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Isolation and Characterisation of Three Fucosyloligosaccharide-1-phosphates from Normal Human Urine

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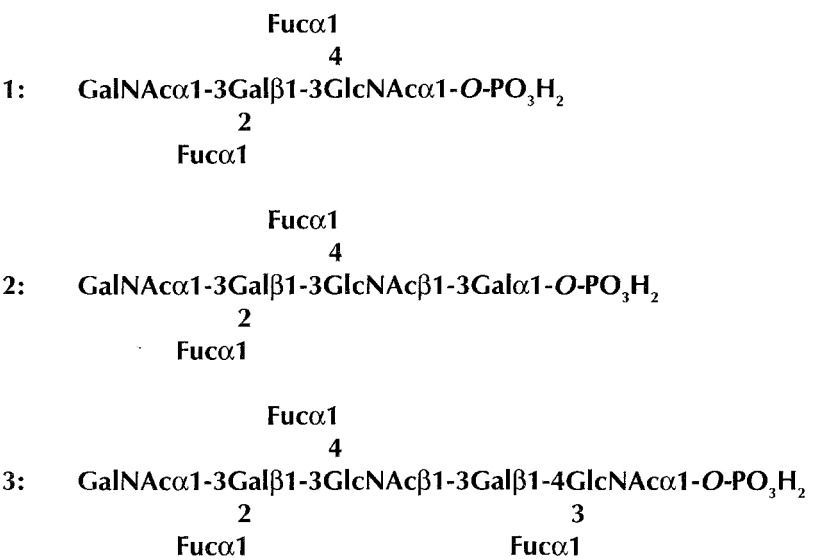
Three phosphate-containing fucosyloligosaccharides were isolated from normal human urine using charcoal adsorption, gel-filtration, ion-exchange chromatography and paper chromatography. Chemical investigations and 400 MHz ^{1}H-NMR spectroscopy analyses led to the following structures :



Their oligosaccharide chains are identical with, or similar to the fucosyloligosaccharides and urinary compounds synthesized by the stepwise transfer of *N*-acetylglucosamine, galactose, fucose and *N*-acetylgalactosamine to free galactose or glucose residues. A transfer reaction of monosaccharides to free hexose-1-phosphates or glycosylnucleotides is proposed for explaining the origin of these sugar-phosphates.

Normal human urine contains a large number of different oligosaccharides which reflect the structure and the metabolism of tissue glycoproteins [1, 2], or which result from the stepwise transfer of monosaccharides to free glucose or galactose [3-6]. Recently, three phosphate-containing sialyloligosaccharides and a phosphate-containing fucosyloligosaccharide have been isolated and characterized from normal urine [7] and from human pregnancy urine [8], respectively.

During the course of our studies on sialyloligosaccharides of normal urine, we characterized, together with sialyllactoses, traces of oligosaccharides of higher molecular weight devoid of sialic acid, but containing a phosphate residue. The present paper describes the isolation and the structural characterization of these compounds.

Materials and Methods

Materials

Bio-Gel P-2 and Dowex 1-X2 were from Bio-Rad Laboratories (Richmond, CA, USA), activated charcoal, *Escherichia coli* alkaline phosphatase (type III) and reference compound *N*-acetyl-α D-glucosamine 1-phosphate from Sigma Chemical Co (St Louis, MO, USA) and deuterium oxide (99.96% atom ^2H) from Aldrich (Milwaukee, WI, USA).

Analytical Methods

The oligosaccharides were analyzed by paper chromatography on Whatman No. 3 paper with ethyl acetate/pyridine/acetic acid/H_2O, 5/5/1/3 by vol. Oligosaccharides were detected with aniline oxalate reagent [10]. The molar ratios of monosaccharides were determined after methanolysis as trifluoroacetate derivatives by gas-liquid chromatography [11]. The reducing-end residues were characterized as alditols after reduction with $NaBH_4$ (4 mg/mg oligosaccharide) at 20°C for 2 h, neutralization with Dowex 50-X8 (20-50 mesh; H^+ form), and distillation of boric acid with methanol.

