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THE STRUCTURE OF UNIT B-TYPE GLYCOPEPTIDES FROM PORCINE THYROGLOBULIN

TOSHIAKI KONDO MINORU FUKUDA, AND TOSHIAKI OSAWA*

Division of Chemical Toxicology and Immunochemistry, Faculty of Pharmaceutical Sciences University of Tokyo, Bunkyo-ku, Tokyo 113 (Japan)

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SUMMARY

The structure of Unit B-type glycopeptides (monosialo-type and disialo-type) was investigated by Smith degradation, methylation, and mass spectral analysis These glycopeptides contain three peripheral sugar chains Two are composed of D-galactose residues linked at C-6 and 2-acetamido-2-deoxy-D-glucose residues linked at C-4, and the other is composed of a D-galactose residue linked at C-6, a 2-acetamido-2-deoxy-D-glucose residue linked at C-4, and a D-mannose residue linked at C-2 Most of these peripheral sugar chains are linked to two inner D-mannose residues which are substituted at C-3 and C-6, and constitute branching points L-Fucose and *N*-acetylneuraminic acid residues are nonreducing terminal groups, and a di-*N*-acetylchitobiose moiety is linked to an asparagine residue in the peptide moiety By methylation analysis of the oligosaccharide obtained by hydrazinolysis of the disialoglycopeptide, the L-fucose residue was found to be linked to C-6 of the 2-acetamido-2-deoxy-D-glucose residue linked to the asparagine residue From these results, and from the previously reported data on the sugar sequence and the anomeric configurations of the linkages between sugar residues, structures for these glycopeptides are proposed

INTRODUCTION

Thyroglobulin, the major protein constituent of the thyroid gland, is a glycoprotein and the stored form of the thyroid hormones The Spiros[1,2] have shown that thyroglobulins from all species studied possess two distinct types of oligosaccharide chains One of them (Unit A-type sugar chain) consists of D-mannose and *N*-acetyl-D-glucosamine (2-acetamido-2-deoxy-D-glucose), and the other (Unit B-type sugar chain) contains D-mannose, *N*-acetyl-D-glucosamine, D-galactose, L-fucose, and sialic acid Arima and Spiro[3] have partially elucidated the structure of the Unit A-type sugar chain of calf and human thyroglobulins

In previous papers[4–7], the structure of the disialo, Unit B-type glycopeptide[4] (Fraction V) of porcine thyroglobulin has been investigated by means of periodate oxidation and sequential enzymic degradation with purified glycosidases, and the

*To whom inquiries should be addressed

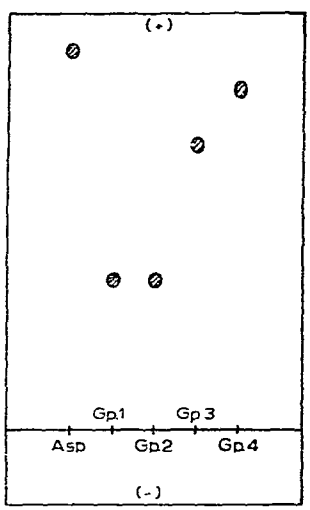
sugar sequence and the anomeric configurations between sugar residues have been elucidated as

$$(\alpha\text{-NeuAc})_2 \rightarrow (\beta\text{-Gal})_3 \rightarrow (\beta\text{-GlcNAc})_3 \rightarrow (\alpha\text{-Man})_2 \rightarrow [(\beta\text{-Man})\,(\alpha\text{-L-Fuc})] \rightarrow \rightarrow (\text{GlcNAc})_2 \rightarrow \text{Asn}$$

However, the positions of the glycosidic linkages and the location of the L-fucose residue have remained unknown In this paper, we report the complete structure of the Unit B-type sugar chain of porcine thyroglobulin

