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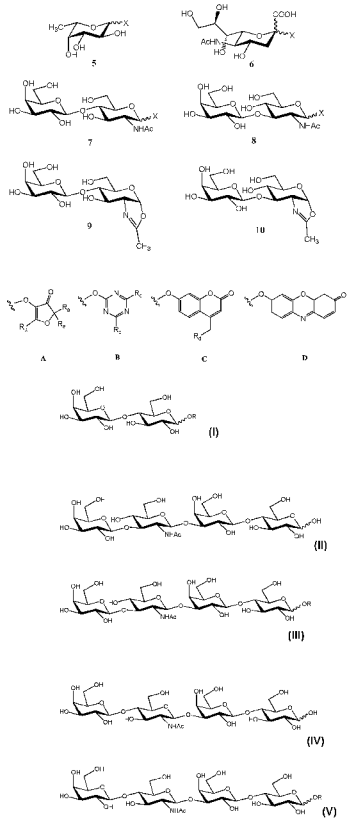
(54) Title: METHOD FOR GENERATING HUMAN MILK OLIGOSACCHARIDES (HMOs) OR PRECURSORS THEREOF



(57) Abstract: A method for generating human milk oligosaccharides (HMOs) or precursors thereof, compounds obtainable by the method and uses and compositions involving such compounds. The method comprising the steps of a) Providing at least one donor selected from the group of compounds of any of formulae 5 to 10, b) Providing at least one acceptor from a group of lactose, LNT, LNnT and derivatives thereof, c) Providing at least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity, d) Preparing a mixture of the at least one donor, at least one acceptor and at least one enzyme provided in steps a),b) and c); and e) Incubating the mixture prepared according to step d).

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METHOD FOR GENERATING HUMAN MILK OLIGOSACCHARIDES (HMOs) OR PRECURSORS THEREOF

FIELD OF THE INVENTION

The present invention relates to a method for generating human milk oligosaccharides (HMOs) or precursors thereof and to compounds suitable for use in or obtainable by the method of the present invention. The invention furthermore describes uses of and products involving such compounds.

BACKGROUND OF THE INVENTION

Human milk oligosaccharides (HMOs) have attracted much interest in the past few years. In particular, commercialization efforts for the synthesis of these complex carbohydrates including secreted oligosaccharides have increased significantly due to their roles in numerous biological processes occurring in the human organism. One prominent natural human source of such complex oligosaccharides is mammalian milk. Mammalian milk contains up to 10% carbohydrate, of which the disaccharide, lactose (Gal(β1-4)Glc), is usually a prominent component. Milk and colostrum also contain lesser amounts of other saccharides, referred to as milk oligosaccharides, nearly all of which have a lactose unit at their reducing end to which GlcNAc, Gal, Fuc and/or Neu5Ac or Neu5Gc residues can be attached (Messer and Urashima, 2002, Trends Glycosci. Glycotech, 14, 153-176; and Urashima *et al.*, Advanced Dairy Chemistry, Volume 3: Lactose, Water, Salts and Minor Constituents, 2009, pp. 295-349).

To date, the structures of at least 115 oligosaccharides of human milk have been determined, while mass spectra (MS) data have suggested the presence of almost 130 oligosaccharides in human milk or colostrums (Newburg and Neubauer, 1995, Carbohydrates in milks: Analysis, quantities and significance. In: Handbook of Milk Composition (R.G.Jensen, ed.), pp. 273-249, Academic Press, San Diego, USA). Moreover, matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOFMS) analyses suggest that polysaccharides, consisting of more than 50 monosaccharide residues as indicated by size exclusion chromatography, are also present in human milk. Therefore, considerably more than 130 different saccharides are probably present in human milk (see also Urashima *et al.*, Advanced Dairy Chemistry, Volume 3: Lactose, Water, Salts and Minor Constituents, 2009,

pp. 295-349; and TADASU URASHIMA et al, MILK OLIGOSACCHARIDES, Nova Biomedical Books, *New York*, 2011, ISBN: 978-1-61122-831-1).

The 115 human milk oligosaccharides of which the structures have been determined to date, can be grouped into 13 series based on their core structures. Such 13 core structures are exemplarily shown in Table 1 below:

No	Core name	Core structure
1	lactose (Lac)	Galβ1-4Glc
2	lacto-N-tetraose (LNT)	Galβ1-3GlcNAcβ1-3Galβ1-4Glc
3	lacto-N-neotetraose (LNnT)	Galβ1-4GlcNAcβ1-3Galβ1-4Glc
4	lacto-N-hexaose (LNH)	Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
5	lacto-N-neohexaose (LNnH)	Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
6	para-lacto-N-hexaose (para-LNH)	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc
7	para-lacto-N-neohexaose (para-LNnH)	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc
8	lacto-N-octaose (LNO)	Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
9	lacto-N-neooctaose (LNnO)	Galβ1-4GlcNAcβ1-3(Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
10	Iso-lacto-N-octaose (iso-LNO)	Galβ1-3GlcNAcβ1-3(Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-6)Galβ1-4Glc
11	para-lacto-N-octaose (para-LNO)	Galβ1-3GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc
12	lacto-N-neodecaose (LNnD)	Galβ1-3GlcNAcβ1-3[Galβ1-4GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-6]Galβ1-4Glc
13	lacto-N-decaose (LND)	Galβ1-3GlcNAcβ1-3[Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-6]Galβ1-4Glc

Table 1: 13 different core structures of human milk oligosaccharides (HMOs)

As found by Urashima *et al.* (see also Urashima *et al.*, Advanced Dairy Chemistry, Volume 3: Lactose, Water, Salts and Minor Constituents, 2009, pp. 295-349; and TADASU URASHIMA et al, MILK OLIGOSACCHARIDES, Nova Biomedical Books, *New York*, 2011, ISBN: 978-1-61122-831-1) the many variations of the oligosaccharides are constructed by the addition of a Neu5Acβ2-3/2-6 residue to Gal or GlcNAc, and of Fucα1-2/1-3/1-4 to

Gal, GlcNAc or a reducing Glc of the core units. The main structural features of human milk oligosaccharides are the presence of oligosaccharides containing the type I unit (Gal(β1-3)GlcNAc), as well as those containing the type II unit (Gal(β1-4)GlcNAc), and oligosaccharides containing the type I predominate over those containing the type II unit. The milk oligosaccharides of other species investigated to date mostly exhibit the type II but not the type I unit.

The large variety of oligosaccharides in human milk and colostrum and the difference to other species, however, makes it difficult to prepare suitable replacements in foods, particularly in infant food formulae, which display at least part of the entire spectrum of human milk oligosaccharides. Furthermore, their recognized importance in the maturation of the immune system and their prognostic use as immunomodulators underlines their importance as a possible immunomodulator.

Accordingly, there is an urgent need in the art for the preparation of complex oligosaccharides and mixtures thereof, which resemble as much as possible or even reproduce the variety of complex oligosaccharides in human milk.

Many attempts have been carried out in this respect to produce individual HMOs via organo-chemical synthesis and, due to its stereoselectivity, via enzymatic means. Enzymatic means have been increasingly explored in the last two decades.

Notably, in biological systems, Leloir-type glycosyltransferases (GTs, EC 2.4.1.-) and glycosidases (also called glycoside hydrolases: GHs, EC 3.2.1.-) constitute the two major classes of carbohydrate-processing enzymes, which may be utilized in the production of HMOs. Both classes of enzymes act to transfer a glycosyl group from a donor to an acceptor resulting in oligosaccharide production. The use of glycosyltransferases for synthesis in industrial processes, however, is limited both by the availability of the desired enzymes due to problems with expression and solubility and the high costs of the activated donor sugars. These nucleotide donors may be typically generated *in situ*, but the process requires additional enzymes (see Hanson, S., et al., Trends Biochem Sci, 2004. **29**(12): p. 656-63). In contrast to glycosyltransferases, glycosidases have a wide range of donor substrates employing usually monosaccharides, oligosaccharides or/and engineered substrates (i.e. substrates carrying various functional groups). They often display activity towards a large variety of carbohydrate

and non-carbohydrats acceptors. Another advantage of the use of glycosidases compared to glycosyltransferases is their robustness and accessibility.

In vivo, glycosidases usually catalyze the hydrolysis of glycosidic linkages with either retention or inversion of stereochemical configuration in the product. In vitro, they can catalyse the formation of a new glycosidic bond either by transglycosylation or by reverse hydrolysis (ie condensation). Under kinetically controlled reactions these enzymes (typically, retaining glycosidases) can be used to form glycosidic linkages using a glycosyl donor activated by a good anomeric leaving group (e.g. nitrophenyl glycoside). In contrast, the thermodynamically controlled reverse hydrolysis uses high concentrations of free sugars. However, even though the appropriate application of glycosidases in the synthetic direction is of considerable interest, it remains challenging as optimal conditions and suitable substrates have to be found to drive the reaction in the desired direction and to avoid hydrolysis of the products.