Phosphate was estimated according to [12], after mild acid hydrolysis (0.1 M HCl ; 80°C ; 1 h). Phosphatase treatment was carried out with 1 U of *E. coli* alkaline phosphatase in 200 µl of 0.1 M Tris-HCl buffer (pH 8) at 37°C for 24 h, as previously described for similar components [7].

The 400 MHz ^1H-NMR experiments were performed on a Bruker AM-400WB spectrometer equipped with a 5 mm $^1H/^{13}C$ mixed probe-head, operating in the pulse Fourier transform mode and controlled by an Aspect 3000 computer. The chemical shifts are given relative to sodium-4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured to methyl of acetone ($\delta = 2.225$ ppm for ^1H and $\delta = 31.55$ ppm for ^{13}C). The details of the experiments have been previously described [13].

The pulse programs for multiple step relayed COSY were from Bruno Perly, CEA Saclay, France (personal communication).

Results

Isolation of the Phosphate-containing Fucosyloligosaccharides

50 l human urine (A Le^b phenotype) were filtered on 3 kg of charcoal-Celite (1:1 by weight) using a vacuum filtration funnel. After washing with 10 l water, carbohydrate material was desorbed with 20 l of 50% ethanol in water. After concentration to 500 ml under reduced pressure, the carbohydrate material was chromatographed on two columns of ion exchanger (4 x 60 cm Dowex 50-X2, H^+, 200-400 mesh ; 4 x 60 cm Dowex 1-X2, CH_3COO^-, 200-400 mesh). After washing with 1 l of water, the columns were separated and the anionic exchanger was eluted with a discontinuous gradient of pyridine acetate (pH 5.4) varying from 10 to 500 mM. The fractions eluting around 10 and 20 mM (2 l for each solution) were collected and concentrated to 12 ml, prior to analytical chromatography. These fractions contained mainly a mixture of NeuAc(α2-3)Gal(β1-4)Glc and NeuAc(α2-6)Gal(β1-4)Glc, and traces of sugar material remaining at the starting point of the chromatogram.

This material of higher molecular weight was isolated by gel filtration on Bio-Gel P-2, and submitted to preparative paper chromatography on Whatman No. 3 paper with ethyl acetate/pyridine/acetic acid/H_2O, 5/5/1/3 by vol, for four days. Three major components were obtained, the molar composition of which indicated the presence of *N*-acetylneuraminic acid, fucose, galactose, mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine and phosphate residues. The mannose- and *N*-acetylneuraminic acid-containing material was quantitatively retained on a ConA-Sepharose column (further investigations showed that the sialylated mannose-containing oligosaccharides retained on ConA-Sepharose were identical to the sialyloligosaccharides which accumulate in the urine of patients suffering from sialidoses [14]). The non-retained material was found to contain exclusively fucose, galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine and phosphate residues. Finally, the phosphate-containing fucosyloligosaccharides were isolated in a relative pure form, as shown by further NMR investigations, i.e. 100% for compound **1** and about 75% for compounds **2** and **3**. The migration rate for each compound is given relative to NeuAc(α2-6)Gal(β1-4)Glc, i. e. 0.27 for **1**, 0.20 for **2** and 0.11 for **3**. The yields were, respectively, 2, 1.3 and 8 mg, starting from 50 l urine.

