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(54) Title: NOVEL FUCOSYLATED OLIGOSACCHARIDES AND PROCESS FOR THEIR PREPARATION

(57) Abstract: The present invention relates to a process for the preparation of oligosaccharides or oligosaccharide containing compounds, especially N-acetyl-chitooligosaccharides having a fucosylated monosaccharide. The invention also relates to novel oligosaccharides or oligosaccharide containing compounds, especially N-acetyl-chitooligosaccharides, which are fucosylated and optionally covalently bound to a carrier molecule.

## Novel fucosylated oligosaccharides and process for their preparation

## FIELD OF INVENTION

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The present invention relates to novel fucosylated oligosaccharides or oligosaccharide containing compounds which are analogues to natural oligosaccharides and which contain at least one fucosylated monosaccharide unit. The invention also relates to a process for the preparation of such oligosaccharides or oligosaccharide

10 containing compounds.

## BACKGROUND OF THE INVENTION

Fucosylated mammalian glycans have functions in fertilization (1), early differen-

15 tiation of embryo (2), brain development (3,4), and leukocyte extravasation (5,6), the  $\alpha$ 1-3/4fucosylated oligo- and polysaccharides being conjugated to lipids and proteins or as free oligosaccharides such as the oligosaccharides of human milk.

The fucosylated N-acetyllactosamines (Lewis x, Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc and Lewis a, Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc) and fucosylated N-acetyllactosamine (Lex-

20 NAc, GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc) occur often as terminal sequences such as Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-2Man $\alpha$ -, Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ 1-2Man $\alpha$ -, Gal-NAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-2Man $\alpha$ -, Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-3Gal $\beta$  - /GalNAc $\alpha$ -, Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ 1-3Gal $\beta$ -, Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)

GlcNAc $\beta$ 1-4Man $\alpha$ -, Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc $\beta$ 1-4Man $\alpha$ -, and

25 Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-6Gal $\beta$ -/GalNAc $\alpha$ -/Man $\alpha$ - In the middle of lactosamine-type chains the Lewis x-sequences, -GlcNAc $\beta$ 1-3Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-3Gal are common, but also Lewis a-structures, Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc, are present with a possibility for repeating the sequence chain. In the free oligosaccharides found mainly in human milk, the  $\alpha$ 1-3fucosylated epitope at the reducing end of the saccharide is commonly lactose (Gal $\beta$ 1-4Glc) or its elongated/substituted form such as Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)Glc, Fuc $\alpha$ 1-2Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)Glc, Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)-Glc, Gal $\beta$ 1-3GlcNAc $\beta$ 1-3Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)Glc, and Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-

3Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)Glc. Analogs of these could be useful for studies of specificities of biological activities of the natural mammalian  $\alpha$ 1-3/4fucosylated sequences.

5 The fucosylation step in the biosynthesis of these glycans is accomplished by the family of  $\alpha$ 1-3/4fucosyltransferases (Fuc-Ts). In man, at least Fuc-Ts III-VII and IX are expressed (7-9), and enzymatically active homologs are known in other animals and bacteria.

10  $\alpha$ 1-3Fucosyltransferases transfer fucose to position 3 of GlcNAc or Glc residues in Gal $\beta$ 1-4GlcNAc (LacNAc), GalNAc $\beta$ 1-4GlcNAc (LacdiNAc) and Gal $\beta$ 1-4Glc (lactose) to synthesize the bioactive epitopes Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc (Lewis x, Lex), GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc (LexNAc), and Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)Glc, respectively. All human Fuc-Ts are known to use Gal $\beta$ 1-4GlcNAc-type acceptors (7,9). GalNAc $\beta$ 1-4GlcNAc serves also as an acceptor for the Fuc-Ts of human 15 milk (10). Human Fuc-Ts III and V have also  $\alpha$ 1-4fucosyltransferase activity using acceptors such as Gal $\beta$ 1-3GlcNAc (type I N-acetyl-lactosamine) to synthesize Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc (Lewis a). At least human Fuc-Ts III and V and (weakly VI) are able to fucosylate lactose and related oligosaccharides to structures containing Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)Glc sequences.

20 Enzymatic  $\alpha$ 1-3fucosylation of N-acetyl-chitobiose has been described, see US 5,759,823, but the product was not characterized. The reducing N-acetyl-chitobiose contains also the epimer in which there is ManNAc at the reducing end, and it is not known from the data of the said US patent if N-acetyl-chitobiose or its 25 reducing-end epimer was fucosylated. Saccharides with fucosylated reducing-end GlcNAc are not considered useful as they are labile and degrade even in aqueous solutions at near neutral pH. The reducing-end fucosylated GlcNAc is very rare, or non-existent, in mammalian natural oligosaccharides, possibly because of the lability which could make it useless also *in vivo* for biological functions.

30 The present invention describes saccharide epitope analogues of the mammalian fucosylated saccharide chains, as well as their synthesis. An effective method to

synthesize such epitopes is to use  $\alpha$ 1-3 fucosyltransferases or  $\alpha$ 1-3/4fucosyltransferases for fucosylation of novel acceptor sequences. Some of the acceptor sequences can be synthesized from cheap natural polysaccharides such as cellulose, chitin, chondroitin/chondroitin sulphates, or hyaluronic acid, also natural polysaccharides with the sequence  $\text{Glc}\beta 1-(3\text{Glc}\beta 1-4\text{Glc}\beta 1-)_n\text{3Glc}$  could be used to synthesize acceptors.  $\beta$ 1-4GlcNAc transferase and UDP-GlcNAc can be used to generate GlcNAc $\beta$ 1-4GlcNAc $\beta$ 1- linked to Gal, GalNac or Man (11) and these can be used to make other analogues. Certain parasites have also been reported to contain N-acetyl-chitooligosaccharides which could be used as acceptors for the fucosylation reaction (12).

Interestingly the novel fucosylations of N-acetyl-chitooligosaccharides (N-acetyl-chitotriose and larger) described here occurred to the non-reducing subterminal residue (forming a terminal Lewis x-like structure with a linkage structure similar to human glycans) and not to the reducing-end GlcNAc as in plant N-glycans.

## SUMMARY OF THE INVENTION

In a first aspect, the present invention is directed to novel fucosylated oligosaccharides and oligosaccharide type compounds, especially N-acetyl-chitooligosaccharides which are  $\alpha$ 1-3 fucosylated in the monosaccharide at the position subterminal to the non-reducing end of the oligosaccharide. The present invention is also directed to a process for the preparation of such compounds, which results in a site-specific fucosylation by the use of  $\alpha$ 1-3fucosyltransferase or  $\alpha$ 1-3/4fucosyltransferase enzyme to glycosylate the oligosaccharide acceptor substrate with L-fucose.

## DESCRIPTION OF THE DRAWINGS

In the drawing, Fig. 1 (A) is a HPAE-chromatography of the neutral Fuc-TV fucosylation products of N-acetyl-chitotetraose. Fucosylated product (Glycan 4, FGN<sub>4</sub>; for structures of Glycans 3-7, see Table 1) eluted at 7.62 min and the

putative acceptor at 9.74 min. in the isocratic run with 40 mM NaOH. (B) shows HPAE-chromatographic purification of fucosylated N-acetyl-chitohexaose (FGN<sub>6</sub>) eluting at 7.09 min., putative N-acetyl-chitohexaose peak at 9.04 min in the isocratic run with 40 mM NaOH. The saccharides were from Fuc-TV reaction.

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Fig. 2 shows MALDI-TOF mass spectra of the key reaction mixtures and purified product saccharides. F is L-Fucose, GN is N-acetyl-D-glucosamine. (A) shows a mixture of FGN<sub>4</sub> and GN<sub>4</sub> produced by human Fuc-TV, (B) shows a mixture of FGN<sub>6</sub> and GN<sub>6</sub> produced by Fuc-TV. (C) shows FGN<sub>6</sub> purified by HPAE-chromatography. (D) shows exo- $\beta$ -N-acetylhexosaminidase and novel endo-chitinase (the jack bean enzyme, mild conditions) reaction to the mixture of FGN<sub>6</sub> and GN<sub>6</sub> produced by Fuc-TV. The major product is surprisingly FGN<sub>5</sub>. (E) shows mild cleavage of the purified FGN<sub>6</sub> to mostly FGN<sub>5</sub> by the jack bean preparation. (F) shows mild treatment of the purified FGN<sub>4</sub> by the jack bean preparation, 91% of the substrate remains intact.

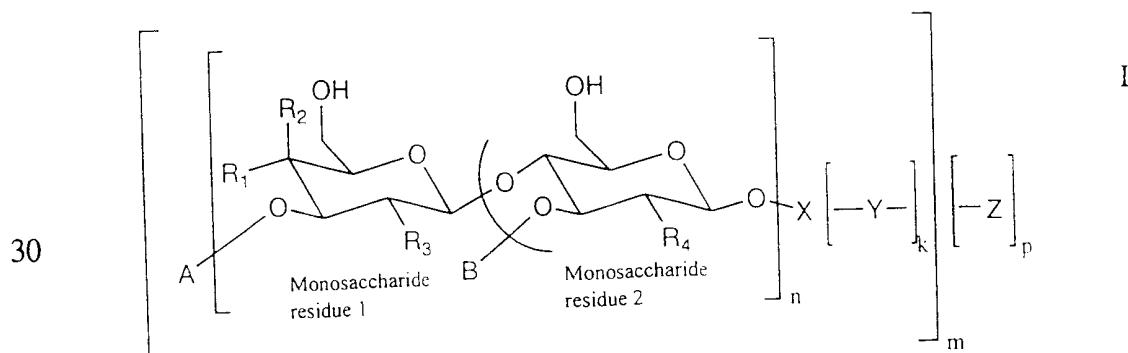
Fig. 3. shows MS/MS spectra of reduced and permethylated fucosylated N-acetyl-chitotriose 3 (A), and N-acetyl-chitotetraose 4 (B). The fragment ion nomenclature of Domon and Costello is used to denote the generated fragments.

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#### DETAILED DESCRIPTION OF THE INVENTION

Specifically the present invention concerns fucosylated oligosaccharide compounds having the formula

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In the formula I, A is H or a glycosidically  $\beta$ 1-3 linked D-glucopyranosyl residue (Glc $\beta$ 1-3), R<sub>1</sub> is OH, R<sub>2</sub> is H and R<sub>3</sub> is OH or acylamido, -NH-acyl (*i.e.* monosaccharide 1 is Glc, or GlcNAcyl) or R<sub>1</sub> is H, R<sub>2</sub> is OH and R<sub>3</sub> is acetamido - NHCOCH<sub>3</sub> (*i.e.* monosaccharide 1 is GalNAc), B is H, or an  $\alpha$ -L-fucosyl or an 5  $\alpha$ -L-fucosyl analogue, and R<sub>4</sub> is OH or acetamido -NHCOCH<sub>3</sub> (*i.e.* monosaccharide 2 is optionally fucosylated Glc or GlcNAc), the curved line between the saccharide units indicating that the monosaccharide 1 is  $\beta$ 1-4 linked to monosaccharide 2 when B is linked to the position 3 of the monosaccharide 2, and the monosaccharide 1 is  $\beta$ 1-3 linked to monosaccharide 2 when B is linked to the position 10 4 of the monosaccharide 2, monosaccharide 1 is GalNAc only when monosaccharide 2 is Glc, n is 1 to 100, with the proviso that there is always at least one  $\alpha$ -fucosyl or  $\alpha$ -fucosyl analogous group present in the molecule, and

i) p and k are 0 and m is 1, in which case X is H, an aglycon residue or a monosaccharide selected from the group consisting of Glc, GlcNAc, Gal or GalNAc, 15 optionally in reduced form, or oligosaccharide containing one or more of said monosaccharide units, the monosaccharide 2 being  $\beta$ 1-2,  $\beta$ 1-3,  $\beta$ 1-4 or  $\beta$ 1-6 linked to saccharide X, with the proviso that X is not H when both monosaccharides 1 and 2 are GlcNAc, B is L-fucosyl and n is 1, or

ii) p is 1, k is 0 or 1 and  $1 \leq m \leq 1000$ , in which case X is a straight bond, or a 20 mono- or oligosaccharide as defined under i),  
Y is a spacer or linking group capable of linking the saccharide 2 or X to Z, and Z is a mono- or polyvalent carrier molecule.