Another approach to overcome this bottleneck and to make glycosidases more suitable for oligosaccharide synthesis has been recently developed by providing modified enzymes (variants). Thus, during these two past decades, protein engineering based on rational or combinatorial techniques has proven to be extremely powerful to generate biocatalysts with improved transglycosylation activity and efficiency.

The synthesis of Fucα1-3Galβ1-4Glc using p-nitrophenyl fucoside, lactose and α-fucosidase from *Alcaligenes* sp. has been reported by Murata et al. *Carbohydr. Res.* **320**, 192 (1999). Synthesis of 2'-fucosyllactose using fucosyl fluoride, lactose and 1,2-α-L-fucosynthase has been disclosed in Wada et al. *FEBS Lett.* **582**, 3739 (2008). Sialylation of lactose with p-nitrophenyl sialoside employing recombinant transsialidase from *T.cruzi* has been described by Neubacher et al. *Org. Biomol. Chem.* **3**, 1551 (2005). Transsialylation of benzyl glycosides of galactosyl oligosaccharides has been claimed in WO 2012/007588. Direct conversion of lactose into LNT with p-nitrophenyl lacto-N-bioside using a lacto-N-biosidase from *Aureobacterium* sp. L-101 has been reported in Murata et al. *Glycoconj. J.* **16**, 189 (1999). However, even though many organo-chemical syntheses or enzyme based syntheses for basic human milk oligosaccharide structures or their precursors have been published meanwhile, such synthesis methods still do not allow the preparation of complex mixtures of naturally occurring oligosaccharides or derivatives thereof. Preparing such mixtures on the basis of

individually designed syntheses of single HMOs is furthermore costly and may not resemble the large variety of naturally occurring HMOs.

Accordingly, it is an object underlying the present invention to provide a method, which allows the provision of a larger variety of human milk oligosaccharides than is obtained by prior art methods, preferably in a cost efficient manner, and preferably on an industrial scale.

BRIEF DESCRIPTION OF THE FIGURES

The following figures are intended to illustrate the invention further. They are not intended to limit the subject matter of the invention thereto.

Figure 1: depicts the presently known 13 core structures of human milk oligosaccharides (HMOs).

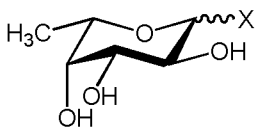
Figure 2: depicts an exemplary synthesis of HMOs using lactose, N-acetyllactosaminyl donor and lacto-N-biosyl donor with the enzymes β-1,3-trans-lacto-N-biosidase, β-1,3-trans-N-acetyllactosaminidase, and β-1,6-trans-N-acetyllactosaminidase.

Figure 3: shows the NMR spectrum of benzyl 3'''-O-sialyl-β-LNnT.

DESCRIPTION OF THE INVENTION

According to a first aspect, the present invention provides a method for generating one or more human milk oligosaccharides (HMOs) or derivatives or precursors thereof, that is making one or more human milk oligosaccharides (HMOs) or derivatives or precursors thereof, the method comprising the steps of

a) providing at least one compound as a universal donor selected from the group consisting of a compound of any of formulae **5** to **10**



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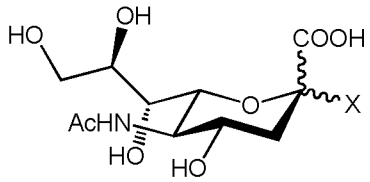

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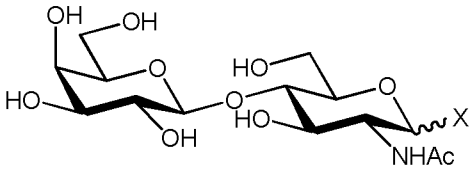

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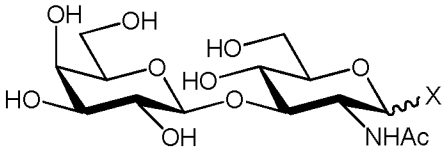

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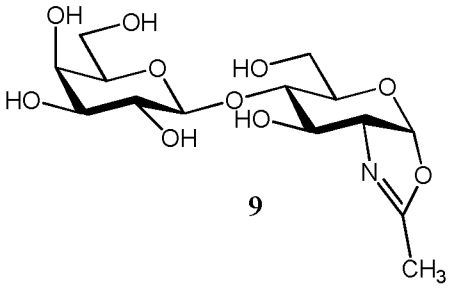

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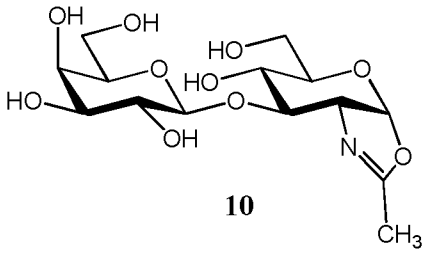

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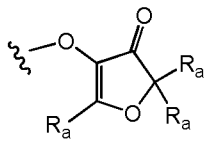
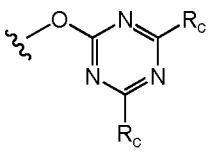
and salts thereof,

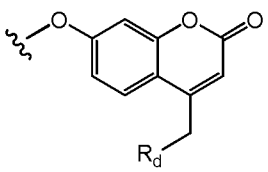
wherein X is selected from the group consisting of azide, fluoro, optionally substituted phenoxy, optionally substituted pyridinyloxy, group **A**, group **B**, group **C** and group **D**



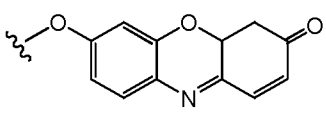
A



B



C



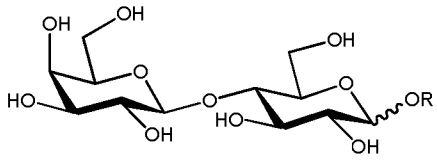
D

wherein R_a is independently H or alkyl, or two vicinal R_a groups represent a $=C(R_b)_2$ group, wherein R_b is independently H or alkyl, R_c is independently selected from the group consisting of alkoxy, amino, alkylamino and dialkylamino, R_d is selected from the group consisting of H, alkyl and $-C(=O)R_e$, wherein R_e is OH, alkoxy, amino, alkylamino, dialkylamino, hydrazino, alkylhydrazino, dialkylhydrazino or trialkylhydrazino.

b) providing at least one compound as an acceptor selected from the group consisting of:

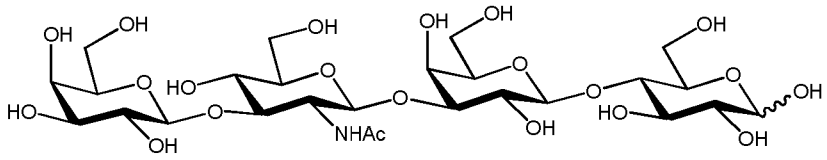
- lactose

- lactose derivatives of the following formula:

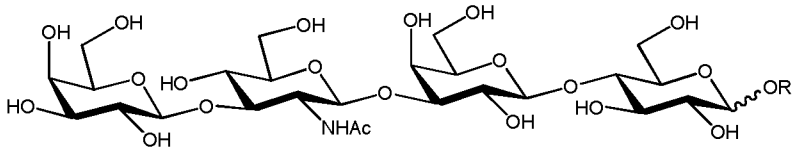


wherein R is a group removable by hydrogenolysis,

- LNT:

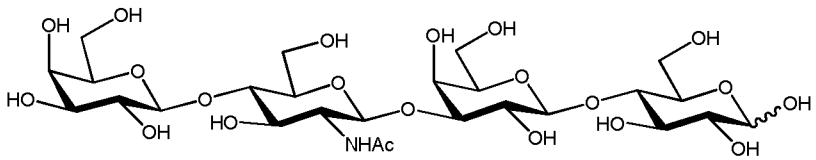


- LNT derivatives of the formula:

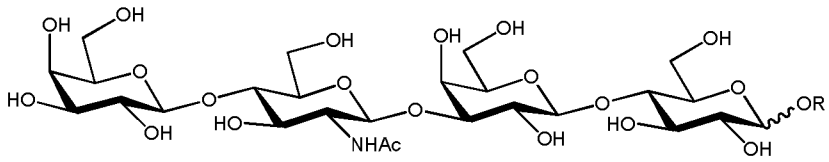


wherein R is a group removable by hydrogenolysis, or salts thereof

- LNnT:



- LNnT derivatives of the formula:



wherein R is a group removable by hydrogenolysis, or salts thereof

c) providing at least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity ;

d) preparing a mixture of the donor(s), acceptor(s) and enzyme(s), provided in steps a), b) and c)

e) incubating the mixture prepared according to step d);

f) optionally: repeating any of steps a) to e), with the mixture obtained according to step e);

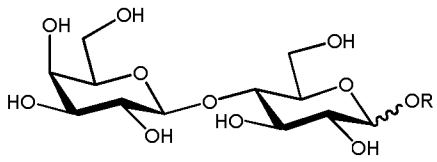
g) optionally subjecting the mixture obtained as a result of step e) or f) to a hydrogenolysis reaction.