Structures of the Compounds Isolated

Compound ***1****:* Determination of the chemical composition of compound **1** indicated the presence of fucose, galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine and phosphate in the ratio 2:1:1:1. Reduction of the native oligosaccharide with $NaBH_4$ had no effect on the monosaccharide composition, whereas reduction of the material treated with alkaline phosphate led to the disappearance of *N*-acetylglucosamine. The aglycon position of the phosphate group was confirmed by examination of the ^{1}H-NMR spectrum (Fig. 1), which also establish its α-linkage ($J_{H1,H2}$ = 3.2 Hz) and the value of the coupling constant ^{1}H1-^{31}P ($J_{H\text{-}1,\ ^{31}P}$ = 7.3 Hz). The typical H-1 and H-6 of the α(1-2)- and α(1-4)-linked fucose residues were observed on the COSY DR spectrum owing the direct H-1 → H-4 and H-4 → H-5 → H-6 correlations. The H-1, H-2 and H-5 resonances of the α(1-3)-linked *N*-acetylgalactosamine residue are identical to those found for blood-group A Le^b active oligosaccharides [15]. For the galactose residue, (δH-1 = 4.599 ppm), the H-4 resonance is downfield shifted at δ

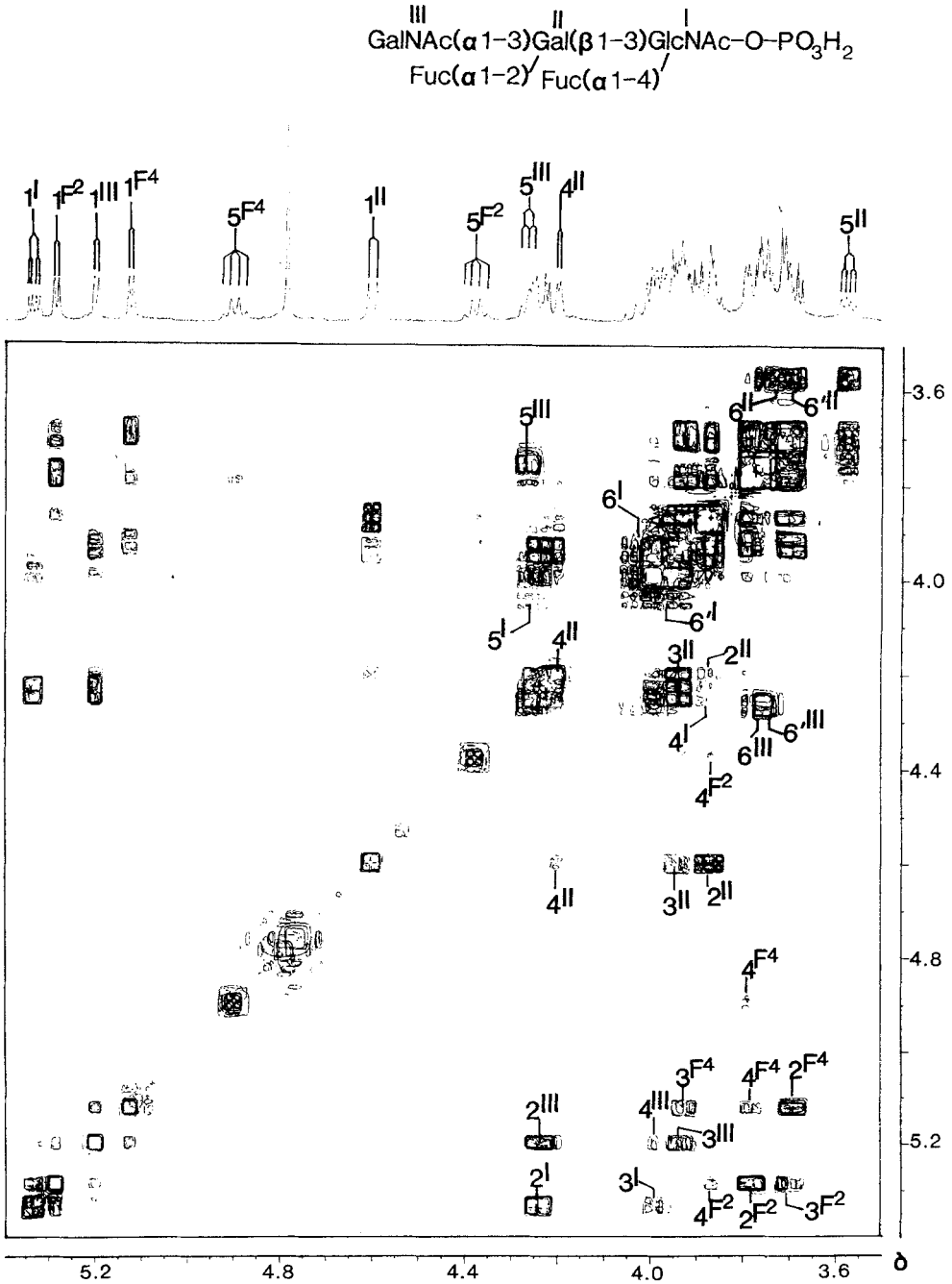


Figure 1. Homonuclear double relayed-COSY spectrum of compound **1**.

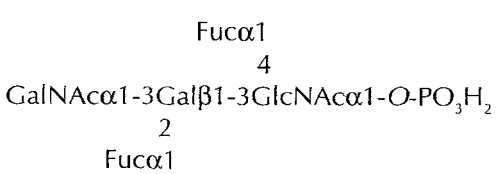
Table 1. ^{1}H-Chemical shifts for compounds **1** to **3**[a]

Compound **1**	H-1	H-2	H-3	H-4	H-5	H-6	H-6'	NAc