In the above formula, as well as below, Glc means a D-glucose residue and Gal means a D-galactose residue. Fuc or F means a L-fucose residue. GlcNAc or GN means a N-acetyl-D-glucose amine residue. The monosaccharides are in pyranose form when glycosidically linked.

B as an analogue to the L-fucosyl residue is preferably a compound that contains a hydroxy-methyl group in place of the methyl group in 6 position of fucosyl, that is L-galactosyl, or a deoxy derivative of L-fucosyl, or an analogue where a di- to

tetrasaccharide is linked to C6. Most preferably, however, B is H or L-fucosyl.

$R_3$  as an acylamido group is preferably an alkanoylamido group with 2 to 24 carbon atoms and 0 to 3 double bonds between carbon atoms in a straight chain.

5 Preferably  $R_3$  is acetamido -NH-COCH<sub>3</sub>, or an alkanoylamido group with 8 to 24 carbon atoms, and 1 to 3 double bonds. m is preferably 1 to 100 and most preferably 1 to 10, and n is preferably 1 to 10.

10 An oligosaccharide in the meaning of X contains preferably from 2 to 10 monosaccharide units, the monosaccharide units preferably being glycosidically  $\beta$ 1-4 or  $\beta$ 1-3 linked Glc or GlcNAc residues.

An aglycon group is preferably a hydrocarbon group, such as a C<sub>1-20</sub> alkyl or C<sub>2-20</sub> alkenyl group, a C<sub>3-10</sub> cycloalkyl or cycloalkenyl group, an aryl or aralkyl

15 group containing up to 10 carbon atoms in the aromatic ring, alkyl having the meaning given above, for example a phenyl group or benzyl group, or a heterocyclic group, that is a cycloalkyl or an aryl group as defined containing one or more heteroatoms O, S or N in the ring(s).

20 A preferred aglycon group is a lower alkyl or alkenyl group of 1 to 7, or 2 to 7 carbon atoms, respectively, or a phenyl or benzyl group. A preferred heterocyclic aglycon group is 4-methylumbelliferyl.

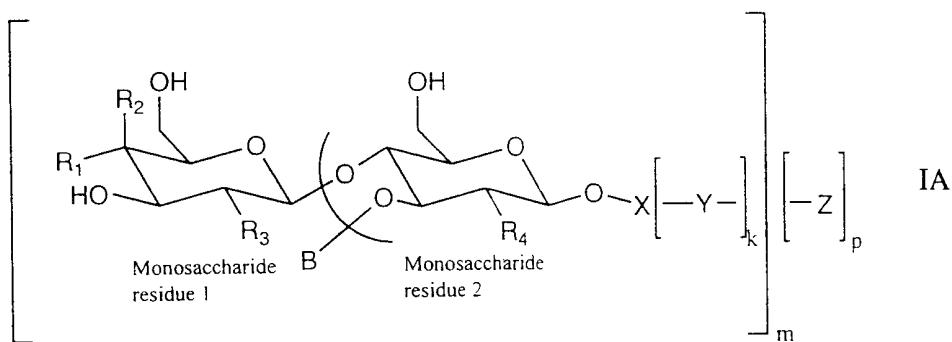
25 The spacer group Y, if present, can be any group that is capable of linking the group X or saccharide 2 to the carrier molecule Z, and such groups and methods of linking are known in the art, and also commercially available. For example when X is a saccharide, a bond between X and Y can be formed by reacting an aldehyde or a carboxylic acid with X at its C<sub>1</sub> or by introducing any aldehyde or carboxylic acid group in X through oxidation, to form a suitable bond such as -  
30 NH-, -N(R)- where R is an alkyl group, or a hydroxyalkylamine, an amide, an ester, a thioester or thioamide. A suitable bond is also -O- or -S-, see e.g. Stowell et al Advances in Carbohydrate Chemistry and Biochemistry, 37 (1980), 225-.

Thus the present invention provides fucosylated oligosaccharide compounds as such or covalently bound to a carrier molecule. The carrier molecule can be mono- or polyvalent, and is preferably selected from polymers, such as polyacrylamides, polyols, polysaccharides, such as agarose, biomolecules, including peptides and proteins, bovine or human serum albumin being commonly used carriers for example in immunoassays.

A preferred group of compounds with the formula I comprises the following

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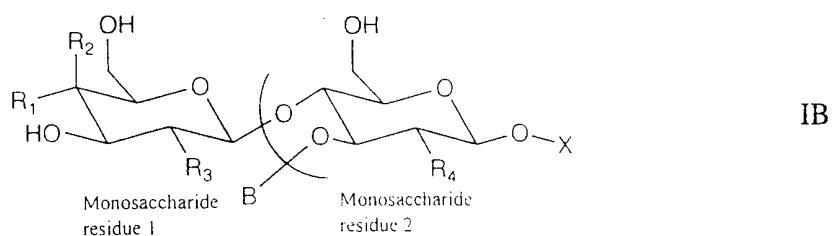
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wherein the symbols have the meanings given in connection with the formula I above. In the above formula, the monosaccharides 1 and 2 are preferably independently Glc and GlcNAc, B is L-fucosyl, and X is Glc or GlcNAc or a  $\beta$ 1-3 or  $\beta$ 1-4 linked oligomer comprising up to 10 units of Glc and/or GlcNAc. When p and k = 0 and m = 1, the compounds have the formula

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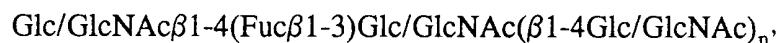


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wherein the symbols have the same meanings as given above in the formula IA, or X can also be H provided the monosaccharides 1 and 2 are both Glc.

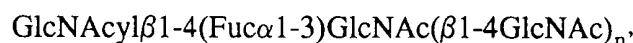
The compounds of the formula IA and IB are thus oligosaccharides that are fucosylated in the subterminal or penultimate non-reducing end monosaccharide. According to the invention it has been discovered that this specific fucosylation results in highly stable oligosaccharides, especially N-acetyl-chitooligosaccharides.

5 According to a further preferred embodiment of the invention, the saccharides have the formula



10 In the above formula,  $n'$  is the integer 1 to 8, preferably 1 to 6.

According to another embodiment of the invention, the saccharides have the formula



15 wherein  $n'$  has the meaning given above and acyl is an alkanoyl group which preferably contains 8 to 24 carbon atoms and 1 to 3 double bonds. Preferably  $1 < n' < 6$ , more preferably  $2 < n' < 4$ .

20 According to the invention the compounds of the formula I are prepared by fucosylating a compound of the formula I wherein B is always H, with a donor nucleotide sugar containing L-fucose, or an analogue thereof, in the presence of a fucosyltransferase enzyme, and optionally recovering the fucosylated saccharide so prepared. Such a reaction may be carried out on the starting oligosaccharide prior to the optional binding the oligosaccharide to the carrier molecule Y. In the alternative, it is also possible to fucosylate a carrier bound oligosaccharide. Such a reaction is carried out essentially in the same manner as with non-bound oligosaccharides. For example, a N-acetyl-chitooligosaccharide such as a N-acetyl-chitotriose, can be linked to bovine serum albumin BSA through a spacer or it can be 25 reductively aminated in a known manner to BSA, the reduced residue forming the spacer. In the fucosylation reaction, preferably an excess GDP-Fuc and a lower concentration of acceptor sites (0.1 - 1 mM) are used. If BSA is the carrier, the 30 concentration of acceptor sites (0.1 - 1 mM) are used. If BSA is the carrier, the

reaction buffer contains no non-glycosylated BSA. The products can be purified by methods of protein chemistry and the level of fucosylation can be checked by MALDI-TOF mass spectrometry, as described in more detail below. The fucosylation can be repeated if the reaction is incomplete.

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Oligosaccharides with an aglycon group as X can be fucosylated essentially as has been described, preferably using lower acceptor concentrations (0.1 - 1 mM). The products can be purified using chromatographic methods including gel filtration and with partial reaction cleavage with N-acetylhexosaminidase from jack beans.

10 As an example, one obtains  $\text{GlcNAc}\beta 1\text{-}4(\text{Fuc}\alpha 1\text{-}3)\text{GlcNAc}\beta 1\text{-benzyl}$  from  $\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-benzyl}$ ,  $\text{GlcNAc}\beta 1\text{-}4(\text{Fuc}\alpha 1\text{-}3)\text{GlcNAc}\beta 1\text{-O-methyl}$  from  $\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-O-methyl}$ , and  $\text{GlcNAc}\beta 1\text{-}4(\text{Fuc}\alpha 1\text{-}3)\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-O-4-methylumbelliferyl}$  from  $\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-O-4-methylumbelliferyl}$ .

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According to a preferred embodiment of the invention, the fucosyltransferase is human  $\alpha 1\text{-}3$ -fucosyltransferase or human  $\alpha 1\text{-}3/4$ fucosyltransferase, especially one of human fucosyltransferases III-VII, IX, or  $\alpha 1\text{-}3/4$ fucosyl transferase of human milk. According to another preferred embodiment, the L-fucose is in the form of

20 GDP-L-fucose.

According to an embodiment for making the preferred compounds described above, the starting oligosaccharide contains 3 to 10, especially 3 to 8  $\beta 1\text{-}4$  saccharide units. Such units are preferably N-acetyl-D-glucosamine residues, GlcNAc, the

25 corresponding starting materials thus being N-acetyl-chitooligomers with formula  $(\text{GlcNAc}\beta 1\text{-}4)_{3\text{-}10(8)}$ . According to a second preferred embodiment, the oligosaccharides contain 2 to 10, especially 2 to 6  $\beta 1\text{-}4$  D-glucose units, Glc, the corresponding starting materials thus being cellobiooligomers with formula  $(\text{Glc}\beta 1\text{-}4)_{2\text{-}10(8)}$ .

30 According to the invention it is also possible to include a further step of reacting the product obtained with a  $\beta$ -N-acetyl-hexosaminidase under sufficiently strong conditions and in an amount sufficient to release the non-reducing terminal mo-

nosaccharide. It is also possible to react the product obtained with a  $\beta$ -N-acetyl-hexosaminidase under less severe conditions in order to release a monosaccharide from its reducing terminal. In this manner, a product is obtained which contains one saccharide unit less than the primary oligosaccharide product. This latter reaction 5 may also be controlled in such a manner that the enzyme primarily degrades any remaining non-fucosylated substrate. This method thus provides a convenient way of purifying the reaction mixture after the fucosylating step.