According to a preferred embodiment of the first aspect, the present invention provides a method for generating one or more human milk oligosaccharides (HMOs) or derivatives or precursors thereof, that is making one or more human milk oligosaccharides (HMOs) or derivatives or precursors thereof, the method comprising the steps of

a) providing at least one compound as a universal donor selected from the group consisting of a compound of any of formulae **5** to **10** defined above, or salts thereof,

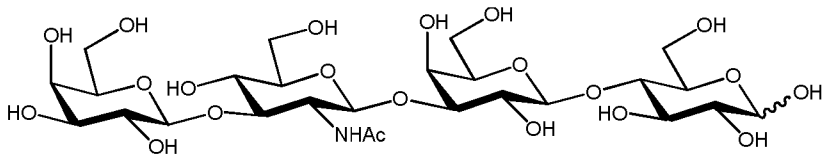
b) providing at least one compound as an acceptor selected from the group consisting of:

- lactose
- lactose derivatives of the following formula:

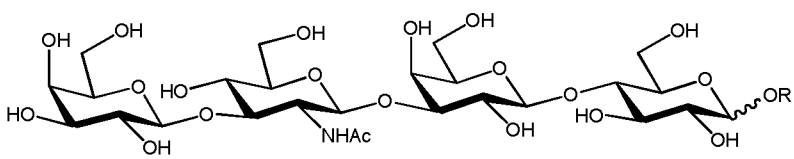


wherein R is a group removable by hydrogenolysis,

- LNT:

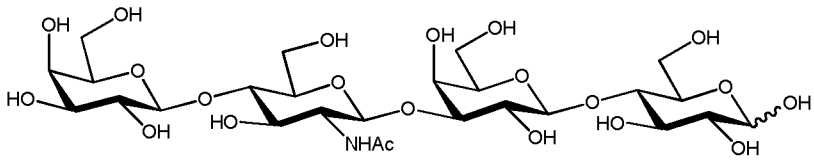


- LNT derivatives of the formula:

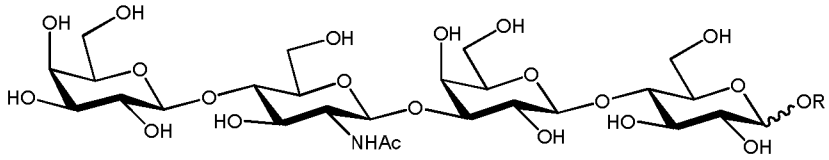


wherein R is a group removable by hydrogenolysis, or salts thereof

- LNnT:



- LNnT derivatives of the formula:



wherein R is a group removable by hydrogenolysis,

c) providing at least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity;

d) preparing a mixture of the donor(s), acceptor(s) and enzyme(s), provided in steps a), b) and c);

e) incubating the mixture prepared according to step d);

f) repeating at least one of the steps a) to c), then repeating step d) and step e) in the case where:

f-a) a single compound of formula **5** is provided as the universal donor in step a), a single acceptor is provided according to step b), the enzyme comprising a transglycosidase activity and/or a glycosynthase activity added in step c) is an enzyme having a trans-fucosidase and/or fucosynthase activity, and a single HMO or derivative or precursor thereof is made as a result of step e); or

f-b) a single compound of formula **6** is provided as the universal donor in step a), a single acceptor is provided according to step b), the enzyme comprising a transglycosidase activity and/or a glycosynthase activity added in step c) is an enzyme having a trans-sialidase activity, and a single HMO or derivative or precursor thereof is made as a result of step e); or

f-c) a single compound of formula **8** is provided as the universal donor in step a), a single acceptor is provided according to step b), the enzyme comprising a transglycosidase activity and/or a glycosynthase activity added in step c) is an

enzyme having a trans-lacto-N-biosidase activity, and a single HMO or derivative or precursor thereof is made as a result of step e);

g) optionally: repeating at least one of the steps a) to c), then repeating step d) and step e) with the mixture obtained according to step e) or f);

h) optionally subjecting the mixture obtained as a result of step e), f) or g) to a hydrogenolysis reaction.

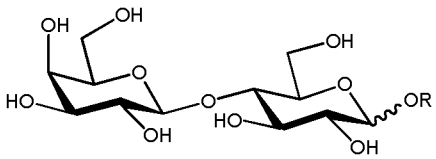
Suitably, the step f) is conducted where a single donor is provided in step a), a single acceptor is provided in step b), the enzyme provided in step c) is a single enzyme comprising a transglycosidase activity and/or a glycosynthase activity, and a single HMO or derivative or precursor thereof is made as a result of step e).

According to a more preferred embodiment of the first aspect, the present invention provides a method for generating more human milk oligosaccharides (HMOs) or derivatives or precursors thereof, that is making one or more human milk oligosaccharides (HMOs) or derivatives or precursors thereof, the method comprising the steps of

a) providing at least one compound as a universal donor selected from the group consisting of a compound of any of formulae **5** to **10** defined above, or salts thereof,

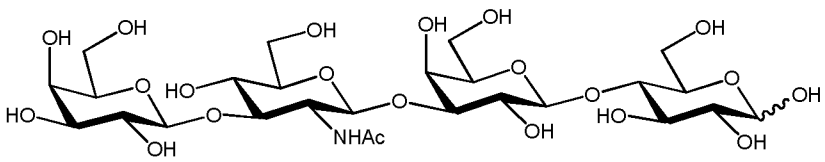
b) providing at least one compound as an acceptor selected from the group consisting of:

- lactose
- lactose derivatives of the following formula:

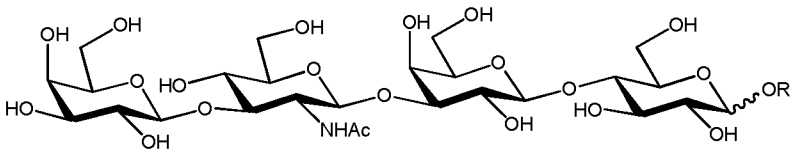


wherein R is a group removable by hydrogenolysis,

- LNT:

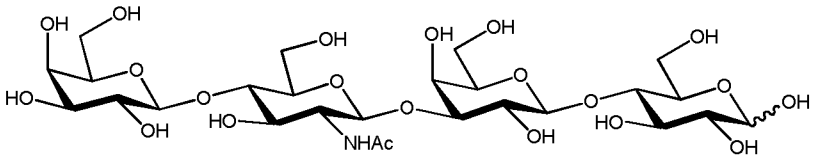


- LNT derivatives of the formula:

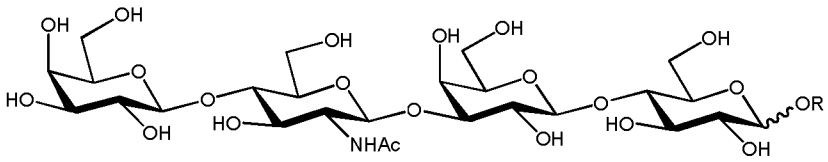


wherein R is a group removable by hydrogenolysis, or salts thereof

- LNnT:



- LNnT derivatives of the formula:



wherein R is a group removable by hydrogenolysis,

c) providing at least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity;

d) preparing a mixture of the donor(s), acceptor(s) and enzyme(s), provided in steps a), b) and c);

e) incubating the mixture prepared according to step d);

f) repeating at least one of the steps a) to c), then repeating step d) and step e) when only one HMO or derivative or precursor thereof is made as a result of step e) above;

g) optionally repeating at least one of the steps a) to c), then repeating step d) and step e), with the mixture obtained as a result of step e) or f);

h) optionally subjecting the mixture obtained as a result of step e), f) or g) to a hydrogenolysis reaction.

According to the preferred and the more preferred embodiments above, at least one more additional incubation cycle is needed when only one product (HMO or HMO derivative or HMO precursor) is formed after the first incubation cycle. Lactose is not herein considered to be an HMO. This case may occur in some donor-acceptor pairs provided in steps a) and b).

This situation includes cases where starting materials, that is compounds provided in steps a) and b) of the method, remain in the reaction mixture after the incubation step along with a single product formed during the incubation step. When repeating at least one of the steps a) to c), then step d) and step e), the at least one compound provided according to step a) is preferably different from that/those provided in the first cycle; the at least one compound provided according to step b) is preferably different from that/those provided in the first cycle, and the at least one enzyme provided according to step c) is preferably different from that provided in the first cycle, but at least one of the steps a) to c) must be different from that carried out in the first cycle.