EXPERIMENTAL

Preparation of Unit B glycopeptides from porcine thyroglobulin — The glycopeptides from porcine thyroglobulin were prepared according to the method of Fukuda and Egami[4] with the following modifications Fraction III (described in the previous publication[4]) was dissolved in mM HCl and applied to a column of SP-Sephadex G-25 (1 4 × 32 cm) The column was eluted with mM HCl, and the unadsorbed glycopeptide fraction was lyophilized (Gp 1) This glycopeptide was



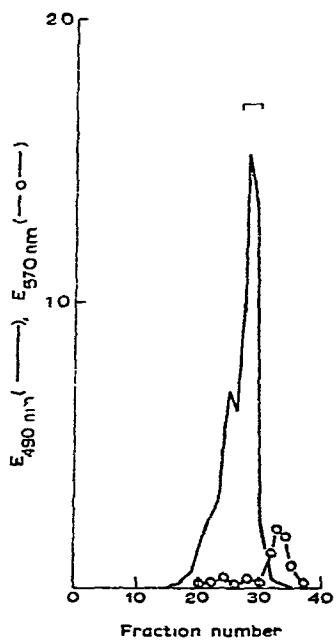


Fig 1 Paper electrophoretograms of fractionated glycopeptides Experimental details are given in the text

Fig 2 Gel filtration of the Pronase digest of glycopeptides[4] (Fraction IV and V) The Pronase digest was applied to a column of Sephadex G-50 (1 2 × 105 cm), and elution was performed with water Fractions of 6 ml were collected at 15 ml/h at 4° and analyzed for neutral sugars with the phenol–H_2SO_4 reaction[8] (———) and for peptides with the ninhydrin reaction[9] (—O—O—) The glycopeptide fractions were pooled as indicated.

homogeneous on electrophoresis in pyridine–acetic acid–water (1 10 89, pH 3 6) (Fig 1) From its chemical composition (Table I), this glycopeptide apparently corresponds to Fraction III-2 described in the previous publication[4] Fraction IV and V of the previous publication[4] were combined and subjected to extensive Pronase digestion by the method of Arima and Spiro[3] The digest was applied to a column of Sephadex G-50 (1 2 × 105 cm) and eluted with water (Fig 2), and the glycopeptide fraction subsequently applied to a column of DEAE-Sephadex A-25 (2 1 × 50 cm) The column was first washed with water (150 ml), and then with 15mM sodium phosphate buffer (pH 7 0), and then eluted with a gradient of 15mM sodium phosphate buffer (pH 7 0) (375 ml in the mixing vessel) and 150mM sodium phosphate buffer (pH 7 0) (375 ml in the reservoir) Fractions of 15 ml, collected at 20 ml/h at 4° (Fig 3) were analyzed for neutral sugars by the phenol–H_2SO_4 reaction Three peaks were detected, which were pooled, desalted by passage through a column of Sephadex G-25 (1 2 × 105 cm), and lyophilized The three glycopeptides (Gp 2, Gp 3, and Gp 4, Fig 1) were found to be homogeneous on electrophoresis in pyridine-acetic acid–water (1 10 89, pH 3 6)

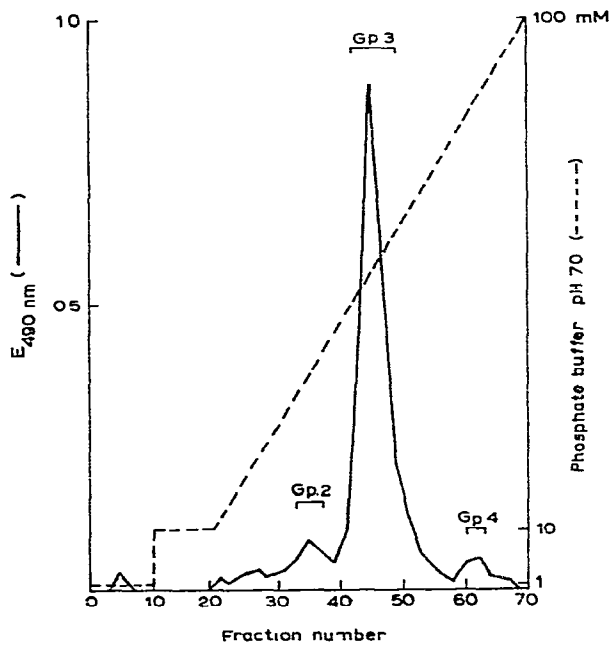


Fig 3 DEAE-Sephadex A-25 column chromatography of the glycopeptide fraction described in Fig 2 Experimental details are given in the text Fractions were analyzed for neutral sugars by the phenol–H_2SO_4 reaction[8] and pooled as indicated