Due to the fucose unit, the novel oligosaccharides are stable compounds and as 10 such useful in a number of applications. As such they can, for example, be used as substrates for testing, identifying and differentiating enzymes, such as chitinases, and when bound to a support they find use in immunoassays and affinity chromatography. They can also find use in a agrobiology, as stable plant protectants, as 15 activators of the defence mechanisms and growth regulators of the plant cell similarly as has been described with N-acetyl-chitooligosaccharides and their acyl derivatives (13). In such use, incorporation of the fucose group in the oligosaccharide will in practice protect the oligosaccharide from enzymatic degradation.

The  $\alpha$ 1-3/4fucosylated analogues of animal oligosaccharides are useful for studies 20 of biological interactions involving their natural counterparts. The natural  $\alpha$ 1-3-/4fucosylated oligosaccharides are known to be ligands or counterreceptor of lectins mediating cell adhesion and other intercellular interactions. These saccharides are also important antigenic epitopes recognized by anti-cancer or allergy-related antibodies. Some of these interactions are of special medical interest such as 25 the leukocyte adhesion to blood vessels mediated by the binding of the selectin proteins to their  $\alpha$ 1-3/4fucosylated counterreceptor oligosaccharides linked to proteins or lipids. The free oligosaccharide analogues can be tested in *in vitro* or *in vivo* assays to find out their abilities to inhibit the binding between the lectins and their counterreceptors or alternatively the direct binding of the oligosaccharides 30 to the lectins can be measured for example by affinity chromatography. The data obtained with the analogues in comparison to free saccharide epitopes identical to the natural counterreceptor oligosaccharides reveal part of the specificity of

the interaction. It shows which modifications in the molecule are useful and which are not tolerated when better medical lead compounds for antagonists of the interaction are designed. In search of better medical derivatives of the oligosaccharides the reducing end of the oligosaccharide chain can be modified by numerous 5 non-carbohydrate structures, aglycons. Free oligosaccharides can also be useful as mixtures of known composition (so called libraries) in the tests of biological activities. Mixtures of positional fucosyl isomers are easily obtained by incubating oligosaccharide with multiple acceptor sites with less GDP-Fuc than the amount of the acceptor sites or by following the reaction level by MALDI-TOF analysis of 10 the reaction mixture and limiting the reaction time (14).

Multi- or polyvalent conjugates of the oligosaccharides can also be more active antagonists of biological interactions when they are of natural and non-antigenic type. Antigenic polyvalent conjugates such as oligosaccharides coupled to bovine 15 serum albumin or to keyhole limpet hemocyanin can be used to raise antibodies against the saccharide epitopes. Polyvalent conjugates conjugated to solid supports, such as agarose affinity chromatography media or plastic micro titer plates, are also useful for assaying and purification of lectins and other proteins binding the saccharides. Fucosylated N-acetyl-chitooligosaccharide epitopes -Man $\beta$ 1-4Glc- 20 NAc(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-Asn of plant and insect proteins (15) are potent and cross reactive human allergens and GlcNAc(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-conjugates can be useful for assaying allergy antibodies recognizing the epitope.

The selectin proteins mediating vascular leukocyte adhesions are known to bind 25 sialyl-Lewis x [sLex, NeuNAc $\alpha$ 2-3Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc], especially in sLex- $\beta$ 1-3Lex $\beta$ 1- (16), and sialyl-Lewis a [NeuNAc $\alpha$ 2-3Gal $\beta$ 1-3(Fuc $\alpha$ 1-4)GlcNAc] oligosaccharides (17) and in some reports also Lewis x [Lex, Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc] (18) or VIM-2 -epitopes [NeuNAc $\alpha$ 2-3Gal $\beta$ 1-4GlcNAc $\beta$ 1-3Gal $\beta$ 1-4(Fuc- $\alpha$ 1-3)GlcNAc] (19). GalNAc-analogue of Lewis x, GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc 30 has been reported to be better selectin ligand than the sialyl-Lewis x (20, 21).

The following examples are intended to illustrate the invention.

The products according to the invention have been characterized by degradation, mass spectrometry and NMR-experiments. The latter suggest that the non-reducing 5 end monosaccharide and the fucose are stacked in a solution conformation reminiscent of that dominating in Lex and LexNAc determinants. This lends significant protection to the fucosylated oligosaccharides.

In the examples the following abbreviations have been used:

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DQFCOSY, double quantum filtered correlation spectroscopy; ESI-CID, electrospray ionization-collision induced decay; Fuc & F, L-fucose; Fuc-Ts III-VIII and IX, human  $\alpha$ 1-3fucosyltransferases/ $\alpha$ 1-3/4fucosyltransferases III-VII and IX; Fuc-Thm,  $\alpha$ 1-3/ $\alpha$ 1-3/4fucosyltransferases of human milk; Gal, D-galactose; GalNAc, 15 D-N-acetylgalacosamine; GlcNAc & GN, D-N-acetylglucosamine; HPAEC-PAD, high pH anion exchange chromatography - pulsed amperometric detection; Lac-NAc, Gal $\beta$ 1-4GlcNAc; LacdiNAc, GalNAc $\beta$ 1-4GlcNAc; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; *m/z*, mass to charge ratio; MOPS, 3-[N-Morpholino]propanesulphonic acid; ROESY, rotating 20 frame nuclear Overhauser spectroscopy; ROE, rotating frame nuclear Overhauser; TOCSY, total correlated spectroscopy

25

In the examples, N-acetyl-chitotriose, N-acetyl-chitotetraose, and N-acetyl-chitohexaose were from Seikagaku (Tokyo, Japan). Cellobiose was from Thomas Kerfoot and Co.Ltd., and  $\beta$ -N-acetylhexosaminidase from jack beans was from Sigma (St. Louis, MO, USA). GDP-fucose (used in human milk experiments) was a kind gift from Prof. B. Ernst (Universität Basel, Switzerland). Human fucosyltransferases V and VI, recombinant proteins expressed in *Spodoptera frugiperda*, and GDP-fucose were from Calbiochem (La Jolla, CA, USA). GDP-[U-<sup>14</sup>C]fucose was from 30 Amersham International (Buckinghamshire, England). Superdex Peptide HR 10/30 HPLC-column was from Pharmacia (Uppsala, Sweden). Dowex AG 1-X8 (AcO<sup>-</sup>, 200-400 mesh) and Dowex AG 50W-X8 (H<sup>+</sup>, 200-400 mesh) and Biogel P-2

were from Bio-Rad (Richmond, CA). D<sub>2</sub>O was from Cambridge Isotope Laboratories (Woburn, MA). Partially purified human milk fucosyltransferases were prepared as described in (22) and assayed as described in (23), 1 mU corresponds to transfer of 1 nmol of fucose to 190 mM lactose / minute at 37 °C, pH 7.5.

5

*Fucosyltransferase reactions.*

Fucosylation of N-acetyl-chitooligosaccharides with human milk fucosyltransferases (EC 2.4.1.152 and EC 2.4.1.65) was carried out essentially as described in (24), but with 2 \* 360 μU of the enzyme (adding half of the enzyme after 2 days)/ 100 μl of reaction mixture and the acceptor concentrations were 5 mM and by incubating reactions at 37°C for four days. Fucosyltransferase V (Fuc-TV, EC 2.4.1.152, recombinant, Calbiochem) reactions were carried out under similar conditions but with 12.5 mU of the enzyme/100 μl, and the reaction mixtures were incubated at room temperature for five days. Vast excesses of GDP-Fuc in comparison to expected amount of products were used. Fucosyltransferase VI (EC 2.4.1.152, recombinant, Calbiochem) reactions were carried out under the same reaction conditions as with Fuc-TV except 2 mM acceptor and 4 mM GDP-Fucose concentration and 10 mU of the enzyme/100 μl, and incubation for 3 days at 15 37°C. The reactions were terminated by boiling for 3 minutes, the reaction mixtures were stored frozen until analysed.

*β-N-acetylhexosaminidase reactions.*

Reactions catalyzed by jack bean β-N-acetylhexosaminidase (EC 3.2.1.30) under 25 mild conditions were performed as described (25), using 0.3 mU of the enzyme / nmol releasable GlcNAc or in endochitinase reactions with Fuc<sub>1</sub>GN<sub>4,6</sub> 6 nmol of saccharides were incubated 11 h with 38 mU of the enzyme.

Reactions performed under exhaustive reactions contained 300 mU of the β-N-acetylhexosaminidase in 4.8 μl of 2.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 nmol of fucosylated N-acetyl-chito-oligosaccharides, and 40.2 μl of 50 mM sodium citrate pH 4.0. The 30 reactions were incubated at 37°C, 300 mU of fresh enzyme in 2.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added on days 2,3,5 and 7, and the reactions were stopped at day 8 by adding

1 vol of ice-cold ethanol and 8 volumes of ice-cold water.

*Reduction of oligosaccharides.*

Oligosaccharides were reduced with NaBH<sub>4</sub> essentially as described in (26). The 5 alditols were purified by gel filtration and the completeness of the reactions was verified by MALDI-TOF mass spectrometry.

*Chromatographic methods.*

Samples from the enzymatic reactions were desalted by passing them in water 10 through 1.5 ml of Dowex AG-50 (H<sup>+</sup>, 200-400 mesh) and 1.5 ml of Dowex AG-1 (AcO<sup>-</sup>, 200-400 mesh) and then purified by gel filtration HPLC in a column of Superdex Peptide HR 10/30, with ultra pure water or 50 mM NH<sub>4</sub>HCO<sub>3</sub> as eluant, at a flow rate of 1 ml/min. Gel filtration in a Biogel P-2 column (1 x 142 cm) was performed with ultrapure water, UV-absorbance was monitored at 214 nm. High- 15 pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was performed on a (4x250 mm) Dionex CarboPac PA-1 column (Dionex, CA), the samples were run isocratically with 40 or 60 mM NaOH.

*Mass spectrometry.*

20 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed in the positive ion delayed extraction mode with a BIFLEX<sup>TM</sup> mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) using 2,5-dihydroxybenzoic acid as the matrix. The matrix peaks at the low mass region were "suppressed" by using unusually high sample concentrations (100 25 pmol/μl).

Electrospray mass spectra (ESI-MS) were collected using an API365 triple quadrupole mass spectrometer (Perkin-Elmer instruments, Thornhill, Ontario, Canada). Samples were dissolved in 50 % aqueous methanol containing 0.5 mM sodium 30 hydroxide, and injected into the mass spectrometer with a nanoelectrospray ion source (Protana A/S, Odense, Denmark) at a flow rate of about 30 nL/min. MS/MS spectra were acquired by colliding the selected precursor ions to nitrogen

collision gas with acceleration voltages of 35 V (doubly-charged precursors) or 55 V (singly charged precursors).