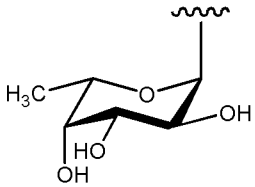
In the context of the present invention the term "group removable by hydrogenolysis" refers to groups whereby a carbon-heteroatom single bond is cleaved or undergoes "lysis" by hydrogen. The heteroatom may vary, but it usually is oxygen, nitrogen, or sulfur. Hydrogenolysis represents an exception among protecting group chemistries, in which water can be used as a solvent. Hydrogenolysis itself is a powerful deprotection process suitable to remove O-benzyl/substituted O-benzyl moieties from an oligosaccharide scaffold in almost a quantitative manner under extremely gentle conditions preventing by-product formations. It is also an advantage of hydrogenolysis as a final deblocking procedure within a complex synthetic pathway that only a catalytic amount of the reagents are required for the completion of the reaction, and that the reaction provides exclusively toluene or substituted toluene derivatives as by-products. Both toluene and substituted toluene derivatives can easily be removed even in multi ton scales from water soluble oligosaccharide products via evaporation and/or extraction processes. Suitable groups for hydrogenolysis may include benzyl, diphenylmethyl (benzhydryl), 1-naphthylmethyl, 2-naphthylmethyl or triphenylmethyl (trityl) groups, each of which may be optionally substituted by one or more groups selected from: alkyl, alkoxy, phenyl, amino, acylamino, alkylamino, dialkylamino, nitro, carboxyl, alkoxycarbonyl, carbamoyl, *N*-alkylcarbamoyl, *N*,*N*-dialkylcarbamoyl, azido, halogenalkyl or halogen. Preferably, such substitution, if present, is on the aromatic ring(s). A particularly preferred protecting group is benzyl optionally substituted with one or more groups selected from alkyl or halogen. More preferably, the protecting group is selected from unsubstituted benzyl, 4-chlorobenzyl and 4-methylbenzyl. These particularly preferred and more preferable protecting groups have the advantage that the by-products of the hydrogenolysis are exclusively toluene or substituted toluene. Such by-products can easily be removed even in

multi ton scales from water soluble oligosaccharide products via evaporation and/or extraction processes. Hydrogenolysis may be carried out by adding catalytic amounts of palladium, Raney nickel or another appropriate metal catalyst known for use in hydrogenolysis, resulting in the regeneration of the OH group. Groups of this type are well known to the skilled man and thoroughly discussed (see e.g. P.G.M. Wuts and T.W. Greene: *Protective Groups in Organic Synthesis*, John Wiley & Sons (2007)).

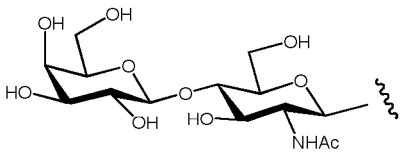
Furthermore, the term "HMO precursor" means an R-glycoside of an HMO which has been modified with a residue R to form a glycoside via a glycosidic bond.

Furthermore, the term "HMO derivative" means an oligosaccharide structurally similar to a HMO and R-glycosides thereof, preferably derivatives according to general formula **1**, **2**, **3** and **4**.

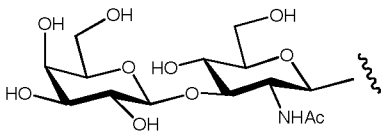
Additionally, the term „fucosyl" within the context of the present invention means a L-fucopyranosyl group attached to the core oligosaccharide with α-interglycosidic linkage:



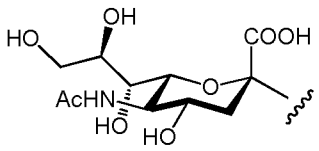
„N-acetyl-lactosaminyl" group within the context of the present invention means the glycosyl residue of N-acetyl-lactosamine (LacNAc, Galpβ1-4GlcNAcp) linked with β-linkage:



Furthermore, the term „lacto-N-biosyl" group within the context of the present invention means the glycosyl residue of lacto-N-biose (LNB, Galpβ1-3GlcNAcp) linked with β-linkage:



The term „sialyl" within the context of the present invention means the glycosyl residue of sialic acid (N-acetyl-neuraminic acid, Neu5Ac) linked with α-linkage:



Additionally, the term „glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl units" within the context of the present invention means a linear or branched structure comprising the said units that are linked to each other by interglycosidic linkages.

The term "optionally substituted phenoxy" means a phenoxy group optionally substituted with 1 or 2 groups selected from nitro, halogen, alkyl, hydroxyalkyl, amino, formyl, carboxyl and alkoxycarbonyl, or two substituents in ortho position may form a methylenedioxy-group. Preferred substituents are nitro (preferably in 2- and/or 4-position), halogen (preferably fluoro and chloro). Especially preferred substituted phenoxy groups are selected from 4-nitrophenoxy, 2,4-dinitrophenoxy, 2-chloro-4-nitrophenoxy, 2-fluoro-4-nitrophenoxy, 3-fluoro-4-nitrophenoxy, 2-hydroxymethyl-4-nitrophenoxy, 3-hydroxymethyl-4-nitrophenoxy, 2-formyl-4-nitrophenoxy, 2-carboxy-4-nitrophenoxy, 2-methoxycarbonyl-4-nitrophenoxy, 5-fluoro-2-nitrophenoxy, 4-methoxycarbonyl-2-nitrophenoxy, 4-carboxy-2-nitrophenoxy, 2-aminophenoxy and 3,4-methylenedioxy-phenoxy.

The term "optionally substituted pyridinyloxy" means a pyridinyloxy, more preferably 2- or 4-pyridyloxy group, optionally substituted with 1 or 2 groups selected from nitro, halogen, alkyl and alkoxy. Especially referred pyridinyloxy groups are selected from 4-pyridinyloxy, 2-pyridinyloxy, 3-nitro-2-pyridinyloxy and 3-methoxy-2-pyridinyloxy.

According to step a) of the first aspect, including the preferred and more preferred embodiments, at least one universal donor is provided, and according to step b) of the first aspect, including the preferred and more preferred embodiments, at least one acceptor is provided. In the context of the present invention, the term "donor" is understood as a compound, which provides or transfers a specific moiety in a chemical reaction, e.g. a nucleophilic or electrophilic substitution reaction, to a further compound, preferably an acceptor. Particularly preferably, a "universal donor" is understood as a compound, which provides or transfers a specific moiety in a chemical reaction, e.g. a nucleophilic or electrophilic substitution reaction, to a further compound, preferably an acceptor, wherein the donor is not restricted to naturally occuring donors for the preparation of HMOs and may also

lead to naturally occuring HMOs and/or to non-naturally occuring HMOs as a product. Such a universal donor is preferably as defined above. Likewise, the term "acceptor" is understood as a compound, which receives a specific moiety in a chemical reaction, e.g. nucleophilic or electrophilic substitution reaction, from a further compound, preferably a universal donor as defined herein. Such an acceptor is preferably as defined above.

Compounds for use as universal donors in step a) of the first aspect, including the preferred and more preferred embodiments, for the preparation of human milk oligosaccharides (HMOs), may preferably be selected from compounds according to formulae **5** to **8**, wherein the substituents are preferably selected depending on the enzymes used during the method of the present invention. Preferably, the donors are selected depending on their a transglycosidase activity from compounds according to formulae **5** to **8**, wherein X is optionally substituted 4-nitrophenoxy, group **A**, group **B**, group **C**, or from compounds **9** and **10**. Likewise preferably, the donors are selected depending on their glycosynthase activity from compounds according to formulae **5** to **8**, wherein X is azide or fluoro. Both selections may be carried out independently of each other or together.

Furthermore, compounds for use as universal donors in step a) of the first aspect, including the preferred and more preferred embodiments, for the preparation of human milk oligosaccharides (HMOs), may preferably be selected from compounds according to formulae **5** to **8**, wherein X is 4-nitrophenoxy, 2,4-dinitrophenoxy, 2-chloro-4-nitrophenoxy, 2,5-dimethyl-3-oxo-(2H)-furan-4-yloxy, 2-ethyl-5-methyl-3-oxo-(2H)-furan-4-yloxy, 5-ethyl-2-methyl-3-oxo-(2H)-furan-4-yloxy, 4,6-dimethoxy-1,3,5-triazin-2-yloxy, 4,6-diethoxy-1,3,5-triazin-2-yloxy, 4-methylumbelliferyloxy.

In step c) of the method of the present invention for generating human milk oligosaccharides (HMOs) at least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity is provided.