Enzymes — Highly purified samples of β-galactosidase (*Charonia lampus*), α-mannosidase (*Turbo cornutus*), and β-*N*-acetylhexosaminidase (*Turbo cornutus*), purchased from Seikagaku Kogyo Co (Tokyo), were assayed with *p*-nitrophenyl

glycosides as described by Fukuda *et al* [10] Each purified enzyme used in this study was found to be devoid of other glycosidase activity One unit of enzyme activity is defined as the amount of enzyme that could liberate 1 0 μmol of *p*-nitrophenol in 1 min from the corresponding *p*-nitrophenyl glycosides

Amino acid analysis — After hydrolysis of glycopeptides for 24 h with 5 7M HCl, at 110° in sealed, evacuated tubes, amino acids were determined, with a Hitachi KLA-3B amino acid analyzer according to the method of Spackman *et al* [11]

Sugar analysis — The total sialic acid content was measured by the thiobarbituric acid method[12] *N*-Glycolylneuraminic acid was determined by the modified method of Klenk and Uhlenbruck[13] according to Spiro[14] Neutral sugars were determined by the phenol method of Dubois *et al* [8] Hexosamines were determined with a Hitachi KLA-3B amino acid analyzer according to the method of Spiro[14], hydrolysis being performed with 4M HCl for 6 h For the identification and the determination of neutral sugars, gas-liquid chromatography was performed after reduction to the respective alditols, followed by acetylation, according to the method of Spiro[15]

Electrophoretic procedure — The homogeneity of the glycopeptides was tested by electrophoresis on Toyo–Roshi No 51 paper at 33 0 V/cm in a pyridine–acetic acid–water (1 10 89) solvent for 2–3 h The glycopeptides were detected with 0 2% ninhydrin in acetone

Sequential degradation of glycopeptides — Removal of sialic acid from the glycopeptides was performed by acid hydrolysis with 0 05M H_2SO_4 at 80° for 1 h The content of fucose in the glycopeptides was not affected by this treatment Sequential enzymic degradation of the glycopeptides was performed by the method of Toyoshima *et al* [6]

Periodate oxidation — A sample of glycopeptide (1 0 μmol) was treated at 4° in the dark with sodium metaperiodate (20 μmol) in 0 05M acetate buffer, pH 4 0 The reaction was followed by measuring[16] the decrease in absorbance at 260 nm When the consumption of periodate reached a plateau (after 30 h), the reaction was terminated by adding 1,2-ethanediol (40 μmol) The salts were removed from the reaction mixture by gel filtration on a small column of Sephadex G-25, and $NaBH_4$ (8 mg) was then added After the solution had been kept in the dark for 16 h at 40°, a few drops of acetic acid were added to terminate the reaction For sequential oxidation, the oxidized and borohydride-reduced glycopeptide was isolated after removal of the salts by gel filtration on Sephadex G-25 The glycopeptide obtained was hydrolyzed with 25mM H_2SO_4 at 80° for 1 h, and applied to a column of Dowex 50 (X-8, 200–400 mesh, H^+) followed by elution with 1 5M NH_4OH The eluate was lyophilized *in vacuo* to remove ammonia and the sugars were determined