*NMR spectroscopy.*

5 Prior to NMR experiments the saccharides were lyophilized twice from D<sub>2</sub>O and then dissolved in 300  $\mu$ L of D<sub>2</sub>O (99.996 atom %, Cambridge Isotope Laboratories, Woburn, MA, USA). The NMR experiments were carried out on a Varian Unity 500 spectrometer at 23°C using Shigemi tubes (Shigemi Co., Tokyo, Japan). In recording 1D proton spectra a modification of the WEFT sequence (27)  
10 was used. For the DQFCOSY (28) and TOCSY (29) experiments (32 scans per  $t_1$  value) matrices of 2k\*256 and 4k\*256 points were collected, and zero-filled to 2k\*512 and 4k\*512 points, respectively. A 90° shifted sine-bell weighting function was employed in both dimensions. In TOCSY, a spin-lock time of 100 ms (MLEV-17) was used. In order to resolve overlap within spin systems 1D selective  
15 TOCSY (30) spectra were recorded with mixing times varying from 10 ms to 140 ms and a gaussian selective pulse. In recording the ROESY spectrum (31, 32) the transmitter was placed outside the signal area at 5.750 ppm and a continuous-wave spin-lock with spin-lock time of 300 ms was employed. A matrix of 2k\*256 was collected and zero-filled to 2k\*512 points. A 90° shifted sine-bell weighting function was used in both dimensions. Additionally, the  $t_1$  time domain data was doubled using forward-backward linear prediction.  
20

For the DEPT(135) (33) spectrum 92,000 points were recorded with a spectral width of 18,000 Hz. For the HSQC (34) and 2D HSQC-TOCSY (35) (48 and 56 scans per  $t_1$  value, respectively), matrices of 2k\*128 and 2k\*256 points were recorded and zero-filled to 2k\*256 and 2k\*512 points, respectively and a shifted sine-bell function was used. In the 2D HSQC-TOCSY a mixing time of 120 ms was employed. The <sup>1</sup>H and <sup>13</sup>C chemical shifts were referenced to internal acetone, 2.225 and 31.55 ppm, respectively.  
25

## EXAMPLE 1

*Human Fuc-TV-catalyzed reaction of N-acetyl-chitotriose and GDP-Fucose*

The oligosaccharide mixture, generated by incubation of *N*-acetyl-chitotriose (1000 nmol, 5 mM) with GDP-Fuc (1000 nmol, 5 mM) and 25 mU Fuc-TV at room temperature for 5 days was desalted and isolated by gel filtration HPLC. MALDI-TOF-MS revealed that the mixture contained 32 mol % fucosylated *N*-acetyl-chitotriose and 68 % *N*-acetyl-chitotriose. This mixture was subjected to the "mild treatment" with jack bean  $\beta$ -N-acetylhexosaminidase, which cleaved most of the surplus *N*-acetyl-chitotriose substrate but only a small amount of the fucosylated *N*-acetyl-chitotriose product. A representative sample of all oligosaccharides was isolated from a 5 % aliquot of the digest by using gel filtration HPLC. MALDI-TOF MS analysis of showed that it consisted of fucosylated *N*-acetyl-chitotriose 67 mol %, *N*-acetyl-chitotriose 7 %,  $\text{Fuc}_1\text{GlcNAc}_2$  12 %, and *N*-acetyl-chitobiose 13 %.

The monofucosylated *N*-acetyl-chitotriose was purified from the rest of the  $\beta$ -N-acetylhexosaminidase digest by gel filtration HPLC. MALDI-TOF-MS analysis of the purified  $\text{Fuc}_1\text{GlcNAc}_3$ , Glycan 3, revealed the presence of only a 5 % *N*-acetyl-chitotriose contamination. The total yield of the purified Glycan 3 was 191 nmol (19 %).

*Mass spectrometric characterization of Fuc-TV generated Glycan 3*

An aliquot of the monofucosylated *N*-acetyl-chitotriose was reduced and permethylated and subjected to ESI-MS. The doubly-charged  $[\text{M}+2\text{Na}]^{2+}$  ion (m/z 508.8) was first selected for MS/MS (Fig. 3A). The fragments obtained could all be assigned to a tetrasaccharide carrying the fucose unit in the middle GlcNAc unit, *i.e.*  $\text{GlcNAc}\beta 1-(\text{Fuc}\alpha 1-)\text{GlcNAc}\beta 1-\text{GlcNAc}\alpha 1$ . A loss of terminal, nonsubstituted GlcNAc unit is evident from the  $\text{B}_1$  ions at m/z 282.2 (sodiated) and m/z 260.0 (protonated). Loss of methanol from the m/z 260.0 ion accounts for the m/z 228.2 and m/z 196.2 ions. The  $\text{Y}_1$  ions at m/z 316.2 and m/z 338.2 (carrying one and two sodiums, respectively) indicate that the GlcNAc alditol carried only one

monosaccharide substituent. Furthermore, the  $Y_{2\alpha}/B_2$  ion at m/z 442.4 can only arise by loss of terminal unsubstituted GlcNAc and the reduced GlcNAc residue. No fragments were observed even in closer inspection, which would represent fucosylated reduced end (*i.e.* Fuc $\alpha$ 1-GlcNAc1, m/z 490.4) or fucosylated nonreducing end GlcNAc (terminal Fuc $\alpha$ 1-GlcNAc, m/z 456.2). The origin of the fairly intense fragment at m/z 455.2 is somewhat complex. Its nature was revealed by producing the  $B_2$ ,  $Y_{2\alpha}$  and  $Y_{2\beta}$ -H<sub>2</sub>O fragments with a high orifice voltage, and by collecting MS/MS/MS data with these skimmer fragments (not shown). Only the Y ions generated the m/z 455 ion, so it must contain the reducing end. We suggest that this is an  $^{0,4}X_1$  ion, arising by a cross-ring cleavage of the penultimate GlcNAc ring.

#### *NMR-spectroscopy of Fuc-TV generated Glycan 3*

The data from 1D 1H-NMR-spectrum of Fuc-TV-generated Glycan 3 is reported in Table 2. Table 1 indicates the numbering of the residues used in Table 2. The H1, H5 and H6 signals of fucose in Glycan 3 resemble their counterparts in GlcNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc glycans (36) and in Glycan 4 analyzed by 2D NMR (Table 2), but are distinct from those in -GlcNAc $\beta$ 1-4(Fuc $\alpha$ 1-6)GlcNAc saccharides (37). Taken together, the NMR-data confirm that the fucosylated N-acetyl-chitotriose, Glycan 3, generated by Fuc-TV is GlcNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-4GlcNAc.

#### *The reaction of N-acetyl-chitotriose and GDP-Fucose catalyzed by human milk Fuc-Ts*

Incubation of N-acetyl-chitotriose (1000 nmol, 5 mM) with GDP-fucose (600 nmol, 3 mM) and 1.4 mU of the enzyme for four days at 37°C gave an oligosaccharide mixture. After ion exchange desalting, mild  $\beta$ -N-acetylhexosaminidase treatment, and repeated gel filtration HPLC runs, a sample of 221 nmol of purified fucosylated N-acetyl-chitotriose was obtained. MALDI-TOF MS revealed that the product contained 92 % of fucosylated N-acetyl-chitotriose and 7 % of N-acetyl-chitotriose. The 1D 1H-NMR-spectrum of the product was almost identical with that of Fuc-TV-generated Glycan 3. The ESI-MS-data of the Glycan 3 in

reduced form, too, were identical to those obtained with reduced derivative of the Fuc-TV-generated Glycan 3 (not shown).

## EXAMPLE 2

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### *The Fuc-TV-catalyzed reaction of N-Acetyl-Chitotetraose and GDP-Fucose*

Incubation of N-acetyl-chitotetraose (1000 nmol, 5 mM) with GDP-Fuc (1000

nmol, 5 mM) and 25 mU human Fuc-TV at room temperature for 5 days, and

isolation of the resulting oligosaccharide mixture by using gel filtration HPLC

10 gave a product consisting of 32 mol % fucosylated N-acetyl-chitotetraose and 68

% of unreacted N-acetyl-chitotetraose (Fig. 2A). The mild treatment of the mixtu-

re with jack bean  $\beta$ -N-acetylhexosaminidase followed by chromatographic removal

of the liberated from GlcNAc gave an oligosaccharide mixture consisting of

Fuc<sub>1</sub>GlcNAc<sub>4</sub> 50 %, GlcNAc<sub>4</sub> 6 %, Fuc<sub>1</sub>GlcNAc<sub>3</sub> 8 %, GlcNAc<sub>3</sub> 14 %, GlcNAc<sub>2</sub>

15 22 mol %. The composition of this mixture implies that also the fucosylated N-

acetyl-chitotetraose was cleaved much slower than N-acetyl-chitotetraose by the  $\beta$ -

N-acetylhexosaminidase, confirming the analogous data on degradation of Glycan

3. The oligosaccharide mixture was then fractionated by gel filtration HPLC,

yielding a purified product (225 nmol) that consisted of 93 mol % of fucosylated

20 N-acetyl-chitotetraose and 7 % N-acetyl-chitotetraose.

Another fucosylation mixture consisting of 11 % of the fucosylated N-acetyl-chito-

tetraose and 89 % of N-acetyl-chitotetraose was separated in an isocratic HPAE

run in a (4 x 250 mm) Dionex CarboPac PA-1 column (Dionex, Sunnyvale, CA)

25 column by using with 40 mM NaOH as the eluent (Fig. 1A). The peak eluting at

7.62 min contained the fucosylated N-acetyl-chitotetraose. The 1D 1H-NMR spec-

trum of the purified product, Glycan 4 synthesized by Fuc-TV was almost identical

with that of Glycan 4 synthesized by the Fuc-Ts of human milk (see bellow).

30 A sample of the purified Glycan 4 was degraded by the exhaustive treatment with

jack bean  $\beta$ -N-acetylhexosaminidase, yielding an oligosaccharide that behaved in

gel filtration as a compound about 1.5 glucose unit smaller than N-acetyl-chitotet-

raose, suggesting that it had lost a GlcNAc unit. This view was confirmed by applying MALDI-TOF-MS analysis, which revealed 89 mol %  $\text{Fuc}_1\text{GlcNAc}_3$  and 11 mol %  $\text{Fuc}_1\text{GlcNAc}_2$ . The minor product species had probably lost both the non-reducing end GlcNAc and the reducing end GlcNAc during the exhaustive 5 cleavage (see below, at the mild jack bean  $\beta$ -N-acetylhexosaminidase treatment of the fucosylated N-acetyl-chitohexaose).

Put together, the analytical data of Fuc-TV-generated Glycan 4 are consistent with the structure of  $\text{GlcNAc}\beta 1\text{-}4(\text{Fuca}1\text{-}3)\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}$ .

10

*Glycan 4-synthesis catalyzed by human milk Fuc-Ts*

N-Acetyl-chitotetraose (6.0  $\mu\text{mol}$ , 5 mM), and GDP-Fuc (3.0  $\mu\text{mol}$ , 2.5 mM) were incubated with 8.7 mU of the enzyme for four days at 37°C. After desalting and gel filtration HPLC-run, the fucosylated product was separated from the non-fucosylated N-acetyl-chitotetraose by HPAEC using isocratic run with 40 mM NaOH (not shown). The purified product was desalted by ion exchange and was further purified by gel filtration HPLC yielding 744 nmol monofucosylated N-acetyl-chitotetraose. MALDI-TOF mass spectrometry of the product revealed that it was uncontaminated by N-acetyl-chitotetraose acceptor (not shown).

20

A sample of the purified Glycan 4 generated by human milk Fuc-Ts was treated exhaustively with jack bean  $\beta$ -N-acetylhexosaminidase. MALDI-TOF-MS analysis of the HPLC-purified oligosaccharides of the digest revealed 91 mol %  $\text{Fuc}_1\text{GlcNAc}_3$  and 9 %  $\text{Fuc}_1\text{GlcNAc}_2$ .

