1</sup>H-NMR spectrum of A2b shows the presence of

one major compound. The NMR features of this oligosaccharide-alditol, presented in table 1, resemble those of reference compound IgM, which is a classical diantennary structure terminated with  $\beta$ 1 $\rightarrow$ 4-linked Gal in the Man $\alpha$ 1 $\rightarrow$ 6 branch and  $\alpha$ 2 $\rightarrow$ 6-linked NeuAc in the Man $\alpha$ 1 $\rightarrow$ 3 branch and having an  $\alpha$ 1 $\rightarrow$ 6-linked L-fucose residue at the Asn-bound *N*-acetylglucosamine [31]. However, of the structural-reporter-group signals the Gal-6' H-1 doublet had shifted downfield from  $\delta$  = 4.470 ppm to  $\delta$  = 4.587 ppm. Furthermore, in contrast to the spectrum of IgM, in that of A2b two additional signals can be observed in the structural-reporter-group region, namely, a doublet of doublets at  $\delta$  = 4.339 ppm (*J* values of 3.2 Hz and 10.0 Hz) and a doublet of doublets at  $\delta$  = 4.294 ppm (*J* values of 3.2 Hz and ~1 Hz). The latter resonance has the typical shape of a Gal H-4 signal. By selective <sup>1</sup>H-decoupling of the signal at  $\delta$  = 4.339 ppm, connected signals at  $\delta$  = 4.294 ppm and  $\delta$  = 3.684 ppm are found by difference spectroscopy. In the same way, selective <sup>1</sup>H-decoupling of the H-1 signal of Gal-6' ( $\delta$  = 4.587 ppm) and difference spectroscopy identified the resonance at  $\delta$  = 3.684 ppm as Gal-6' H-2. When a similar experiment is carried out for the H-1 signal of Gal-6, the corresponding H-2 resonance is found at  $\delta$  = 3.534 ppm. In conclusion, the signals at  $\delta$  = 4.339 ppm and  $\delta$  = 4.294 ppm can be attributed to Gal-6' H-3 and H-4, respectively.

Since the major compound in subfraction A2b is a monosialylated structure, an additional acidic substitution has to be present. In view of the literature data on acidic carbohydrate chains, it is obvious to suppose that either a phosphate or a sulfate group is involved [10,15–18]. The most obvious positions of attachment are Gal-6' C-3 or C-4, as can be concluded from the downfield positions of Gal-6' H-3 and H-4. Because of the absence of <sup>31</sup>P couplings on these signals, the occurrence of phosphate can be excluded. In the case of sulfate, the chemical shift values at  $\delta$  = 4.339 ppm and  $\delta$  = 4.294 ppm were compared to those of sulfated monosaccharide references. In table 2, the <sup>1</sup>H-NMR data of the methyl glycosides of  $\alpha$ -D-galactopyranose,  $\beta$ -D-galactopyranose, 3-*O*-SO<sub>3</sub>Na- $\alpha$ -D-galactopyranose and 4-*O*-SO<sub>3</sub>Na- $\alpha$ -D-galactopyranose [28] are summarized. Going from methyl  $\alpha$ -D-galactopyranoside to methyl

Table 1

<sup>1</sup>H chemical shifts of structural-reporter-group protons of the constituent monosaccharides of fractions A2b and A3c obtained from porcine thyroglobulin, together with those of reference compounds IgM and PT

Reporter group	Residue <sup>a</sup>	Chemical shifts <sup>b</sup> in <sup>c</sup>			
		A2b	IgM	A3c <sup>d</sup>	PT