Hydrazinolysis — Hydrazinolysis of one of the glycopeptides was performed according to the method of Fukuda *et al* [17] The dry glycopeptide was heated at 105° for 4 h with freshly distilled, anhydrous hydrazine containing 1% of hydrazine sulfate in a sealed, evacuated tube The amount of hydrazine was the minimum amount required to dissolve the glycopeptide, and the glycopeptide concentration

was generally 0 5–1 0 g per ml of hydrazine Immediately after opening the tube, the reaction mixture was evaporated *in vacuo* to remove the hydrazine with occasional additions of toluene, and then the residue was dried over H_2SO_4 in a vacuum desiccator The dry hydrazinolyzate was dissolved in water, and the solution was subjected to gel filtration on Sephadex G-25 with water The phenol–H_2SO_4-positive fractions were pooled and concentrated by lyophilization, and the concentrated solution was stored at −20° The oligosaccharide thus obtained was *N*-acetylated according to the method described by Spiro[14] immediately after thawing the frozen solution of the oligosaccharide and diluting the solution with 10% methanol–4 5M sodium acetate to a concentration of about 3 μmol/ml After *N*-acetylation, the reaction mixture was applied to a column of Sephadex G-25 with water to separate the *N*-acetylated oligosaccharide from sodium acetate, acetic acid, and acetic anhydride The *N*-acetylated oligosaccharide thus obtained was then reduced with $NaBH_4$ (5 mg/ml) overnight at room temperature After addition of Dowex 50 (H^+) to destroy residual $NaBH_4$ and repeated additions and evaporations of methanol, the reduced oligosaccharide was permethylated

Methylation analysis — Glycopeptides or the oligosaccharide obtained by hydrazinolysis of one of the glycopeptides were methylated by the method of Hakomori[18] as described by Lindberg[19] The permethylated product was subjected to acetolysis, hydrolysis, reduction, and reacetylation as described by Stellner *et al* [20] Alditol acetates of partially methylated sugars were analyzed by gas-liquid chromatography performed on a column of Gas-Chrom Q (80–100 mesh) coated with 3% OV-225 for neutral sugars or 2% OV-17 for amino sugars Each peak was identified by g l c –m s with a gas chromatograph–mass spectrometer Model JMS-D 100 (Japan Electric Optic Laboratory) with a column (2 mm × 1 5 m) of 2% OV-17 on Gas-Chrom Q (80–100 mesh)[21 22] Conditions for recording the mass spectra were ionization potential, 36 eV, ion source temperature, 210°, and separation temperature, 280°

RESULTS AND DISCUSSION

Chemical composition of the glycopeptides — The results of the amino acid and sugar analyses of the purified glycopeptides (see Table I) indicate that both Gp 1 and Gp 2 contain one sialyl residue and Gp 3 two residues Gp 4, which was obtained in the smallest yield, contains three sialyl residues The molecular weight of Gp 3 was estimated to be 2,800 by gel filtration on a calibrated column of Sephadex G-50 (super fine) according to the method of Fukuda and Egami[4] Although the molar ratio of *N*-acetylneuraminic acid to *N*-glycolylneuraminic acid in the disialoglycopeptide had been estimated to be ~3 2 with the thiobarbituric acid method[12] (after paper chromatographic separation[23]) in the previous publication[4], the determination of glycolic acid[14] indicated that *N*-glycolyneuraminic acid comprised less than 10% of the total sialic acid of the disialoglycopeptide Gp 3

Sequential periodate degradation of the glycopeptides — After the first periodate

degradation of Gp 3 (see Table II), all of the sialic acid and D-galactose residues, and one residue each of *N*-acetyl-D-glucosamine and D-mannose were removed The second periodate degradation removed all of the remaining D-mannose residues and two residues of *N*-acetyl-D-glucosamine, leaving only the two terminal *N*-acetyl-D-glucosamine residues (Table II), one of which is linked to asparagine in the peptide moiety

TABLE I

CARBOHYDRATE AND AMINO ACID RESIDUES OF PORCINE THYROGLOBULIN GLYCOPEPTIDES[a]