25

*Mass spectrometric characterization of Glycan 4 generated by human milk Fuc-Ts*

An aliquot of the monofucosylated N-acetyl-chitotetraose was reduced and permethylated and subjected to ESI-MS. The doubly-charged  $[\text{M}+2\text{Na}]^{2+}$  ion (m/z 631.6) was selected for MS/MS (Fig. 3B). The low-mass region of the MS/MS 30 spectrum resembles that of the monofucosylated N-acetyl-chitotriose, showing the same  $\text{B}_1$  fragment s for the unsubstituted terminal GlcNAc unit (m/z 282.2, m/z 260.2), and  $\text{Y}_1$  ions for the reducing end GlcNAc alditol (m/z 316.2, m/z 338.2).

In addition, the  $Y_{2\alpha}/B_2$  ion (m/z 442.4), the  $B_3$  ion (m/z 946.8), and the  $Y_{3\alpha}$  ion (m/z 980.6) verify that the fucose unit is linked to either of the midchain GlcNAc units. The  $Y_2$  ion at m/z 561.4 indicates that this disaccharide fragment only carried one monosaccharide substituent, and thus the fucose must reside at the 5 penultimate GlcNAc residue. This is directly confirmed by the  $B_2$  ion at m/z 701.6, carrying the non-reducing terminus structure  $\text{GlcNAc}\beta 1\text{-(Fuc}\alpha 1\text{-)}\text{GlcNAc}$ . Even closer investigation of the minute fragments in the MS/MS data did not reveal any specific fragments for other pentasaccharide isomers, and we therefore conclude that N-acetyl-chitotetraose is fucosylated solely to the penultimate 10 GlcNAc residue next to the non-reducing end.

*NMR-spectroscopic analysis of Glycan 4 produced by human milk Fuc-Ts*

The 1D  $^1\text{H-NMR}$  spectrum of the purified product, Glycan 4 (Table 3) revealed fucose H1, H5 and H6 signals characteristic to  $\alpha 3$ -linked fucose rather than  $\alpha 6$ -linked fucose in unconjugated N-glycans (36, 37). The H4 signal of the distal GlcNAc6 was similar to its analogs in Glycan 3, confirming that the fucose is linked to the penultimate GlcNAc unit close to the non-reducing end. The proton signals of the fucose and the non-reducing end GlcNAc6 were assigned from DQFCOSY and TOCSY spectra of Glycan 4. The 2D NMR-data strengthen the 15 notion that the non-reducing end elements of Glycan 4 resemble those of Glycan 3. 20

Taken together, the NMR-data confirm the MS-results and the degradation data, establishing that Glycan 4 generated by human milk Fuc-Ts represented also 25  $\text{GlcNAc}\beta 1\text{-4(Fuc}\alpha 1\text{-3)}\text{GlcNAc}\beta 1\text{-4GlcNAc}\beta 1\text{-4GlcNAc}$ .

**EXAMPLE 3**

*Human FucTV-Catalyzed Reaction of N-Acetyl-Chitohexaose and GDP-Fucose*

30 N-Acetyl-chitohexaose (1.0  $\mu\text{mol}$ , 5 mM) was fucosylated with GDP-Fucose (1.0  $\mu\text{mol}$ , 5 mM) and 25 mU Fuc-TV at room temperature for 5 days. MALDI-TOF MS of the purified representative mixture of the product oligosaccharides showed

that 31 % of N-acetyl-chitohexaose was fucosylated (Fig. 2B). The product saccharides were further purified in HPAE-chromatography run isocratically with 40 mM NaOH (Fig 1B). The peak eluting at 7.09 min was pooled, was desalted and was further purified by gel filtration HPLC. The product heptasaccharide (195 5 nmol) showed in MALDI-TOF analysis 96 % of monofucosylated N-acetyl-chitohexaose, Glycan 6, and 4 % of N-acetyl-chitohexaose, Fig. 2C. The 1D 1H NMR spectrum (Table 2) shows the H1 signals in the GlcNAc5, the GlcNAc6 and the fucose units in Glycan 6, which are nearly identical with their counterparts in Glycan 4. This suggests that the Glycan 6 is GlcNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-10 4GlcNAc $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc. This notion is further supported by the identical H4-signals of the distal GlcNAc6 units and the fucose H5 resonances in the two glycans. The similarity of this latter pair of signals in the two glycans is particularly significant, because they are known to interact in Glycan 4 (see above), and are likely to be very sensitive to stuctural differences. Likewise, 15 the similarity of fucose H5 signals in Glycan 6, and Glycan 4 speaks for the identity of the nonreducing area in these three saccharides.

#### *Treatment of Glycan 6 with jack bean $\beta$ -N-acetylhexosaminidase*

A Fuc-TV-generated mixture of Glycan 6 and N-acetyl-chitohexaose (27:73 20 mol/mol (Fig. 2B)) was subjected to the mild jack bean  $\beta$ -N-acetylhexosaminidase treatment as described earlier. The resulting oligosaccharides were isolated as a representative mixture by using gel filtration HPLC. MALDI-TOF MS revealed that mixture consisted of Fuc<sub>1</sub>GlcNAc<sub>6</sub> (3.5 mol %), GlcNAc<sub>6</sub> (1.7 %), Fuc<sub>1</sub>GlcNAc<sub>5</sub> (40 %), Fuc<sub>1</sub>GlcNAc<sub>4</sub> (3.5 %); small N-acetyl-chitooligosaccharides ranging 25 from N-acetyl-chitobiose to N-acetyl-chitopentaose were also observed (Fig. 2D). The principal product of the digest, the monofucosylated N-acetyl-chitopentaose, Glycan 5, was purified in a gel filtration HPLC-run. MALDI-TOF MS suggested that Glycan 5 was obtained hereby in a purity of 83 %; it was contaminated by several penta- and hexasaccharides. The chemical shifts are presented in Table 2. 30 The NMR data show nearly identical H1 signals in the GlcNAc5, the GlcNAc6 and the fucose units in Glycan 5 and Glycan 4, suggesting, that the structure of the former is GlcNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc.

This notion is further supported by the identical H4-signals of the distal GlcNAc6 units and the fucose H6 resonances in the two glycans. Hence, the mild jack bean  $\beta$ -N-acetylhexosaminidase treatment had surprisingly released a GlcNAc unit from the reducing end of Glycan 6, but had not attacked the reducing end of the product, Glycan 5, as efficiently.

*Treatments of purified Glycan 6 and Glycan 4 by the endo-chitinase activity of jack bean  $\beta$ -N-acetylhexosaminidase*

When the purified Fuc-TV-generated Glycan 6 was subjected to the mild treatment with jack bean  $\beta$ -N-acetylhexosaminidase, the resulting digest showed in MALDI-TOF MS only 5 mol % of intact substrate; 85 % of the material was converted into Fuc<sub>1</sub>GlcNAc<sub>5</sub> and 11 % into Fuc<sub>1</sub>GlcNAc<sub>4</sub> (Fig. 2E). The data confirm that the endochitinase cleaves fast Glycan 6, releasing mainly one GlcNAc unit from the reducing end and the enzyme may be able to cleave off also a N-acetyl-chitobiose unit from the reducing end of Glycan 6, generating Glycan 4. Alternatively, Glycan 4 may have been formed by the release of a GlcNAc unit from the reducing end of Fuc<sub>1</sub>GlcNAc<sub>5</sub>.

When the purified Fuc-TV-generated Glycan 4 was subjected to the mild treatment with jack bean  $\beta$ -N-acetylhexosaminidase, the resulting digest showed in MALDI-TOF MS that most (91 mol %) of the substrate had survived and only 6 % of Fuc<sub>1</sub>GlcNAc<sub>3</sub> had been formed; 3 % of the material was found as fucose-free N-acetyl-chitotetraose (Fig. 2F). Hence, the endo-chitinase activity appeared to cleave off slowly one GlcNAc unit from the reducing end of Glycan 4.

25

**EXAMPLE 4**

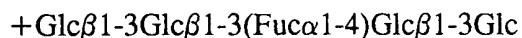
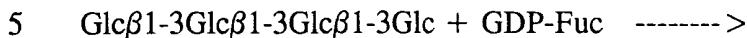
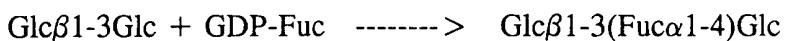
*Fucosylation of cellobiose*

Cellobiose (4000 nmol, 20 mM, from Thomas Kerfoot and Co. ltd) was incubated with GDP-[<sup>14</sup>C]Fuc (1000 nmol, 5 mM, 100 000 cpm) and recombinant fucosyltransferase V (25 mU, 50 ml of the commercial enzyme preparation from Calbioc-hem) in 50 mM MOPS-NaOH pH 7.5 containing 8 mM MnCl<sub>2</sub>, 100 mM NaCl, 1

mg/ml BSA and 0.02 % NaN<sub>3</sub> in total volume of 200 ml at room temperature for 4 days and 16 hours. The reaction products were desalted by running through 1.5 ml beds of Dowex AG 1-X8 and Dowex AG 50W-X8. Part of the products were run in paper chromatography which showed a trisaccharide like product migrating 5 close to maltotriose (Rmaltotriose= 0.97 marker, glycerol in the sample may affect the migration) and a broad peak (broadened by glycerol in the sample) probably corresponding to free [<sup>14</sup>C]Fuc. Solvent A was used as described in (38) , using the upper phase of n-butanol-acetic acid:water (4:1:5, v/v, solvent A) and scintillation counted from small strips of paper. Major part (75 %) of the products were 10 run in gel filtration chromatography in Biogel P-2 (Biorad) column. A peak of radioactive products (256 nmol by radioactivity, calculated total yield 340 nmol) eluting at position expected for monofucosylated cellobiose was obtained and another radioactive peak containing [<sup>14</sup>C]Fuc-like material. Fractions in the first half of the product peak were analyzed to contain saccharide with size corresponding 15 to fucosylated cellobiose in MALDI-TOF mass spectrometry, monoisotopic m/z [M+Na]<sup>+</sup> close to 511.2 and m/z [M+K]<sup>+</sup> close to 527.2, the fractions contained very minor amounts or no other possible saccharides like cellobiose or cellotriose. The pooled saccharide (150 nmol by radioactivity) were analyzed by NMR-spectroscopy. NMR-spectroscopy revealed signals of Fuc H1 $\alpha$  at 5.425 ppm, 20 Fuc H1 $\beta$  at 5.369 ppm and signals of the reducing Glc H1a at 5.189 ppm and Glc H1 $\beta$  at 4.655 ppm similarly as described for -Gal $\beta$ 1-4(Fuc $\alpha$ 1-3)Glc (39).

A product (<0.5 pmol) migrating similarly in paper chromatography (Rmaltotriose=1.04) can be obtained by incubating lysate (40 mg of protein) of CHO-cells 25 expressing full length Fuc-TV with GDP-[<sup>14</sup>C]Fuc (1 nmol, 100 000 cpm) and 100 nmol of cellobiose for 1 hour as described in (40).

## EXAMPLE 5

*Reactions with laminaribiose and laminaritetraose*

Laminaribiose (1000 nmol, 5 mM, Seikagaku) and laminaritetraose (1000 nmol, 5

10     mM, Seikagaku) were reacted with GDP-[<sup>14</sup>C]Fuc (1000 nmol, 5 mM, 100 000

cpm) and recombinant fucosyltransferase V (25 mU, 50 ml of the commercial

enzyme preparation from Calbiochem) as above. The products of both reactions

were desalted as above. The products from laminaribiose were purified by P-2 gel filtration chromatography and radioactive trisaccharide-like product was obtained.