H1	GlcNAc-2	4.713	4.714	n.d.	4.715
	Man-3	4.778	4.760	n.d.	4.786
	Man-4	5.136	5.136	5.139	5.135
	Man-4'	4.924	4.924	4.942	4.942
	GlcNAc-5	4.607	4.605	4.602	4.605
	GlcNAc-5'	4.580 <sup>e</sup>	4.581	4.643	4.605
	Gal-6	4.444	4.445	4.445	4.443
	Gal-6'	4.587 <sup>e</sup>	4.470	4.486	4.443
	Fuc	4.898	4.896	4.896	4.896
H-2	GlcNAc-1-ol	4.213	4.220	4.212	4.219
	Man-3	4.255	4.258	4.258	4.260
	Man-4	4.198	4.197	4.205	4.198
	Man-4'	4.108	4.110	4.112	4.114
H-3a	NeuAc	1.718	1.720	1.719 <sup>f</sup>	1.718 <sup>f</sup>
H-3e	NeuAc	2.669	2.668	2.671 <sup>f</sup>	2.669/2.673
H-3	Gal-6'	4.339	n.d.	n.d.	n.d.
H-4	Gal-6'	4.294	n.d.	n.d.	n.d.
H-5	Fuc	4.072	4.071	4.073	4.072
H-6	GlcNAc-5'	n.d.	n.d.	4.434	n.d.
H-6'	GlcNAc-5'	n.d.	n.d.	4.314	n.d.
CH <sub>3</sub>	Fuc	1.223	1.224	1.225	1.224
NAc	GlcNAc-1-ol	2.056	2.057	2.056	2.056
	GlcNAc-2	2.089	2.088	2.089	2.090
	GlcNAc-5	2.070	2.071	2.072	2.071
	GlcNAc-5'	2.048	2.048	2.066	2.065
	NeuAc	2.030	2.031	2.030 <sup>f</sup>	2.030 <sup>f</sup>

<sup>a</sup> For numbering of the monosaccharide residues, see text

<sup>b</sup> Chemical shifts are given for neutral solutions at 27°C, in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate in <sup>2</sup>H<sub>2</sub>O, acquired at 500 MHz (but were actually measured relative to internal acetone:  $\delta = 2.225$  ppm)

<sup>c</sup> Structures are represented by short-hand notation [27]: ●, GlcNAc; ◆, Man; ■, Gal; ○, NeuAc $\alpha$ 2→6; □, Fuc; S, sulfate

<sup>d</sup> For reasons of simplicity, the  $\delta$  values for GlcNAc-5/5' H-1 and Gal-6/6' H-1 have been grouped for a sulfate group at GlcNAc-5'

<sup>e</sup> Assignments may have to be interchanged

<sup>f</sup> Signal stems from two NeuAc residues

3-O-SO<sub>3</sub>Na- $\alpha$ -D-galactopyranoside, similar downfield shifts for H-3 and H-4 ( $\Delta\delta = +0.666$  ppm and  $\Delta\delta = +0.356$  ppm, respectively) were observed, as going from methyl  $\beta$ -D-galactopyranoside to Gal-6' in subfraction A2b (H-3,  $\Delta\delta = +0.695$  ppm; H-4,  $\Delta\delta = +0.372$  ppm). In the case of the 4-O-sulfated analog quite a different NMR

peak pattern was observed. It has to be noted that similar positions for Gal H-3 and H-4 have recently been reported for a terminal 3-sulfated Gal- $\beta$ 1→4 residue in an O-linked chain [32]. Summarizing, the terminal Gal residue in the Man $\alpha$ 1→6 antenna (Gal-6') in A2b is sulfated at position C-3, leading to the following structure:

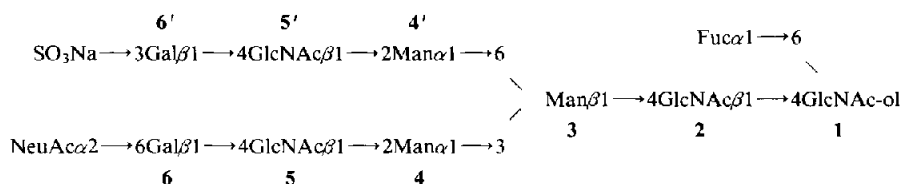


Table 2

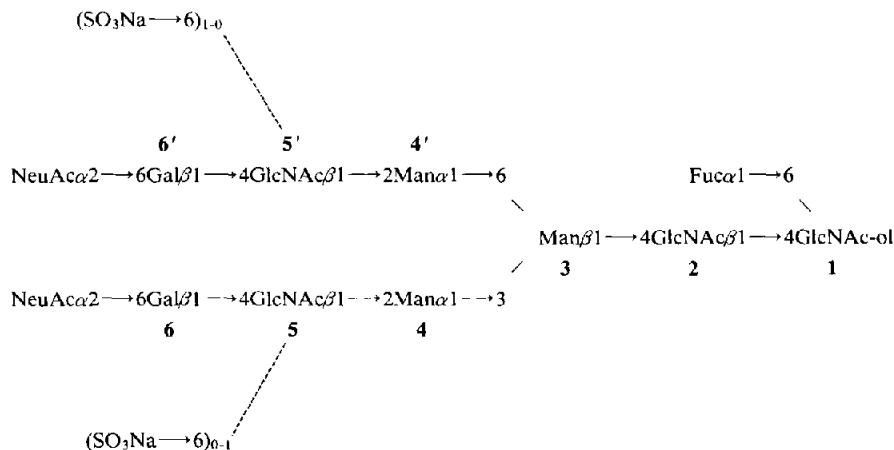
$^1\text{H}$ -NMR data for the methyl glycosides of  $\alpha$ -D-galactopyranose,  $\beta$ -D-galactopyranose, 3-O-SO<sub>3</sub>Na- $\alpha$ -D-galactopyranose and 4-O-SO<sub>3</sub>Na- $\alpha$ -D-galactopyranose [28]