Residues	*Glycopeptides*			
	Gp 1	*Gp 2*	*Gp 3*	*Gp 4*
Galactose	3 0	3 2	3 0	3 4
Mannose	3 3	3 0	3 0	3 0
Fucose	1 1	0 9	1 0	1 1
N-Acetylglucosamine	5 3	4 7	5 0	4 5
N-Acetylneuraminic acid	1 1	1 0	1 9	2 6
N-Glycolylneuraminic acid	0 1	0 0	0 1	0 1
Aspartic acid	1 0	[b]	1 0	[b]
Threonine	0 3	[b]	0 3	[b]
Serine	0 3	[b]	0 1	[b]
Glutamic acid	0 0	[b]	0 0	[b]
Proline	0 0	[b]	0 0	[b]
Glycine	0 2	[b]	0 1	[b]
Yield (%)[c]	4 1	0 2	2 2	0 1

[a]Expressed as mol/mol of glycopeptide in relation to aspartic acid taken as 1 0 (Gp 1, Gp 3) or mannose taken as 3 0 (Gp 2, Gp 4) [b]Not determined [c]Percent (w/w) on the basis of the crude glycopeptide fraction[4]

TABLE II

SEQUENTIAL PERIODATE OXIDATION OF GP 3[a]

Carbohydrate residues	*Untreated*[b]	*After degradation*	
		First[c]	*Second*[d]
Galactose	2 9	0	0
Mannose	3 0	2 0	0 2
Fucose	1 0	0	0
N-Acetylglucosamine	5 0	3 7	2 0
N-Acetylneuraminic acid	1 8	0	0

[a]Carbohydrate residues per glycopeptide [b]Mannose taken as 3 [c]Mannose taken as 2 [d]*N*-Acetylglucosamine taken as 2

Methylation studies — In order to determine accurately the position of the linkages between sugar residues, Gp 1 (monosialoglycopeptide), Gp 3 (disialoglycopeptide), and the derivatives obtained by periodate degradation (one step), glycosidase treatment, or hydrazinolysis were permethylated The methylated sugars obtained

after acid hydrolysis were analyzed as alditol acetates by g l c–m s The sugar compositions of the compounds subjected to the permethylation studies are listed in Table III, and the results of the permethylation in Table IV

As expected, Gp 1, which has only one residue of sialic acid, gave terminal 2,3,4,6-tetra-*O*-methyl-D-galactitol and 2,3,4-tri-*O*-methyl-D-galactitol in a ratio of

TABLE III

CARBOHYDRATE RESIDUES OF THE GLYCOPEPTIDES AND OLIGOSACCHARIDES SUBJECTED TO PERMETHYLATION

Residues	*Compounds*					
	Gp 1[a]	*Gp 3*[a]	*S-Gp 3*[b,c]	*E-Gp 1*[b,d]	*E-Gp 3*[b,e]	*H-Gp 3*[a,f]
Galactose	2 7	2 9		1 1	1 9	3 0
Mannose	3 0	3 0	2 0	2 0	2 0	3 0
Fucose	1 0	1 0		1 0	1 1	1 1
N-Acetylglucosamine	4 8	5 0	3 7	3 1	3 9	5 0
N-Acetylneuraminic acid	1 0	1 8		0 9	1 7	1 9

[a]Mannose taken as 3 [b]Mannose taken as 2 [c]Gp 3 degraded once with periodate [d]Gp 1 treated with β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase [e]Gp 3 treated with β-galactosidase β-*N*-acetylhexosaminidase, and α-mannosidase [f]The $NaBH_4$-reduced oligosaccharide that was obtained by hydrazinolysis of Gp 3

TABLE IV

METHYLATION ANALYSES OF GLYCOPEPTIDES AND THEIR DERIVATIVES[a]