15

The desalted products from laminaritetraose were also purified by P-2 gelfiltration chromatography and a radioactive (174 nmol by radioactivity) peak eluting a pentasaccharide product as expected, was obtained. 131 nmol of product with monoisotopic m/z [M+Na]<sup>+</sup> close (difference less than 0.1 %) to 835.3 and m/z [M+-

20     K]<sup>+</sup> close to 851.3 were pooled from front and middle fractions of the peak, the fractions contained less than 4 % of the acceptor, no other saccharide products were observed in analysis by MALDI-TOF mass spectrometry. The purified product saccharides were analyzed by NMR spectroscopy.

25     EXAMPLE 6

*Desulfation, reduction, and fucosylation of chondroitin sulfate*

Chondroitin sulfate (shark cartilage, Sigma) was cleaved by hyaluronidase (bovine

testes, Sigma; chondroitin sulfates can be also cleaved with chondroitinases giving

30     oligosaccharides with delta-uronic acid at non reducing end) to sulfated oligosaccharides such as [GlcA $\beta$ 1-3(sulf-6)GalNAc $\beta$ 1-4]<sub>n</sub>GlcA $\beta$ 1-3(sulf-6)GalNAc. Fractions containing tetrasaccharide-like materials were purified and desulfated as

essentially as described in (41). Tetrasaccharide and hexasaccharide fractions were purified by gel filtration and anion exchange chromatographies. Similar oligosaccharides were also produced by first desulfating and then cleaving by hyaluronidase and purifying the products. The GlcA-residues of the tetrasaccharide were reduced 5 to Glc by EDAC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and NaBH<sub>4</sub>. The reduced tetrasaccharide Glc $\beta$ 1-3GalNAc $\beta$ 1-4Glc $\beta$ 1-3GalNAcol was purified and fucosylated using GDP-Fuc and human milk fucosyltransferase(s). The MAL-10 DI-TOF mass spectrometry revealed peaks at m/z 919.8 and m/z 935.8 corresponding to the product Glc $\beta$ 1-3GalNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)Glc $\beta$ 1-3GalNAcol (calc. m/z [M+Na]<sup>+</sup> is 919.7 and m/z [M+K]<sup>+</sup> is 935.7).

#### EXAMPLE 7

15 GlcNAcyl $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc, wherein Acyl is trans-9-octadecenoyl, synthesis of the acceptor being described in (13), can be incubated with Fuc-TVI and MnCl<sub>2</sub> under conditions described above for Fuc-TVI, but acceptor concentrations between 0.1-0.5 mM are preferred. The product GlcNAcyl $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc is purified chromatographically and analyzed by NMR-spectroscopy and mass spectrometry.

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#### EXAMPLE 8

25 N-Acetyl-chitotetraose can be incubated with human Fuc-TVI and MnCl<sub>2</sub> under conditions described above for Fuc-TVI. The product GlcNAc $\beta$ 1-4(Fuc $\alpha$ 1-3)GlcNAc $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc can be obtained with almost quantitative yield and purified by HPAE-chromatography. The NMR-data and mass spectrometry data of the product are practically identical with the fucosylated N-acetyl-chitotetraose obtained by Fuc-TV.

Table 1. Structures of the fucosylated N-acetyl-chito-oligosaccharides in present study. GN is GlcNAc and Fuc is L-fucose, line - indicates  $\beta$ 1-4-linkage and line / is  $\alpha$ 1-3-linkage, residue numbering is in italics.

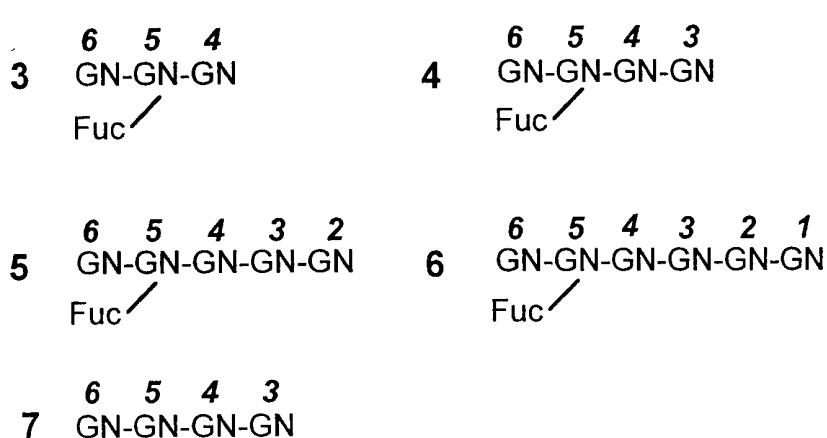


Table 2.  $^1\text{H}$  Chemical shifts (ppm) of structural reporter groups for Glycans 3-7 at 23 °C.

n.d. = not determined

Proton Residue	Glycans				
	3	4	5	6	7
H1 1	-	-	-	5.181( $\alpha$ )/4.688( $\beta$ )	-
2	-	-	5.182( $\alpha$ )/4.688( $\beta$ )	4.578/4.565 <sup>a</sup>	-
3	-	5.184( $\alpha$ )/4.689( $\beta$ )	4.579/4.567 <sup>a</sup>	4.565	5.185( $\alpha$ )/4.689( $\beta$ )
4	5.185( $\alpha$ )/4.688( $\beta$ )	4.579/4.570 <sup>a</sup>	4.567	4.565	4.58
5	4.580/4.571 <sup>a</sup>	4.570	4.567	4.565	4.58
6	4.522	4.519	4.517	4.516	4.58
fucose	5.124	5.119	5.117	5.116	-
H4	6	3.238	3.237	3.236	3.235
H5	fucose	4.764	4.762	4.762	n.d.
CH3	fucose	1.268	1.267	1.265	1.264

<sup>a</sup> The two chemical shift values given arise from signals representing the  $\alpha$ - and  $\beta$ -pyranosidic forms of the oligosaccharides respectively.

Table 3.  
NMR-data of the Glycan 4.

Proton	GlcNAc6	Fucose
H1	4.519	5.119
H2	3.735	3.707
H3	3.535	3.938
H4	3.237	3.803
H5	3.428	4.762
H6	3.607	-
H6'	3.963	-
CH3	-	1.267

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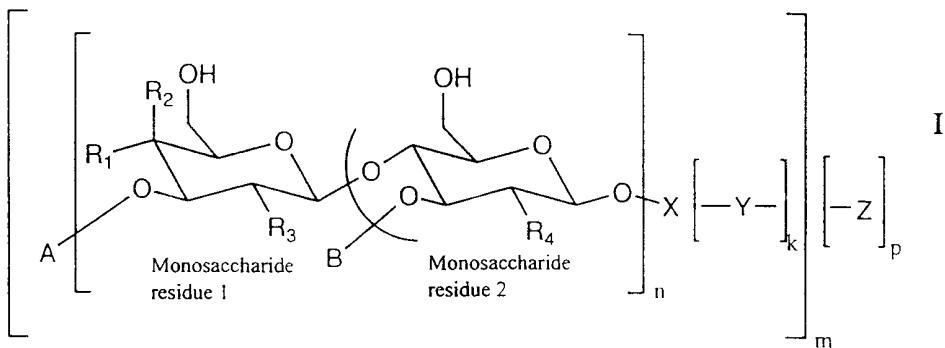
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## Claims

## 1. Oligosaccharides having the formula

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wherein A is H or a glycosidically  $\beta$ 1-3 linked D-glucopyranosyl residue (Glc $\beta$ 1-3), R<sub>1</sub> is OH, R<sub>2</sub> is H and R<sub>3</sub> is OH or acylamido, -NH-acyl (*i.e.* monosaccharide 1 is Glc, or GlcNAcyl) or R<sub>1</sub> is H, R<sub>2</sub> is OH and R<sub>3</sub> is acetamido -NHCOCH<sub>3</sub> (*i.e.* monosaccharide 1 is GalNAc), B is H, or an  $\alpha$ -L-fucosyl or an  $\alpha$ -L-fucosyl analogue, and R<sub>4</sub> is OH or acetamido -NHCOCH<sub>3</sub> (*i.e.* monosaccharide 2 is optionally fucosylated Glc or GlcNAc), the curved line between the saccharide units indicating that the monosaccharide 1 is  $\beta$ 1-4 linked to monosaccharide 2 when B is linked to the position 3 of the monosaccharide 2, and the monosaccharide 1 is  $\beta$ 1-3 linked to monosaccharide 2 when B is linked to the position 4 of the monosaccharide 2, monosaccharide 1 is GalNAc only when monosaccharide 2 is Glc, n is 1 to 100, with the proviso that there is always at least one  $\alpha$ -fucosyl or  $\alpha$ -fucosyl analogous group present in the molecule, and

25

i) p and k are 0 and m is 1, in which case X is H, an aglycon residue or a monosaccharide selected from the group consisting of Glc, GlcNAc, Gal or GalNAc, optionally in reduced form, or oligosaccharide containing one or more of said monosaccharide units, the monosaccharide 2 being  $\beta$ 1-2,  $\beta$ 1-3,  $\beta$ 1-4 or  $\beta$ 1-6 linked to

30

saccharide X, with the proviso that X is not H when both monosaccharides 1 and 2 are GlcNAc, B is L-fucosyl and n is 1, or

ii) p is 1, k is 0 or 1 and  $1 \leq m \leq 1000$ , in which case X is a straight bond, or a

mono- or oligosaccharide as defined under i),

Y is a spacer or linking group capable of linking the saccharide 2 or X to Z,  
and Z is a mono- or polyvalent carrier molecule.

5 2. The oligosaccharides according to claim 1, characterized in that B is  $\alpha$ -L-fu-  
cosyl.

3. The oligosaccharides according to claim 1 and 2, characterized in that A and/or  
X are/is H.

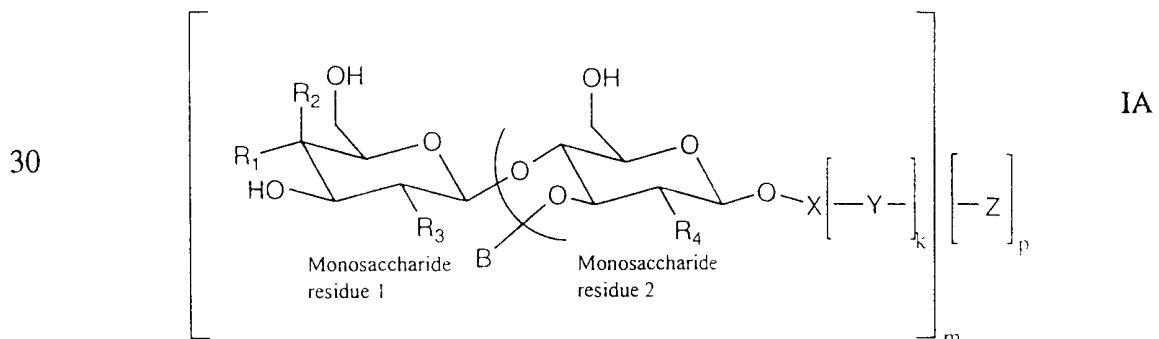
10 4. The oligosaccharides according to claim 1, 2 or 3, characterized in that mo-  
nosaccharide 1 is Glc or GlcNAc.