Enzymes suitable in step c) of the first aspect, including the preferred and more preferred embodiments, for the generation of human milk oligosaccharides (HMOs) typically comprise at least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity, preferably selected from enzymes having, e.g. a fucosidase, trans-fucosidase or fucosynthase activity, a sialidase (neuraminidase) or trans-sialidase (transneuraminidase) activity, a lacto-N-biosidase or trans-lacto-N-biosidase activity and/or a N-acetyllactoaminidase or trans-N-

acetyllactoaminidase activity, or any further enzyme having such an activity. Even more preferably, enzymes suitable in step d) of the first aspect, including the preferred and more preferred embodiments, for the generation of human milk oligosaccharides (HMOs) may be selected from the group comprising wild type or mutated glycosidases or transglycosidases, preferably wild type or mutated glycosidases or transglycosidases having a fucosidase, trans-fucosidase or fucosynthase activity, a sialidase (neuraminidase) or trans-sialidase (transneuraminidase) activity, a lacto-N-biosidase or trans-lacto-N-biosidase activity and/or a N-acetyllactoaminidase or trans-N-acetyllactoaminidase activity, or preferably having α-trans-fucosidase, α-trans-sialidase, β-trans-lacto-N-biosidase and/or β-trans-N-acetyllactosaminidase activity.

Enzymes suitable in step c) of the first aspect, including the preferred and more preferred embodiments, for the generation of human milk oligosaccharides (HMOs) furthermore may be selected from any genus known to a skilled person, to express or secrete at least one enzyme as defined above, e.g. an enzyme having a transglycosidase activity and/or a glycosynthase activity, preferably an enzyme having a fucosidase, trans-fucosidase or fucosynthase activity, a sialidase (neuraminidase) or trans-sialidase (transneuraminidase) activity, a lacto-N-biosidase or trans-lacto-N-biosidase activity and/or a N-acetyllactoaminidase or trans-N-acetyllactoaminidase activity, or preferably having α-trans-fucosidase, α-trans-sialidase, β-trans-lacto-N-biosidase and/or β-trans-N-acetyllactosaminidase activity, or any further enzyme having such an activity. Even more preferably, such enzymes suitable in step c) of the first aspect, including the preferred and more preferred embodiments, for the generation of human milk oligosaccharides (HMOs) may be selected from non-pathogenic bacteria selected from *Bacillus, Bifidobacterium, Lactobacillus, Leuconostoc, Lactococcus, Streptococcus, Streptomyces, Sulfolobus, Thermotoga, or Trypanosoma.*

Even more preferably, such enzymes suitable in step c) of the first aspect, including the preferred and more preferred embodiments, for the generation of human milk oligosaccharides (HMOs) are selected from the group comprising the non-pathogenic bacteria *Bacillus circulans*, lactic acid bacteria, such as *Bifidobacterium bifidum JCM 1254, Bifidobacterium bifidum NCIMB 41171, Bifidobacterium bifidum NCIMB 41171, Bifidobacterium bifidum* JCM1254, *Bifidobacterium bifidum JCM1254, Bifidobacterium bifidum PRL2010, Bifidobacterium bifidum* PRL2010, *Bifidobacterium bifidum S17,*

Bifidobacterium bifidum S17, *Bifidobacterium dentium Bd1, Bifidobacterium longum subsp. infantis ATCC 15697, Bifidobacterium longum subsp longum JDM 301, Bifidobacterium longum subsp. infantis JCM 1222, Lactobacillus casei BL23, Streptomyces sp., Sulfolobus solfataricus P2, Thermotoga maritima MSB8, and Trypanosoma cruzi.*

Particularly preferred microorganisms in the above context, particularly for targeted glycosidases / transglycosidases / glycosynthases, comprise lactic acid bacteria. Lactic acid bacteria, and more particularly non-pathogenic bacteria from the genus *Bifidobacterium* contain a series of glycosidases including α-2,3/6 sialidases (GH33), α-1,2/3/4 fucosidases (GH29 and GH95), lacto-N-biosidases (GH20), β-galactosidases (GH2) and β-N-acetylhexosaminidases (GH20) that are able to recognize human milk oligosaccharides. Depending on the bifidobacteria strains, these glycosidases are intra- or extracellular enzymes.

A further aspect regarding the use of glycosidases and/or glycosynthases from lactic acid bacteria concerns the industrial importance of such bacteria since they have the GRAS (generally recognized as safe) status. According to another more preferred aspect the glycosidase and/or glycosynthases displaying a trans-fucosidase, trans-sialidase, trans-lacto-N-biosidase and/or trans-N-acetyllactosaminidase activity, preferably a α-trans-fucosidase, α-trans-sialidase, β-trans-lacto-N-biosidase and/or β-trans-N-acetyllactosaminidase activity, is a wild type or an engineered glycosidase, most preferably the wild type glycosidase is taken from the group consisting of lactic acid bacteria, wherein the glycosidase is converted to a transglycosidase by rational engineering or/and directed evolution. A glycosidase and/or glycosynthase selected from the group consisting of lactic acid bacteria is most preferably a glycosidase from *Bifidobacterium, Lactobacillus, Lactococcus, Streptococcus or Leuconostoc.* A glycosidase selected from the genus *Bifidobacterium* is most preferably a glycosidase from *Bifidobacterium longum subsp. Infantis, Bifidobacterium longum subsp. Longum, Bifidobacterium breve, Bifidobacterium bifidum* and *Bifidobacterium catenulatum.*

Furthermore, engineered fucosidases from thermophilic organisms such as *Sulfolobus solfataricus* and *Thermotoga maritima* have recently been developed, which may be used in the method of the present invention. These thermostable glycosidases have considerable potential for industrial applications since they can be used in biotechnological processes at

elevated temperatures, and so facilitating the process, preventing risk of contamination, and increasing the solubility of the compounds used in the reaction.

According to another more preferred embodiment, the glycosidase and/or glycosynthase enzyme displaying a trans-fucosidase, trans-sialidase, trans-lacto-N-biosidase and/or trans-N-acetyllactosaminidase activity, preferably an α-trans-fucosidase, α-trans-sialidase, β-trans-lacto-N-biosidase and/or β-trans-N-acetyllactosaminidase activity, is a wild type or an engineered glycosidase. Most preferably, the wild type glycosidase is taken from the group consisting of thermophilic organisms, which glycosidase is converted to a transglycosidase by rational engineering or/and directed evolution. An α-L-fucosidase selected from thermophilic organisms is most preferably an α-L-fucosidase from *Thermotoga maritima* and *Sulfolobus solfataricus.*

Preferably, the at least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity may be selected from an enzyme exhibiting a fucosidase, trans-fucosidase or fucosynthase activity, preferably as described in the following. In this context, enzymes having a fucosidase, trans-fucosidase or fucosynthase activity, more preferably an α-trans-fucosidase activity, are preferably selected from fucosidases in general, and more preferably from α-L-fucosidases, e.g. α-L-fucosidases as classified according to EC 3.2.1.38 and 3.2.1.51. α-L-Fucosidases are widely spread in living organisms such as mammals, plants, fungi and bacteria. These enzymes belong to the families 29 and 95 of the glycoside hydrolases (GH29 and GH95) as defined by the CAZY nomenclature (http://www.cazy.org). Fucosidases from GH 29 are retaining enzymes (3D structure: $(\beta/\alpha)_8$) whereas fucosidases from GH 95 are inverting enzymes (3D structure: $(\alpha/\alpha)_6$). The substrate specificity of the GH29 family is broad whereas that of the GH95 family is strict to α1,2-linked fucosyl residues. The GH29 family seems to be divided into two subfamilies. One subfamily typically has strict specificity towards α1,3- and α1,4-fucosidic linkages. The members of a further subfamily have broader specificity, covering all α-fucosyl linkages. α-L-fucosidases generally hydrolyse the terminal fucosyl residue from glycans. These enzymes are also capable of acting as catalysts for fucosylation reactions due to their transfucosylation activity and thus may be used in the context of the method of the present invention, preferably under kinetically controlled conditions.

Fucosidases, which may be employed in the context of the present invention, may also comprise engineered fucosidases. Such engineered fucosidases preferably comprise engineered α-L-fucosidases, preferably engineered fucosidases derived from fucosidases as described above, e.g. an engineered α-1,2-L-fucosynthase from *Bifidobacterium bifidum*, α-L-fucosynthases from *Sulfolobus solfataricus* and *Thermotoga maritime, etc*. Such engineered fucosidases show an acceptor dependent regioselectivity and are devoid of product hydrolysis activity. Furthermore, engineered fucosidases preferably comprise α-L-fucosidase from *Thermotoga maritima*, which has also been recently converted into an efficient α-L-trans-fucosidase by directed evolution (see Osanjo, G., et al., *Directed evolution of the alpha-L-fucosidase from Thermotoga maritima into an alpha-L-trans-fucosidase.* Biochemistry, 2007, 46(4): p. 1022-33).