Compound peracetates	*Glycopeptides and derivatives*					
	Gp 1	*Gp 3*	*S-Gp 3*[b]	*E-Gp 1*[b]	*E-Gp 3*[b]	*H-Gp 3*[b]
2,3,4-Tri-*O*-methylfucitol	0 6	0 9	0 0	0 6	0 7	0 8
2,3,4,6-Tetra-*O*-methylgalactitol	1 6	0 8	0 0	0 1	0 2	0 8
2,3,4-Tri-*O*-methylgalactitol	1 0	2 3	0 0	0 8	2 1	1 6
2,3,4-Tri-*O*-methylmannitol	0 0	0 0	0 8	2 0	1 2	0 0
3,4,6-Tri-*O*-methylmannitol	1 3	1 2	0 0	0 0	0 0	1 0
2,4-Di-*O*-methylmannitol	1 8	1 8	0 9	0 2	1 0	2 0
3,6-Di-*O*-methylmannitol	0 2	0 2	0 1	0 0	0 0	0 0
2-Deoxy-1,3,5-tri-*O*-methyl-2-(*N*-methylacetamido)glucitol						0 7
2-Deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol		0 4				4 0
2-Deoxy-3-*O*-methyl-2-(*N*-methylacetamido)glucitol		0 9				

[a]The methylated sugars were analyzed as alditol acetates The amount of each sugar was determined by total ion chromatography[22] and expressed as mol/mol of sample by taking the value of di-*O*-methylmannitol as 2 0 for Gp 1, Gp 3, H-Gp 3, and 1 0 for S-Gp 3, E-Gp 3 and the value of 2,3 4-tri-*O*-methylmannitol as 2 0 for E-Gp 1 For amino sugars, the values were calculated by taking the values of 2-deoxy-3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol as 4 0 [b]For abbreviations see legend to Table III

2 1, and Gp 3, which has two residues of sialic acid, gave those methylated D-galactitol derivatives in the ratio of 1 2 These results also show that the sialic acid residues are linked to C-6 of the D-galactose residues From the five residues of partially methylated derivatives of 2-acetamido-2-deoxy-D-glucitol detected, four were 2-deoxy-3,6-di-*O*-methyl-2-*N*-(methylacetamido)-D-glucitol, indicating that four residues of *N*-acetyl-D-glucosamine are substituted at C-4 These results suggest the existence in Gp 1 and Gp 3 of three peripheral sugar chains containing a D-Gal*p*-(1→4)-D-GlcNAc*p* sugar sequence

Since two residues of di-*O*-methyl-D-mannose are present in permethylated Gp 1 and Gp 3, these D-mannose residues constitute branching points, and the preponderance of 2,4-di-*O*-methyl-D-mannitol (see Table IV) indicates that most of the D-mannose residues at the branching points are substituted at C-3 and C-6 However, some heterogeneity at the branching point was shown by the small proportion (~10%) of 3,6-di-*O*-methyl-D-mannitol observed after permethylation (Table IV), which suggests that some D-mannose residues at the branching points are substituted at C-2 and C-4 After treatment with β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase in order to remove the chains devoid of sialic acid, Gp 1 was permethylated to give two moles of 2,3,4-tri-*O*-methyl-D-mannitol This indicates that both sialic acid-free sugar chains are linked to C-3 of the D-mannose residues at the branching points Similarly, the permethylation of Gp 3 after treatments with β-galactosidase, β-*N*-acetylhexosaminidase, and α-mannosidase gave 2,3,4-tri-*O*-methyl-D-mannitol and 2,4-di-*O*-methyl-D-mannitol in the ratio of 1 1, indicating that the sialic acid-free sugar chain of Gp 3 is linked to C-3 of the D-mannose residue at the branching point Furthermore, the presence of a 3,4,6-tri-*O*-methyl-D-mannose residue in the permethylated, original glycopeptide shows that the D-mannose residue in the sialic acid-free sugar chain is substituted at C-2 These methylation data suggest that three residues of D-mannose have the structure α-D-Man*p*-(1→6)-[(α-D-Man*p*-(1→3)]-D-Man*p* or α-D-Man*p*-(1→3)-α-D-Man*p*-(1→6)-D-Man*p* However, the observation that the second step of the Smith degradation of Gp 3 gave $(GlcNAc)_2$-Asn eliminates the latter structure