5. The oligosaccharides according to claim 1, characterized in that X is an oli-  
15 gosaccharide containing from 2 to 10 monosaccharide units, the monosaccharide  
units being glucosidically  $\beta$ 1-4 or  $\beta$ 1-3 linked Glc or GlcNAc residues.

6. The oligosaccharides according to claim 1, characterized in that m is 1 to 100,  
preferably 1 to 10, and n is 1 to 10.

20 7. The oligosaccharides according to claim 1, characterized in that X is an aglycon  
group selected from lower alkyl or alkenyl group 1 to 7, or 2 to 7 carbon atoms,  
respectively, or a phenyl or benzyl group, or 4-methylumbelliferyl.

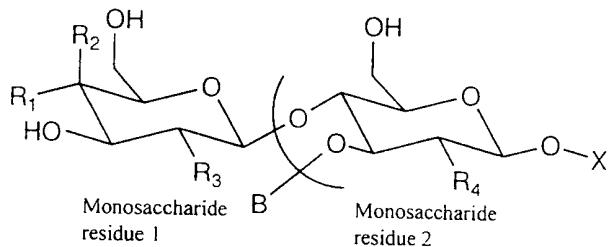
25 8. The oligosaccharides according to claim 1, characterized in that they have the  
formula



wherein the symbols have the meanings given in connection with the formula I in claim 1, preferably the monosaccharides 1 and 2 are independently Glc and GlcNAc, B is L-fucosyl, and X is Glc or GlcNAc or a  $\beta$ 1-3 or  $\beta$ 1-4 linked oligomer comprising up to 10 units of Glc and/or GlcNAc.

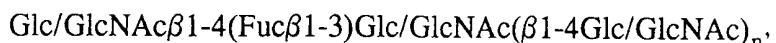
9. The oligosaccharides according to claim 1, characterized in that A is H and the monosaccharides 1 and 2 are independently Glc, or GlcNAc, B is L-fucosyl, p,k = 0 and n = m = 1, and X is Glc or GlcNAc or a  $\beta$ 1-3 or  $\beta$ 1-4 linked oligomer comprising up to 10 units of Glc and/or GlcNAc having the formula

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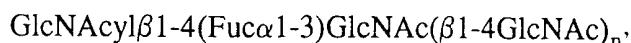
10. The oligosaccharides according to claim 1 having the formula



wherein n' is the integer 1 to 8, preferably 1 to 6.

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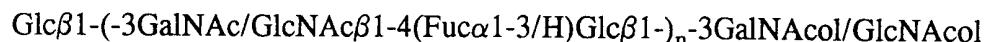
11. The oligosaccharides according to claim 1 having the formula



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wherein n' has the meaning given above and acyl is an alkanoyl group which preferably contains 8 to 24 carbon atoms and 1 to 3 double bonds.

12. The oligosaccharides according to claim 1, characterized in that  $1 \leq n \leq 1000$ , monosaccharide residue 1 is GlcNAc or GalNAc and monosaccharide residue 2 is optionally fucosylated Glc, such as the compound



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13. A process for the preparation of the oligosaccharides according to claim 1, characterized in that a compound of the formula I wherein B is always H, is fucosylated with donor nucleotide sugar containing L-fucose, or an analogue thereof, in the presence of a fucosyl transferase enzyme, and the fucosylated saccharide so prepared is optionally recovered.

14. The process according to claim 13, characterized in that the fucosyltransferase is mammalian  $\alpha 1-3$  or  $\alpha 1-3/4$  fucosyltransferase.

15

15. The process according to claim 13 or 14, characterized in that a N-acetylchitooligosaccharide is used as the starting material.

16. The process according to any one of claims 13 to 15, characterized in that the donor nucleotide sugar containing L-fucose is GDP-L-fucose.

20

17. The process according to any one of the claims 14 to 16, characterized in that the fucosyltransferase is human  $\alpha 1-3$  fucosyltransferase or  $\alpha 1-3/4$ fucosyltransferase III-VII, IX or  $\alpha 1-3/\alpha 1-3/4$ fucosyltransferase of human milk.

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18. The process according to any one of the preceding claims 13 to 17, characterized in that it comprises the further step of reacting the product obtained with the formula I with  $\beta$ -N-acetyl-hexosaminidase.

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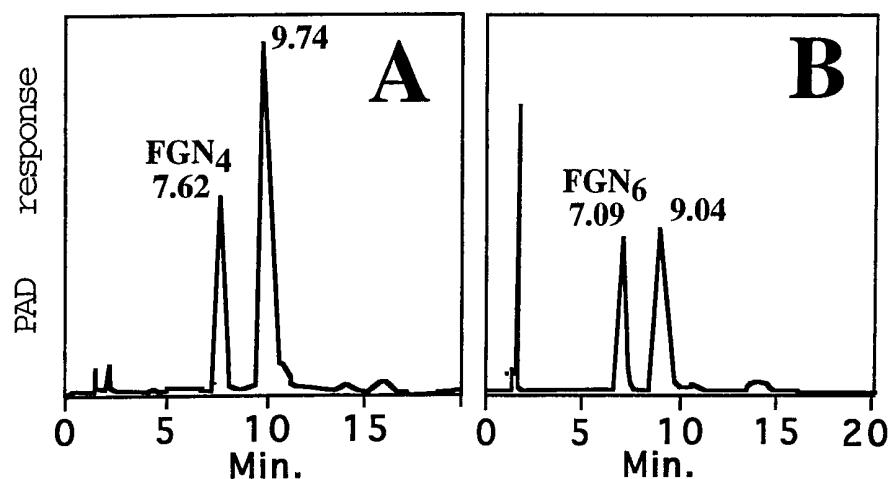


Fig. 1

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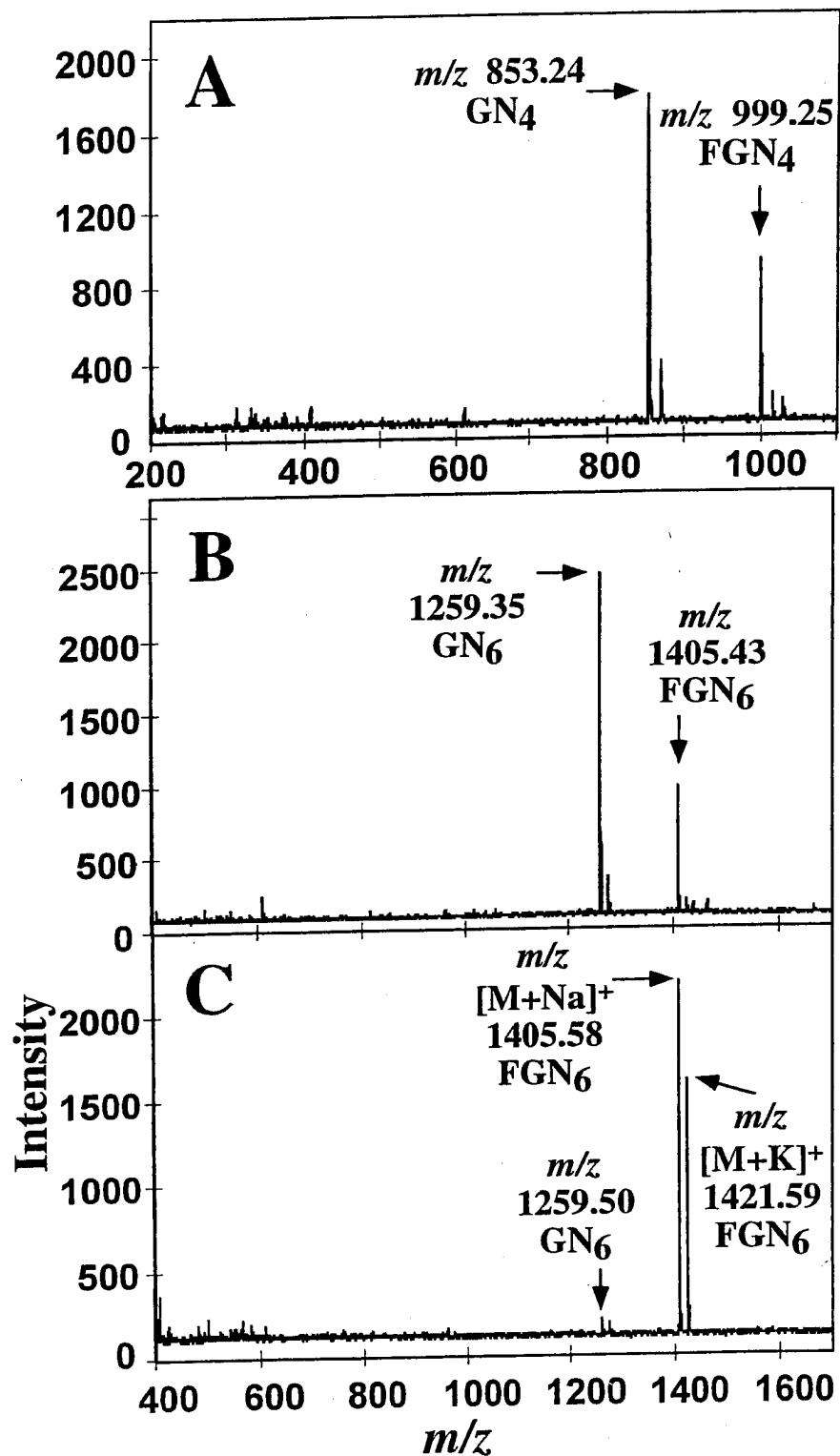