Even more preferably, the at least one enzyme having a fucosidase and/or trans-fucosidase and/or fucosynthase activity may be selected from α-L-fucosidases derived from *Thermotoga maritima* MSB8, *Sulfolobus solfataricus* P2, *Bifidobacterium bifidum* JCM 1254, *Bifidobacterium bifidum* JCM 1254, *Bifidobacterium longum subsp. infantis* ATCC 15697, *Bifidobacterium longum subsp. infantis* ATCC 15697, *Bifidobacterium longum subsp. Infantis* JCM 1222, *Bifidobacterium bifidum* PRL2010, *Bifidobacterium bifidum* S17, *Bifidobacterium longum subsp longum* JDM 301, *Bifidobacterium dentium* Bd1, or *Lactobacillus casei* BL23, etc.

Even more preferably the at least one enzyme having a fucosidase and/or trans-fucosidase and/or fucosynthase activity may be selected from following *a*-L-fucosidases as defined according to the following deposit numbers gi|4980806 (*Thermotoga maritima* MSB8, SEQ ID NO: 1), gi|13816464 (*Sulfolobus solfataricus* P2, SEQ ID NO: 2), gi|34451973 (*Bifidobacterium bifidum* JCM 1254, SEQ ID NO: 3), gi|242345155 (*Bifidobacterium bifidum*, JCM 1254, SEQ ID NO: 4), gi|213524647 (*Bifidobacterium longum subsp. infantis*, ATCC 15697, SEQ ID NO: 5), gi|213522629 (*Bifidobacterium longum subsp. infantis* ATCC 15697), gi|213522799 (*Bifidobacterium longum subsp. infantis* ATCC 15697), gi|213524646 (*Bifidobacterium longum subsp. infantis* ATCC 15697), gi|320457227 (*Bifidobacterium longum subsp. infantis* JCM 1222), gi|320457408 (*Bifidobacterium longum subsp. infantis* JCM 1222), gi|320459369 (*Bifidobacterium longum subsp. infantis* JCM 1222), gi|320459368 (*Bifidobacterium longum subsp. infantis* JCM 1222), gi|310867039 (*Bifidobacterium bifidum* PRL2010), gi|310865953 (*Bifidobacterium bifidum* PRL2010), gi|309250672

(*Bifidobacterium bifidum* S17), gi|309251774 (*Bifidobacterium bifidum* S17), gi|296182927 (*Bifidobacterium longum subsp longum* JDM 301), gi|296182928 (*Bifidobacterium longum subsp longum* JDM 301), gi|283103603 (*Bifidobacterium dentium* Bd1), gi|190713109 (*Lactobacillus casei* BL23, SEQ ID NO: 6), gi|190713871 (*Lactobacillus casei* BL23, SEQ ID NO: 7), gi|190713978 (*Lactobacillus casei* BL23, SEQ ID NO: 8), etc., or a sequence exhibiting a sequence identity with one of the above mentioned enzyme sequences having a fucosidase and/or trans-fucosidase activity of at least 70%, more preferably at least 80%, equally more preferably at least 85%, even more preferably at least 90% and most preferably at least 95% or even 97%, 98% or 99% as compared to the entire wild type sequence on amino acid level.

Particularly preferred α-L-fucosidases with fucosidase/trans-fucosidase/fucosynthase activity are listed in the following Table 2:

GI number in GenBank Database	Organisms	SEQ ID NO:
gi|4980806	*Thermotoga maritima* MSB8	1
gi|13816464	*Sulfolobus solfataricus* P2	2
gi|34451973	*Bifidobacterium bifidum* JCM 1254	3
gi|242345155	*Bifidobacterium bifidum* JCM 1254	4
gi|213524647	*Bifidobacterium longum subsp. infantis* ATCC 15697	5
gi|213522629	*Bifidobacterium longum subsp. infantis* ATCC 15697	-
gi|213522799	*Bifidobacterium longum subsp. infantis* ATCC 15697	-
gi|213524646	*Bifidobacterium longum subsp. Infantis* ATCC 15697	-
gi|320457227	*Bifidobacterium longum subsp. infantis* JCM 1222	-
gi|320457408	*Bifidobacterium longum subsp. infantis* JCM 1222	-
gi|320459369	*Bifidobacterium longum subsp. infantis* JCM 1222	-
gi|320459368	*Bifidobacterium longum subsp. infantis* JCM 1222	-
gi|310867039	*Bifidobacterium bifidum* PRL2010	-
gi|310865953	*Bifidobacterium bifidum* PRL2010	-
gi|309250672	*Bifidobacterium bifidum* S17	-
gi|309251774	*Bifidobacterium bifidum* S17	-
gi|296182927	*Bifidobacterium longum subsp longum* JDM 301	-

GI number in GenBank Database	Organisms	SEQ ID NO:
gi|296182928	*Bifidobacterium longum subsp longum* JDM 301	-
gi|283103603	*Bifidobacterium dentium* Bd1	-
gi|190713109	*Lactobacillus casei* BL23	6
gi|190713871	*Lactobacillus casei* BL23	7
gi|190713978	*Lactobacillus casei* BL23	8

Table 2: Preferred α-L-fucosidases

Likewise preferably, the at least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity may be selected from an enzyme exhibiting a sialidase or trans-sialidase activity, preferably as described in the following. In this context, enzymes having a sialidase or trans-sialidase activity are preferably selected from a sialidase or trans-sialidase as described in the following, e.g. sialidases (EC 3.2.1.18) and trans-sialidases (EC 2.4.1.-) as classified according to the the GH33 family. They are retaining enzymes. Sialidases and trans-sialidases are widely distributed in nature. They are found particularly in diverse virus families and bacteria, and also in protozoa, some invertebrates and mammals. These enzymes differ in their biochemical properties, e.g., kinetics, binding affinity or substrate preference. Nevertheless, they possess conserved domains and structural similarities. Trans-sialidases differ from sialidases since they can transfer sialic acids, preferably α-2,3-bonded sialic acids, from a donor molecule to an acceptor derivative, which is preferably a terminal galactose moiety with a β-interglycosidic linkage. As a result of this transfer, an α-glycosidic bond is be formed between the sialic acid and the acceptor. However, if there is no suitable acceptor, the trans-sialidase hydrolyses the sialic acid.

The first trans-sialidase enzyme described was found in *Trypanosoma cruzi*, a protozoa which causes Chagas disease. This trans-sialidase (TcTS) has been extensively studied. Since that time trans-sialidases have been detected in several other trypanosome types such as *Trypanosoma brucei gambiense*, *Trypanosoma brucei rhodesiense*, *Trypanosoma brucei brucei* and *Trypanosoma congolense*. Moreover, the existence of trans-sialidases has been shown in *Endotrypanum* types, in *Corynebacterium diphtheriae* and even in human plasma.

Sialidases can be classified into two different subgroups, endo- and exo-sialidases. The endo-sialidases hydrolyze sialic acid linkages internal to macromolecules, while the exo-sialidases attack terminal sialic acid linkages, and desialylate glycoproteins, glycopeptides, gangliosides, oligosaccharides and polysaccharides. Recently, sialidases from

Bifidobacterium bifidum and *Bifidobacterium longum subsp. infantis* have been identified, cloned and characterized. These sialidases can cleave and so recognize both α-2,3- and α-2,6-linked sialosides. Sialidases from *Bifidobacterium longum subsp. infantis* have a consistent preference for α-2,6-linkage whereas sialidases from *Bifidobacterium bifidum* have a consistent preference for α-2,3-linkage. These enzymes are also capable of acting as catalysts for sialylation reactions due to their trans-sialidase activity and thus may be used in the context of the method of the present invention, preferably under kinetically controlled conditions.

Sialidases, which may be employed in the context of the present invention, may also comprise engineered sialidases. Based on sequence and structure comparisons, sialidase from *Trypanosoma rangeli* may be mutated at six positions, wherein the resulting mutant is able to display a significant level of trans-sialidase activity (see Paris, G., et al., *A sialidase mutant displaying trans-sialidase activity.* J Mol Biol, 2005. 345(4): p. 923-34).

Even more preferably, the at least one enzyme having a sialidase and/or trans-sialidase activity may be selected from sialidases or trans-sialidases derived from *Bifidobacterium longum subsp. infantis ATCC 15697, Bifidobacterium bifidum* JCM1254, *Bifidobacterium bifidum* S17, *Bifidobacterium bifidum* PRL2010, *Bifidobacterium bifidum NCIMB 41171, Trypanosoma cruzi,* etc.