In a previous publication[6], the location of an L-fucose residue in the core di-*N*-acetylchitobiose moiety was suggested from the results of the sequential enzymic degradation with purified glycosidases In order to ascertain which of the *N*-acetyl-D-glucosamine residues of the di-*N*-acetylchitobiose moiety is linked to the L-fucose residue, Gp 3 was subjected to hydrazinolysis, and the oligosaccharide released was separated, reduced with $NaBH_4$, and permethylated Since a 1-*O*-methyl derivative can be obtained only from the reducing terminal residue, after hydrolysis, the linkage position of the reducing terminal sugar residue could be determined unequivocally by this technique As shown in Table IV, 2-deoxy-1,3,5-tri-*O*-methyl-2-*N*-(methylacetamido)-D-glucitol (Fig 4) was obtained from the reduced and permethylated oligosaccharide, suggesting that the L-fucose residue is linked to C-6 of the *N*-acetyl-D-glucosamine residue linked to an asparagine residue

Total structure of glycopeptides — From the series of experiments just described,

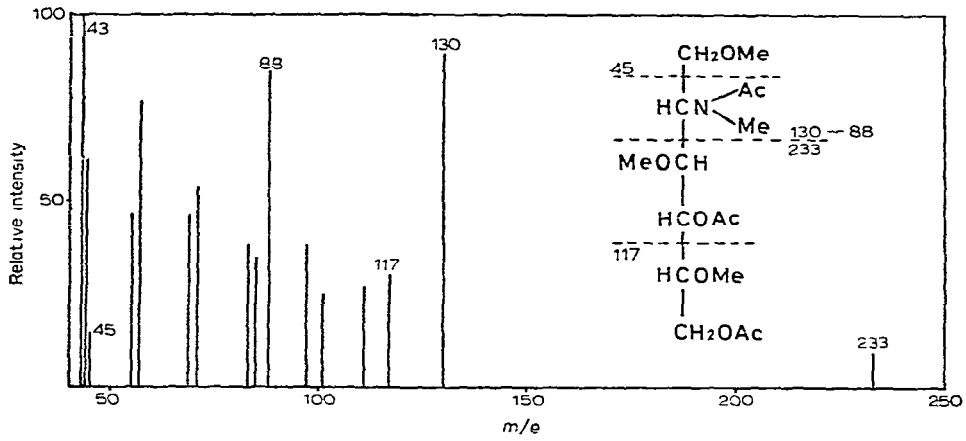


Fig 4 Mass spectrum of the acetate of 2-deoxy-1 3,5-tri-*O*-methyl-2*N*-(methylacetamido)-D-glucitol

and also from the previous observations[5–7] on the sugar sequence and the anomeric configuration between sugar residues, structures are proposed for Gp 1 and Gp 3 (see Fig 5) Although Gp 1 and Gp 3 comprise only 6–7% of the crude glycopeptide fraction[4] (Table I), these glycopeptides possibly constitute the major portion of the Unit B glycopeptides obtained from porcine thyroglobulin, because this glycoprotein has been found to contain larger amounts of Unit A-type sugar chains[4]

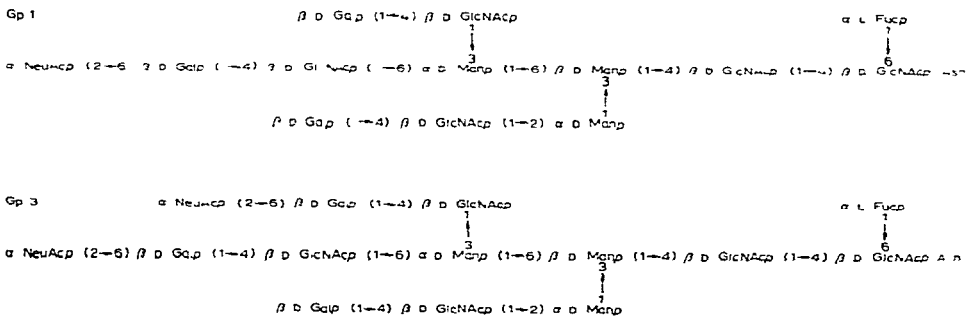


Fig 5 Proposed structure of Unit-B-type glycopeptides of porcine thyroglobulin (Gp 1 and Gp 3)

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