Fucα1-2	5.285	3.783	3.700	3.867	4.376	1.314		
GalNAcα1-3	5.198	4.240	3.932	3.990	4.262	3.75	3.77	2.037
Galβ1-3	4.599	3.871	3.941	4.198	3.574	3.726	3.691	
Fucα1-4	5.123	3.691	3.919	3.792	4.897	1.284		
GlcNAcα1*P*	5.333	4.240	3.976	3.87	4.25	4.02	3.97	2.046
Compound **2**								
Fucα1-2	5.208	3.737	3.651	3.728	4.409	1.294		
GalNAcα1-3	5.218	4.172	3.970	3.967	4.307	3.72	3.74	2.024
Galβ1-3	4.718	3.816	3.873	4.166	3.57	3.71	3.75	
Fucα1-4	5.046	3.803	3.91	3.80	4.861	1.292		
GlcNAcβ1-3	4.661	3.829	4.176	3.732	N.D.[b]	N.D.	N.D.	2.063
Galα1*P*	5.436	3.746	N.D.	4.17	4.186	(≈3.74)		
Compound **3**								
Fucα1-2	5.207	3.739	3.652	3.731	4.396	1.292		
GalNAcα1-3	5.218	4.172	3.966	3.972	4.307	3.73	3.75	2.023
Galβ1-3	4.719	3.813	3.880	4.165	3.545	3.72	3.74	
Fucα1-4	5.042	3.803	3.915	3.801	4.857	1.292		
GlcNAcβ1-3	4.613	3.832	4.174	3.739	3.515	3.84	3.93	2.062
Galβ1-4	4.425	3.485	3.704	4.065	3.592	3.70	3.73	
Fucα1-3	5.076	3.678	3.875	3.766	4.861	1.154		
GlcNAcα1*P*	5.276	4.156	3.97	3.85	4.175	3.98	3.94	2.041

[a] Values determined at pD 7, 27°C. GlcNAc-1*P* H-1 and H-2 chemical shifts are strongly pD-dependent.

[b] N. D. not determined

= 4.198 ppm indicating an O-3 substitution. All these NMR parameters enabled proof of the presence of the A Le^b blood-group determinant, and therefore, the structure of compound **1** has been established as follows.



The COSYDR spectrum of compound **1** furnished all the resonances, which are reported on Table 1.