Fig. 2

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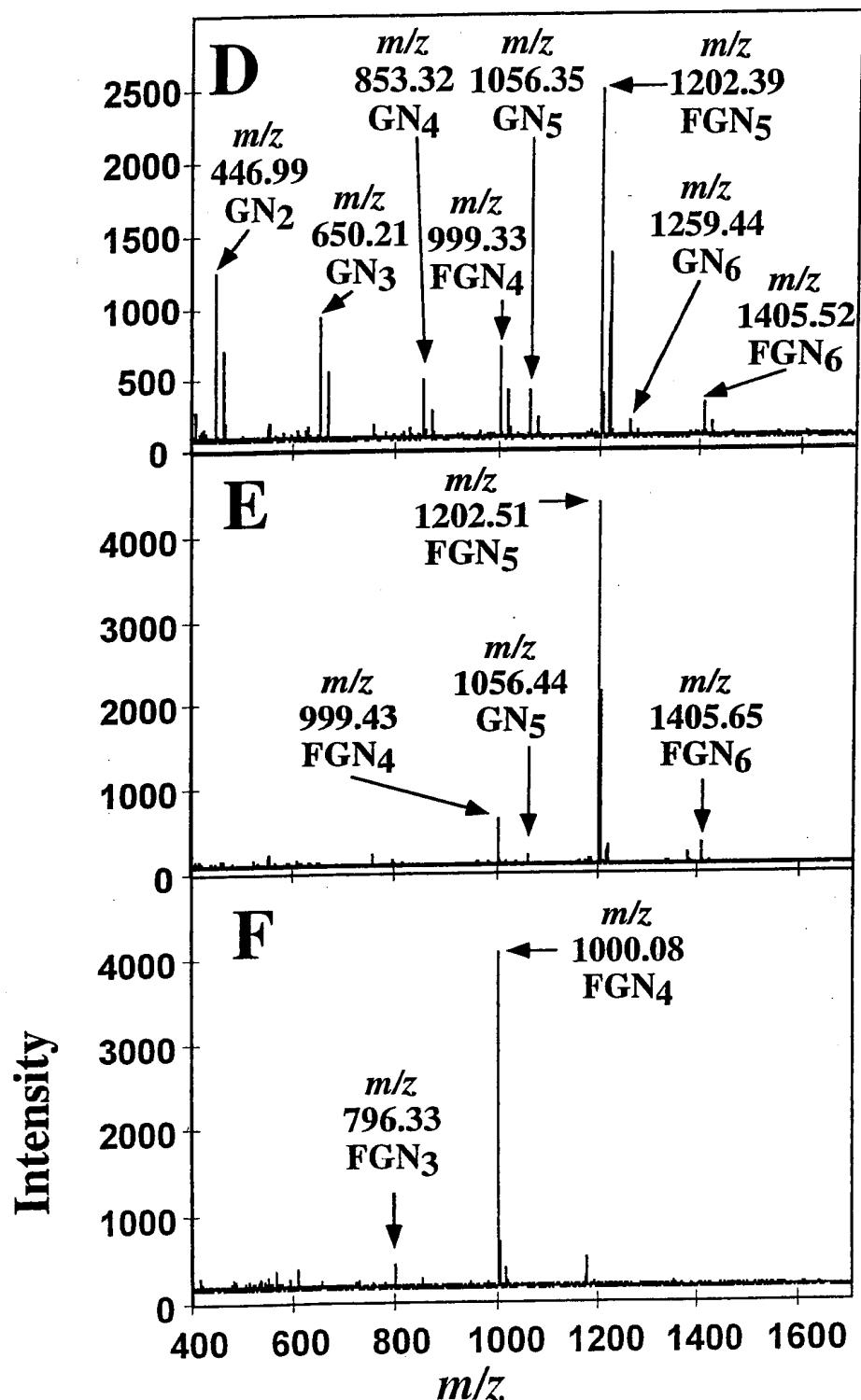


Fig. 2 (continued)

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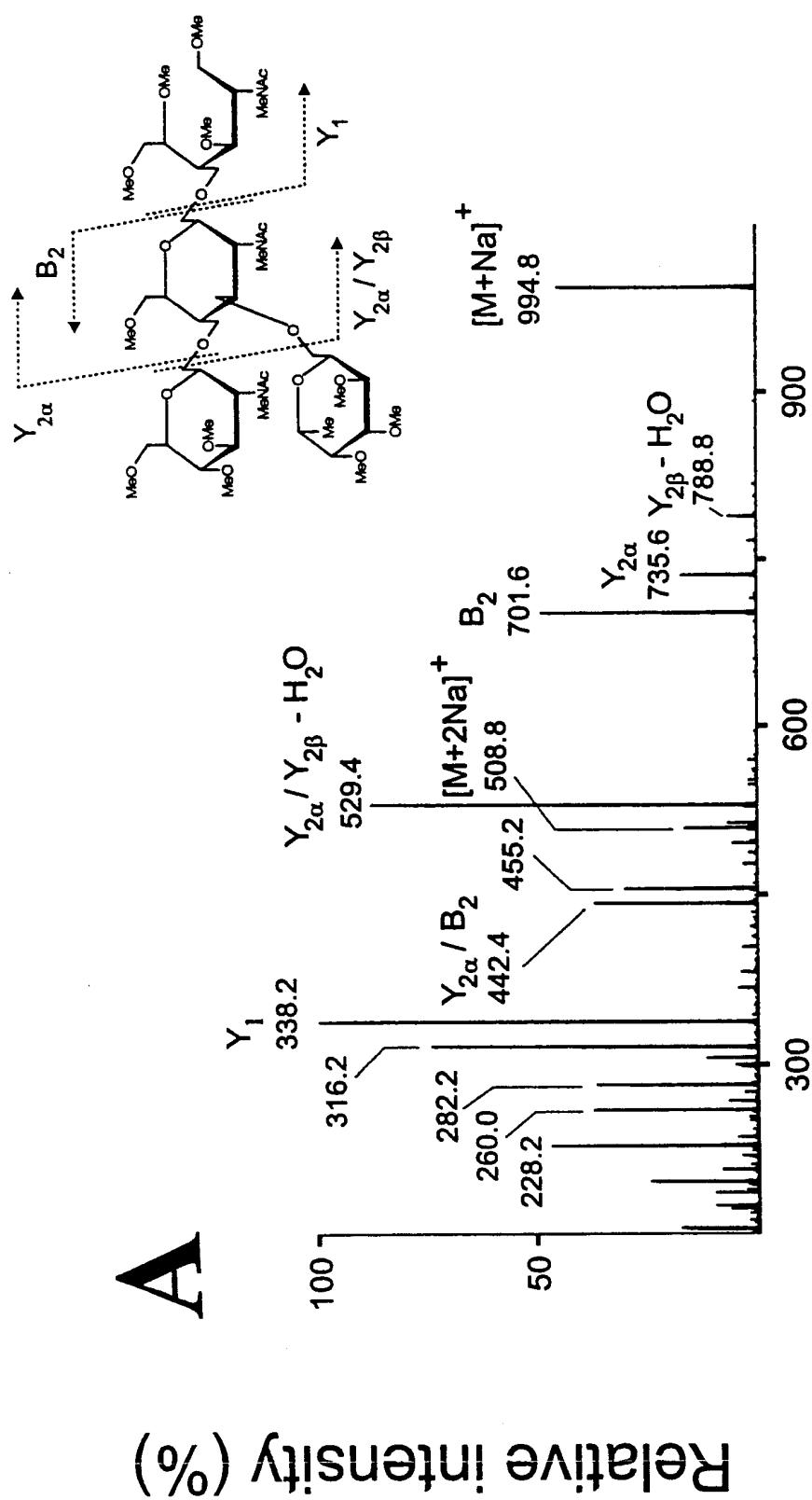


Fig. 3

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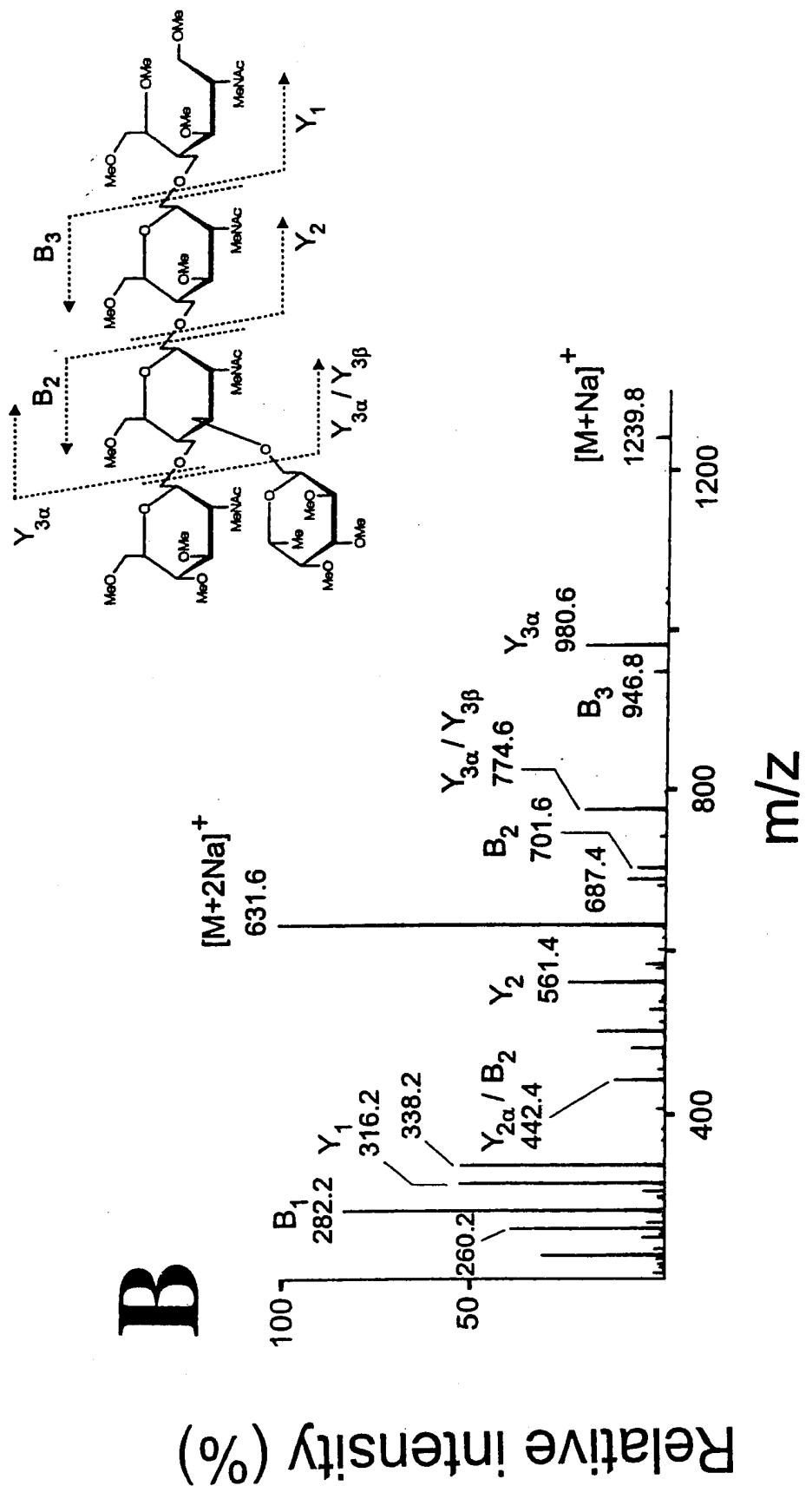


Fig. 3 (continued)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 00/00803

## A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C07H 3/06, C07H 15/04, C08B 37/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C07H, C08B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5922577 A (DEFREES ET AL), 13 July 1999 (13.07.99), column 1, line 51 - column 2, line 2; column 9, line 57 - column 10, line 13; column 10, line 46 - line 64, figure 3, abstract  --	1-18
X	US 5374655 A (KASHEM ET AL), 20 December 1994 (20.12.94), column 4, line 60 - column 5, line 9, abstract, figures, claims  --	1-18

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family

Date of the actual completion of the international search

25 January 2001

Date of mailing of the international search report

29-01-2001

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 00/00803

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. Am. Chem. Soc. 1998, 120, 2184-2185 "Unexpected Enzymatic Fucosylation of the Hindered Tertiary Alcohol of 3-C-Methyl-N-Acetylglucosamine Produces a Novel Analogue of the LeX-Trisaccharide". Xiangping Qian, et al See the whole article  --	1-18
A	Glycoconjugate Journal 15, 873-883 (1998) "In vitro a 1-3 or a 1-4 fucosylation of type I and II oligosaccharides with secreted forms of recombinant human fucosyltransferases III and VI". Manfred Nimtz, et al  --	1-18
A	Tetrahedron Letters, Vol 38, No 33, pp. 5861-5864, 1997 "Chemoenzymatic Synthesis of GM3, Lewis x and Sialyl Lewis x Oligosaccharides in 13C-Enriched Form". Mark A. Probert, et al  -- -----	1-18

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

27/12/00

International application No.

PCT/FI 00/00803

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		JP 2823358 B	11/11/98
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