Protons	Chemical shifts <sup>a</sup> in			
	$\alpha$ -D-Gal	3-O-SO <sub>3</sub> Na- $\alpha$ -D-Gal	4-O-SO <sub>3</sub> Na- $\alpha$ -D-Gal	$\beta$ -D-Gal
H-1	4.837	4.899	4.867	4.315
H-2	3.819	3.982	3.855	3.501
H-3	3.811	4.477	3.941	3.644
H-4	3.968	4.324	4.713	3.922
H-5	3.897	3.935	4.030	3.695
H-6	3.741	3.751	3.810	3.751
H-6'	3.752	3.759	3.755	3.796
OMe	3.414	3.430	3.425	3.573

<sup>a</sup> Chemical shifts are given at 27°C, in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate in  $^2\text{H}_2\text{O}$ , acquired at 500 MHz (but were actually measured relative to internal acetone:  $\delta = 2.225$  ppm)

Subfractionation of fraction A3, isolated from the 'triple-negatively-charged' region of the paper electropherogram, on Lichrosorb-NH<sub>2</sub> resulted in several peaks, of which only fraction A3c is of interest. The  $^1\text{H}$ -NMR spectrum of subfraction A3c shows a nearly pure compound. Comparison of the various resonances in the anomeric region of

the spectrum as well as in the region  $\delta < 2.8$  ppm, with the spectral features of a classical  $\alpha 2 \rightarrow 6$  disialylated diantennary carbohydrate chain with an  $\alpha 1 \rightarrow 6$ -linked Fuc residue at the Asn-bound GlcNAc unit, denoted PT, shows that the basic structure of A3c is similar to PT. The intensities of the GlcNAc-5/5' and Gal-6/6' H-1 doublets in A3c have only half the intensities of those of the corresponding signals in PT. But additionally, for GlcNAc as well as for Gal, a more downfield doublet is observed, namely, at  $\delta = 4.643$  ppm and at  $\delta = 4.486$  ppm, respectively. This means that in one of the antennae a structural element has changed, as compared to PT. Furthermore, two additional non-anomeric downfield signals, resonating at  $\delta = 4.314$  ppm ( $J$  values of 4.8 Hz and 11.0 Hz) and at  $\delta = 4.434$  ppm ( $J$  values of 1.2 Hz and 11.0 Hz) are observed. By selective irradiation it was found that both resonance patterns are coupled with each other. In view of earlier reports [29,33], such a typical coupled pattern belongs to H-6 and H-6' of one of the monosaccharide residues, bearing a sulfate group at C-6. Methylation analysis of fraction A3c yielded a 4,6-disubstituted GlcNAc residue, indicating that GlcNAc-5 or GlcNAc-5' bears a sulfate group at C-6. Combining the various data, the following two structures for A3c are possible:



Also the occurrence of a mixture of both structures cannot be excluded so far. The recently reported  $\delta$  values of GlcNAc H-6 and H-6' in the Gal $\beta$ 1 $\rightarrow$ 4(SO<sub>3</sub>Na $\rightarrow$ 6)GlcNAc element, present in an O-linked chain, are in the same range as the values included here [34]. Recent studies on carbohydrate chains liberated from various <sup>35</sup>S-labeled mammalian cell lines suggested the occurrence of a NeuAc $\alpha$ 2 $\rightarrow$ 6/3Gal $\beta$ 1 $\rightarrow$ 4(SO<sub>3</sub>Na $\rightarrow$ 6)GlcNAc element in N-linked oligosaccharides [35].

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