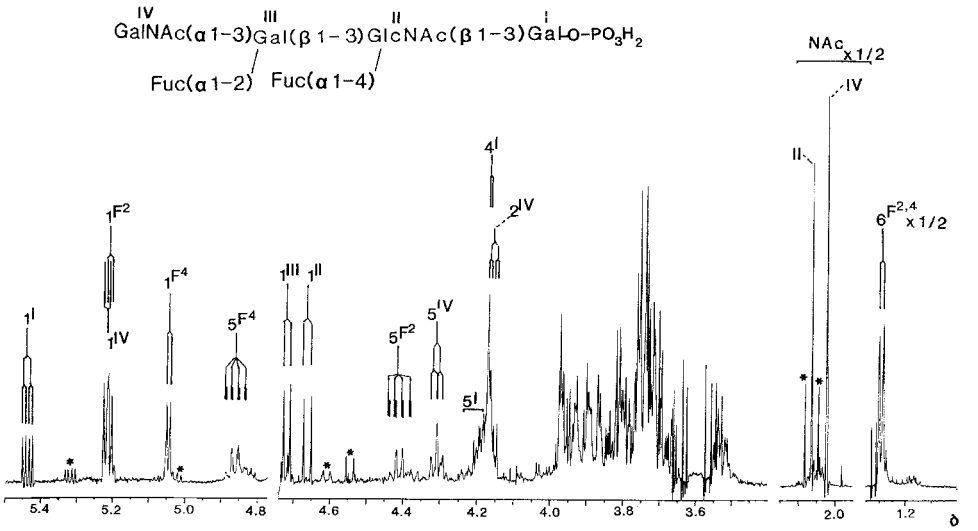
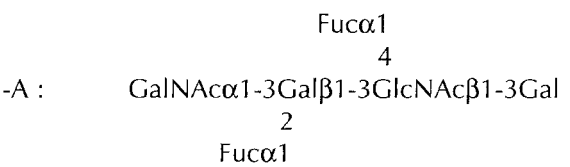


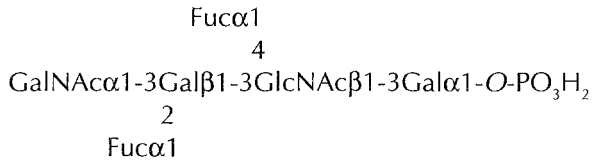
Figure 2. ^{1}H-NMR Spectrum of compound **2**. Asterisks indicate the signals related to a contaminant of undetermined structure.

Compound 2: The chemical composition of compound **2** (1 *N*-acetylgalactosamine, 2 fucose, 1 *N*-acetylglucosamine, 2 galactose, 1 phosphate) suggests the material to be an extension of compound **1** with an additional galactose residue. Compound **2** was partially dephosphorylated during storage, leading to a neutral hexasaccharide (purified by preparative paper chromatography), the ^{1}H-NMR spectrum of which was exactly identical to that of oligosaccharide VI-A previously described [15].



The acidic compound, re-isolated by paper chromatography, was a mixture of two components, in the ratio 4:1. The structure of the minor component was not investigated. The major component is characterized by an anomeric proton resonating at $\delta = 5.436$ ppm (Fig. 2), coupled with an H-2 atom at $\delta = 3.746$ ppm (COSY spectrum, not shown). This upfield value for the H-2 atom indicates that a Galα-*O*-PO_3H_2 residue occurs at the terminal position of the molecule. The low coupling constant $J_{H1,H2}$ is indicative of the α-linkage of

the phosphate group. The other NMR parameters are quite superimposable on those of oligosaccharide VI-A (see above). Therefore, the structure of the major component present in fraction 2 was established as follows.

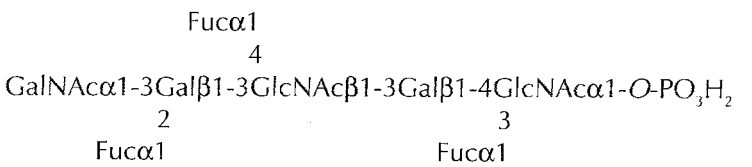


The NMR data, obtained by COSY Relayed experiments, are compiled in Table 1.