Even more preferably the at least one enzyme having a sialidase and/or trans-sialidase activity may be selected from sialidases or trans-sialidases as defined according to the following deposit numbers: gi|213524659 (*Bifidobacterium longum subsp. infantis ATCC 15697*, SEQ ID NO: 9), gi|213523006 *Bifidobacterium longum subsp. infantis ATCC 15697*, SEQ ID NO: 10), siab2 (*Bifidobacterium bifidum* JCM1254), further sialidases or trans-sialidases from *Bifidobacterium bifidum* JCM1254), gi|309252191 (*Bifidobacterium bifidum* S17, SEQ ID NO: 11), gi|309252190 (*Bifidobacterium bifidum* S17, SEQ ID NO: 12), gi|310867437 (*Bifidobacterium bifidum* PRL2010, SEQ ID NO: 13), gi|310867438 (*Bifidobacterium bifidum* PRL2010, SEQ ID NO: 14), gi|224283484 (*Bifidobacterium bifidum NCIMB 41171*), gi|313140638 (*Bifidobacterium bifidum NCIMB 41171),* gi|47252690 (*Trypanosoma cruzi*, SEQ ID NO: 15), gi|432485 (*Trypanosoma cruzi*, SEQ ID NO: 16), gi|343957998 (*Trypanosoma congolense*, SEQ ID NO:20), gi|343958004 (*Trypanosoma congolense*, SEQ ID NO:21) etc., or a sequence exhibiting a sequence identity with one of the above mentioned

enzyme sequences having a sialidase and/or trans-sialidase activity of at least 70%, more preferably at least 80%, equally more preferably at least 85%, even more preferably at least 90% and most preferably at least 95% or even 97%, 98% or 99% as compared to the entire wild type sequence on amino acid level.

Particularly preferred sialidases with sialidase/trans-sialidase activity are listed in the following Table 3:

GI number in GenBank Database	Organisms	SEQ ID NO:
gi|213524659	*Bifidobacterium longum subsp. infantis ATCC 15697*	9
gi|213523006	*Bifidobacterium longum subsp. infantis ATCC 15697*	10
gi|309252191	*Bifidobacterium bifidum* S17	11
gi|309252190	*Bifidobacterium bifidum* S17	12
gi|310867437	*Bifidobacterium bifidum* PRL2010	13
gi|310867438	*Bifidobacterium bifidum* PRL2010	14
gi|224283484	*Bifidobacterium bifidum NCIMB 41171*	-
gi|313140638	*Bifidobacterium bifidum NCIMB 41171*	-
gi|47252690	*Trypanosoma cruzi*	15
gi|432485	*Trypanosoma cruzi*	16
gi|343957998	*Trypanosoma congolense*	20
gi|343958004	*Trypanosoma congolense*	21

Table 3: Preferred sialidases/trans-sialidases

Additionally, the at least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity may be preferably selected from an enzyme exhibiting a lacto-N-biosidase or trans-lacto-N-biosidase activity, preferably as described in the following. In this context, enzymes having a lacto-N-biosidase or trans-lacto-N-biosidase activity are preferably selected from a lacto-N-biosidase or trans-lacto-N-biosidase as described in the following, e.g. lacto-N-biosidases (EC 3.2.1.140) as classified according to the GH20 family. Lacto-N-biosidases typically proceed through a retaining mechanism. Only two lacto-N-biosidases from *Streptomyces* and *Bifidobacterium bifidum* have been described and characterized up to now, which may be utilized in the present invention as a lacto-N-biosidase or trans-lacto-N-biosidase (see Sano, M., K. Hayakawa, and I. Kato, Proc Natl Acad Sci U S A, 1992. 89(18): p. 8512-6; Sano, M., K. Hayakawa, and I. Kato, J Biol Chem, 1993. 268(25): p. 18560-6; Wada, J., et al., Appl Environ Microbiol, 2008. 74(13): p. 3996-4004.). Lacto-N-biosidases

specifically hydrolyse the terminal lacto-N-biosyl residue (β-D-Gal-(1→3)-D-GlcNAc) from the non-reducing end of oligosaccharides with the structure β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→R). Wada et al. (supra) and Murata et al. (*Glycoconj. J.* **16**, 189 (1999)) also demonstrated the ability of the lacto-N-biosidase from *Bifidobacterium bifidum* and *Aureobacterium* sp. L-101, respectively, to catalyze the transglycosylation by incubating donor substrates (such as lacto-*N*-tetraose and *p*NP-β-LNB) with acceptors (such as various 1-alkanols and lactose).

Even more preferably, the at least one enzyme having a lacto-N-biosidase or trans-lacto-N-biosidase activity may be selected from lacto-N-biosidases or trans-lacto-N-biosidases derived from *Bifidobacterium bifidum* JCM1254, *Bifidobacterium bifidum* PRL2010, *Bifidobacterium bifidum* NCIMB 41171, *Aureobacterium* sp. L-101 or *Streptomyces sp.*, etc.

Even more preferably the at least one enzyme having a lacto-N-biosidase or trans-lacto-N-biosidase activity may be selected from lacto-N-biosidases or trans-lacto-N-biosidases as defined according to the following deposit numbers: gi| 167369738 (*Bifidobacterium bifidum* JCM1254, SEQ ID NO: 17), gi|4096812 (*Streptomyces sp., SEQ ID NO:* 18), gi|310867103 (*Bifidobacterium bifidum* PRL2010), gi|313140985 (*Bifidobacterium bifidum NCIMB 41171)*, etc., or a sequence exhibiting a sequence identity with one of the above mentioned enzyme sequences having a lacto-N-biosidase or trans-lacto-N-biosidase activity of at least 70%, more preferably at least 80%, equally more preferably at least 85%, even more preferably at least 90% and most preferably at least 95% or even 97%, 98% or 99% as compared to the entire wild type sequence on amino acid level.

Particularly preferred lacto-N-biosidases with lacto-N-biosidase or trans-lacto-N-biosidase activity are listed in the following Table 4:

GI number in GenBank Database	Organisms	SEQ ID NO:
gi| 167369738	*Bifidobacterium bifidum* JCM1254	17
gi|4096812	*Streptomyces sp.*	18
gi|310867103	*Bifidobacterium bifidum* PRL2010	-
gi|313140985	*Bifidobacterium bifidum NCIMB 41171*	-

Table 4: Preferred lacto-N-biosidases or trans-lacto-N-biosidases

Furthermore, the at least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity may be preferably selected from an enzyme exhibiting a N-

acetyllactosaminidase or trans-N-acetyllactosaminidase activity, preferably as described in the following. In this context, enzymes having a N-acetyllactosaminidase or trans-N-acetyllactosaminidase activity are preferably selected from a N-acetyllactosaminidase or trans-N-acetyllactosaminidase as described in the following, e.g. lacto-N-biosidases (EC 3.2.1.140) as classified according to the GH20 family. Particularly preferably, chitinase from bacillus circulans, more preferably chitinase A1 from *Bacillus Circulans* WL-12 as deposited under gi|142688 (SEQ ID NO: 19), may be used as a N-acetyllactosaminidase or trans-N-acetyllactosaminidase, or a sequence exhibiting a sequence identity with one of the above mentioned enzyme sequences having a N-acetyllactosaminidase or trans-N-acetyllactosaminidase activity of at least 70%, more preferably at least 80%, equally more preferably at least 85%, even more preferably at least 90% and most preferably at least 95% or even 97%, 98% or 99% as compared to the entire wild type sequence on amino acid level. Notably, Shoda et al. showed that chitinase A1 from *B. Circulans* WL-12 is able to transfer N-acetyllactosamine with a β-1,6 glycosidic linkage using a 1,2-oxazoline derivative of N-acetyllactosamine (see Shoda, S.-i., et al., Cellulose, 2006. **13**(4): p. 477-484.).

Particularly preferred N-acetyllactosaminidases or trans-N-acetyllactosaminidases are listed in the following Table 5:

GI number in the GenBank Database	Organisms	SEQ ID NO:
gi|142688	Bacillus circulans	19

Table 5: Preferred N-acetyllactosaminidases or trans-N-acetyllactosaminidases

As defined above, proteins comprising a transglycosidase and/or a glycosynthase activity as defined above may also comprise engineered proteins comprising a transglycosidase and/or a glycosynthase activity. It is particularly envisaged that wild type or mutated glycosidases displaying a transfucosidase, transsialidase, trans-lacto-N-biosidase and/or trans-N-acetyllactosaminidase activity, preferably a α-transfucosidase, α-transsialidase, β-trans-lacto-N-biosidase and/or β-trans-N-acetyllactosaminidase activity, can be used in the present invention to produce such oligosaccharides. Preparation of such enzymes is preferably carried out via site directed mutagenesis approaches or directed evolution.