Compound 3: As for compound **1**, the reduction of the oligosaccharide leads to the disappearance of an *N*-acetylglucosamine residue only after treatment with alkaline phosphatase. The determination of the molar ratio indicated the presence of *N*-acetylgalactosamine, fucose, galactose, *N*-acetylglucosamine and PO_4H_3 in the ratio 1:3:2:2:1. The ^{1}H-NMR spectrum of this octasaccharide (Fig. 3) showed the presence of a major quadruplet in the region of the anomeric proton at δ = 5.276 ppm, the low coupling constant of which allowed assignation to the H-1 atom of GlcNAcα-*O*-PO_3H_2. The downfield shift of the coupled H-2 atom, at δ = 4.156 ppm, confirmed the identification of an *N*-acetylglucosamine residue. A second quadruplet, at δ = 5.174 ppm, revealed the presence of a minor contaminant, the structure of which has not been established.

The fusion of the H-6 resonances of the α(1-2)- and α(1-4)-linked fucose residues, at δ = 1.292 ppm, is characteristic of the A Le^b blood-group determinant [15]. The third fucose is α(1-3)-linked [Le^x(X) determinant] as indicated by the chemical shift of its CH_3 group, at δ =1.156 ppm. For Gal-II, the H-4 resonance is downfield shifted at δ = 4.065 ppm, indicating its O-3 substitution. The ^{1}H-NMR parameters of GalNAc-V, Gal-IV and GlcNAc-III are identical to those established for hexa- and heptasaccharides bearing A Le^b blood-group determinants [15]. The H-5 resonance of GlcNAc-I is downfield shifted at δ = 4.175 ppm, due to the proximity of the phosphate group (for the reference compound GlcNAcα-*O*-PO_3H_2, δH5 = 3.975 ppm).

According to these NMR data, the structure of the octasaccharide 1-phosphate was established as follows.



The ^{13}C-NMR spectrum of compound **3** was also recorded (Fig. 4 and Table 2). The assignments of the carbons were made by comparison with the ^{13}C-NMR spectra of similar A Le^b blood-group active hexa- and heptasaccharides [15]. For GlcNAc-I, the C-1, C-2, C-

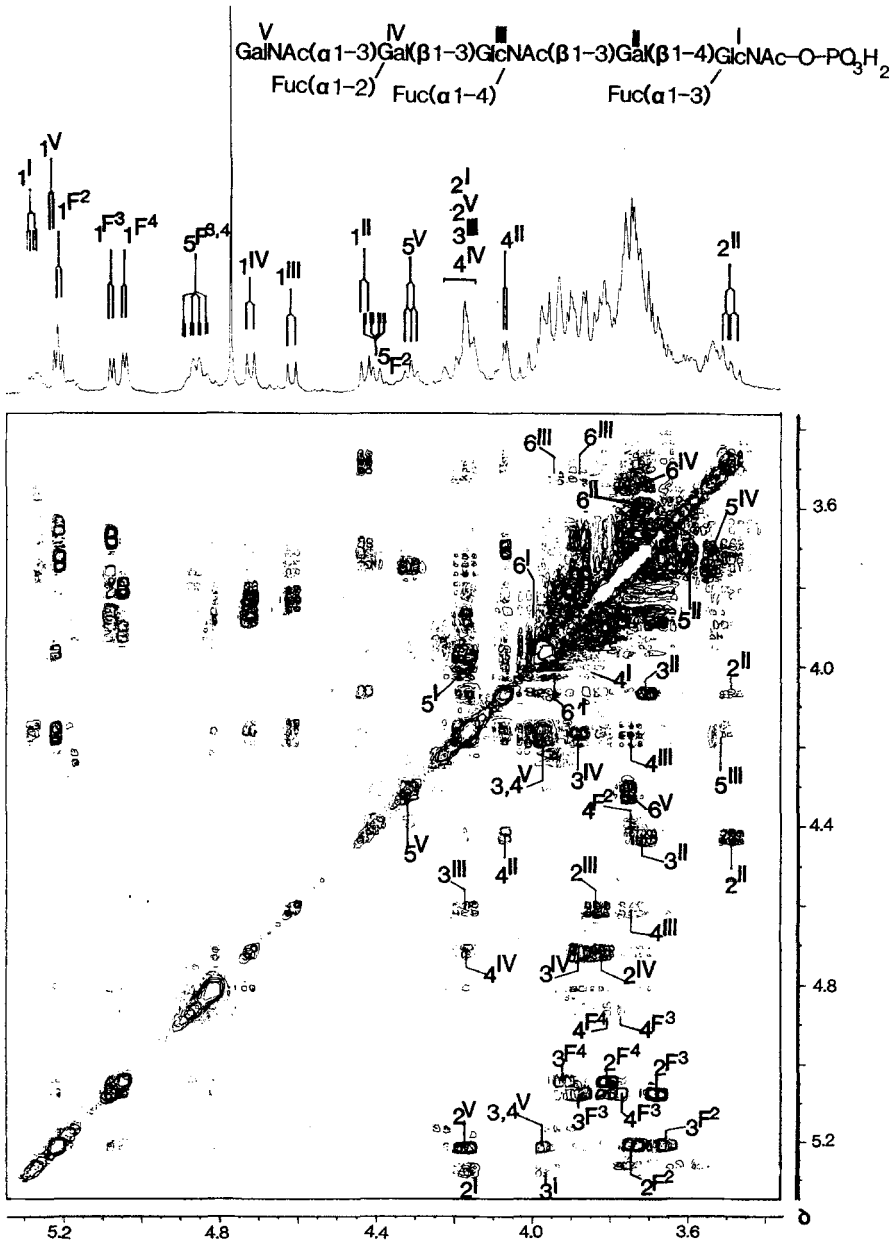


Figure 3. Homonuclear double relayed-COSY spectrum of compound **3**.

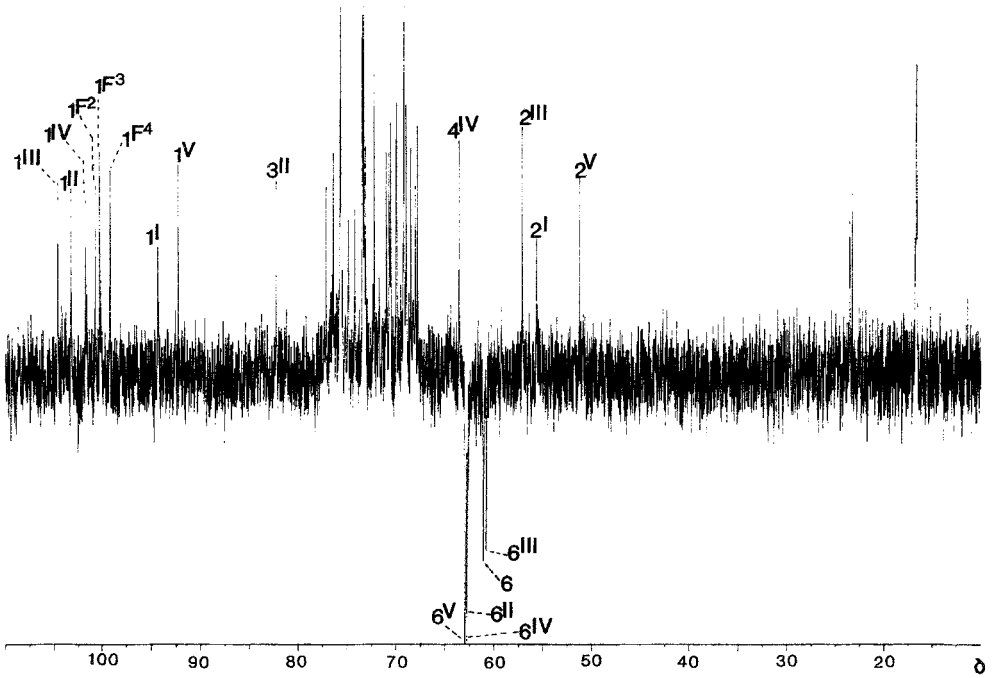


Figure 4. ^{13}C-NMR Spectrum (DEPT) of compound 3.

6 and CH_3 resonances were easily extracted from the 1D spectrum. For the C-3, C-4 and C-5 atoms, the results of the heteronuclear COSY experiment being not exploitable, the chemical shifts were deduced from the fact that the C-4 resonance should be probably not influenced by the phosphate group, in opposition to the neighbouring C-3 and C-5 atoms. For the Le^x determinant, the C-3, C-4 and C-5 resonances of the *N*-acetylglucosamine residue are observed at δ = 76.1, 73.9-74.6 and 76.4-76.6 ppm, respectively [16, 17]. Consequently, among the three last ^{13}C-resonances to be assigned, the two downfield chemical shifts (73.74 and 74.44 ppm) were assigned to C-3 and C-5 atoms whereas the upfield shift value (δ = 72.53 ppm) was attributed to the C-4 atom.

Discussion

The characterization of phosphate-containing fucosyloligosaccharides represents the second description of such a carbohydrate material isolated from human urine [8]. Parkkinen and Finne [7] isolated three sialyloligosaccharide 1-phosphates and discussed their origin. The first possibility is the stepwise transfer of monosaccharides to free hexose 1-phosphate,

Table 2. ^{13}C-Chemical shifts for compound **3**.

	C-1	C-2	C-3	C-4	C-5	C-6	CH_3
Fucα1-2	100.61	69.11	70.92	73.34	67.70	16.73	
GalNAcα1-3	92.19	51.19	68.87	69.87	72.16	62.86	23.62
Galβ1-3	101.63	75.60	77.09	63.49	75.71	62.81	
Fucα1-4	99.09	69.06	70.63	73.34	68.39	16.80	
GlcNAcβ1-3	104.50	57.06	75.60	73.06	76.36	60.69	23.30
Galβ1-4	103.14	72.16	82.22	69.90	75.60	62.65	
Fucα1-3	100.16	68.84	70.45	73.22	67.91	16.62	
GlcNAcα1*P*	94.26	55.59	74.34[a]	72.53	74.44[a]	61.09	23.52

[a] Values given for C-3 and C-5 GlcNAc-O-P may have to be interchanged.

nucleotide or dolichol-linked hexose residues, and then cleaved by a pyrophosphatase. Uridine diphosphate sugar compounds [Fuc(α1-2)Gal(β1-4)GlcNAc-UDP and Gal(β1-4)GlcNAc-UDP] have been previously characterized in human milk and colostrum (18, 19). The second possibility could be "the catabolism of glycoconjugates through a phosphorolysis reaction or alternatively phosphorylation of free oligosaccharides" [7]. The third hypothesis is the occurrence of a new class of phosphodiester-linked fucosylated glycans which remains to be characterized.

Although this type of phosphodiester-linkage has already been described [20-23], the first explanation appears as the most probable. Indeed, urinary oligosaccharides, as well as milk oligosaccharides, are essentially synthesized by the action of glycosyltransferases on free galactose, glucose or lactose. It may be inferred by the observation that individuals on a lactose and galactose diet in urine excrete additional oligosaccharides having galactose at the reducing end [4, 6]. In the same way, the xylose diet induces the excretion of Gal-Xyl, Gal-Gal-Xyl and NeuAc-Gal-Gal-Xyl (G. Strecker, unpublished results). Urinary oligosaccharides with terminal inositol and glycerol have also been characterized [15, 22, 23]. Further investigations are necessary to obtain detailed information in order to determine the origin of these substances, and eventually to verify the existence of phosphodiester-linked glycans.

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