In rational engineering novel altered enzymes (mutants) are created via site directed mutagenesis approaches, preferably by introduction of point mutations. This technique generally requires reliance on the static 3D protein structure. The mutations generally affect

the active site of the enzymes such that they lose their ability to degrade their transglycosylation products but remain capable of synthesis. A preferred strategy consists of the replacement of the catalytic nucleophile by a non-nucleophilic residue. This modification results in the formation of an inactive mutant or an altered enzyme with reduced transglycosylation activity due the lack of appropriate environment for the formation of the reactive host-guest complex for transglycosylation. However, in the presence of a more active glycosyl donor (e.g. glycosyl fluoride) that mimics the glycosyl enzyme intermediate, the mutated enzyme is able to transfer efficiently the glycosyl moiety to a suitable acceptor generating a glycoside with inverted anomeric stereochemistry. Such a mutant glycosidase is termed a glycosynthase and their development represents one of the major advances in the use of glycosidases for synthetic purposes. In principle, the glycosynthase concept can be applied to all GH specificities and offer a large panel of enzymes potentially able to synthesize various oligosaccharides with very high yields, up to 95%.

The second preferred technique is called directed evolution. This strategy comprises random mutagenesis applied to the gene of the selected glycosidase and generates thus a library of genetically diverse genes expressing glycosidase. Generation of sequence diversity can be performed using well-known methodologies, the most preferable being the error prone polymerase chain reaction (epCR) method. This gene library may be inserted into suitable microorganisms such as *E. coli* or *S. cerevisiae* for producing recombinant variants with slightly altered properties. Clones expressing improved enzymes are then identified with a fast and reliable screening method, selected and brought into a next round of mutation process. The recursive cycles of mutation, recombination and selection are continued as far as mutant(s) with the desired activity and/or specificity is/are evolved. To date, different high-throughput screening methodologies for glycosidases including glycosynthases have been developed. Applying these approaches, effective engineered transglycosidases, including new and more efficient glycosynthases can and have been created and isolated. An α-L-fucosidase from *Thermotoga maritima* has been recently converted into an efficient α-L-transfucosidase by directed evolution. The transferase/hydrolysis ratio of the evolved enzyme was 30 times higher than the native enzyme (see Osanjo, G., et al., Biochemistry, 2007. 46(4): p. 1022-33).

Proteins comprising a transglycosidase and/or a glycosynthase activity as defined above may also comprise fragments or variants of those protein sequences. Such fragments or variants may typically comprise a sequence having a sequence identity with one of the above

mentioned proteins sequences of at least 70%, more preferably at least 80%, equally more preferably at least 85%, even more preferably at least 90% and most preferably at least 95% or even 97%, 98% or 99% as compared to the entire wild type sequence on amino acid level.

"Fragments" of proteins or peptides in the context of the present invention may also comprise a sequence of a protein or peptide as defined herein, which is, with regard to its amino acid sequence N-terminally, C-terminally and/or intrasequentially truncated compared to the amino acid sequence of the original (native) protein. Such truncation may thus occur either on the amino acid level or correspondingly on the nucleic acid level. A sequence identity with respect to such a fragment as defined herein may therefore preferably refer to the entire protein or peptide as defined herein or to the entire (coding) nucleic acid molecule of such a protein or peptide. Likewise, "fragments" of nucleic acids in the context of the present invention may comprise a sequence of a nucleic acid as defined herein, which is, with regard to its nucleic acid molecule 5'-, 3'- and/or intrasequentially truncated compared to the nucleic acid molecule of the original (native) nucleic acid molecule. A sequence identity with respect to such a fragment as defined herein may therefore preferably refer to the entire nucleic acid as defined herein.

"Variants" of proteins or peptides as defined in the context of the present invention (e.g. as encoded by a nucleic acid as defined herein) may be encoded by the nucleic acid molecule of apolymeric carrier cargo complex. Thereby, a protein or peptide may be generated, having an amino acid sequence which differs from the original sequence in one or more mutation(s), such as one or more substituted, inserted and/or deleted amino acid(s). Preferably, these fragments and/or variants have the same biological function or specific activity compared to the full-length native protein, e.g. its specific antigenic property.

"Variants" of proteins or peptides as defined in the context of the present invention (e.g. as encoded by a nucleic acid as defined herein) may also comprise conservative amino acid substitution(s) compared to their native, i.e. non-mutated physiological, sequence. Those amino acid sequences as well as their encoding nucleotide sequences in particular fall under the term variants as defined herein. Substitutions in which amino acids, which originate from the same class, are exchanged for one another are called conservative substitutions. In particular, these are amino acids having aliphatic side chains, positively or negatively charged side chains, aromatic groups in the side chains or amino acids, the side chains of which can

enter into hydrogen bridges, e.g. side chains which have a hydroxyl function. This means that e.g. an amino acid having a polar side chain is replaced by another amino acid having a likewise polar side chain, or, for example, an amino acid characterized by a hydrophobic side chain is substituted by another amino acid having a likewise hydrophobic side chain (e.g. serine (threonine) by threonine (serine) or leucine (isoleucine) by isoleucine (leucine)). Insertions and substitutions are possible, in particular, at those sequence positions which cause no modification to the three-dimensional structure or do not affect the binding region. Modifications to a three-dimensional structure by insertion(s) or deletion(s) can easily be determined e.g. using CD spectra (circular dichroism spectra) (Urry, 1985, Absorption, Circular Dichroism and ORD of Polypeptides, in: Modern Physical Methods in Biochemistry, Neuberger *et al.* (ed.), Elsevier, Amsterdam).

Furthermore, variants of proteins or peptides as defined herein may also comprise those sequences, wherein nucleotides of the nucleic acid are exchanged according to the degeneration of the genetic code, without leading to an alteration of the respective amino acid sequence of the protein or peptide, i.e. the amino acid sequence or at least part thereof may not differ from the original sequence in one or more mutation(s) within the above meaning.

In order to determine the percentage to which two sequences are identical, e.g. nucleic acid sequences or amino acid sequences as defined herein, preferably the amino acid sequences encoded by a nucleic acid sequence of the polymeric carrier as defined herein or the amino acid sequences themselves, the sequences can be aligned in order to be subsequently compared to one another. Therefore, e.g. a position of a first sequence may be compared with the corresponding position of the second sequence. If a position in the first sequence is occupied by the same component as is the case at a position in the second sequence, the two sequences are identical at this position. If this is not the case, the sequences differ at this position. If insertions occur in the second sequence in comparison to the first sequence, gaps can be inserted into the first sequence to allow a further alignment. If deletions occur in the second sequence in comparison to the first sequence, gaps can be inserted into the second sequence to allow a further alignment. The percentage to which two sequences are identical is then a function of the number of identical positions divided by the total number of positions including those positions which are only occupied in one sequence. The percentage to which two sequences are identical can be determined using a mathematical algorithm. A preferred, but not limiting, example of a mathematical algorithm which can be used is the algorithm of

Karlin *et al.* (1993), PNAS USA, 90:5873-5877 or Altschul *et al.* (1997), Nucleic Acids Res., 25:3389-3402. Such an algorithm is integrated in the BLAST program. Sequences which are identical to the sequences of the present invention to a certain extent can be identified by this program.

The proteins as added in step c) of the first aspect, including the preferred and more preferred embodiments, may be provided in a free form or alternatively be bound to or are immobilized onto a surface. In this specific case, the order of steps a), b) and c) is preferably inverted. Binding to or immobilization onto a surface may be carried out e.g. via electrostatic bonds, van der Waals-bonds, covalent bonds, etc. Binding to or immobilization onto a surface may be furthermore carried out, using a covalent linker or a crosslinker, or a Tag, as known to a skilled person for purification of proteins. Such tags comprise, inter alia, e.g. affinity tags or chromatography tags. Affinity tags may include e.g. chitin binding protein (CBP), maltose binding protein (MBP), glutathione-S-transferase (GST), or the Strep-Tag. The poly(His) tag is a widely-used protein tag, that binds to metal matrices. Chromatography tags are used to alter chromatographic properties of the protein to afford different resolution across a particular separation technique, and include e.g. polyanionic amino acids based tags, such as the FLAG-tag. The surface may be the surface of a bioreactor, or any suitable reaction chamber.

According to step d) a mixture is prepared from substances provided by steps a), b) and c). Preferably, such a mixture according to step d) represents a mixture of one, two, three, four, five, one to five, three to ten, five to ten or even more different donors as defined according to step a), one, two, three, four, five, one to five, three to ten, five to ten or even more different acceptors as defined according to step b), and one, two, three, four, five, two to five, two to ten, two to twenty, five to ten or even more different enzymes comprising transglycosidase activity and/or glycosynthase activity.

In a further step e) of the first aspect, including the preferred and more preferred embodiments, for the generation of human milk oligosaccharides (HMOs), the mixture containing at least one compound as defined according to step a), at least one compound as defined according to step b), and at least one enzyme as added according to step c), together forming a mixture according to step d), are incubated to allow generation of human milk oligosaccharides (HMOs) or derivatives thereof via enzymatic means using the at least one

enzyme comprising a transglycosidase activity and/or a glycosynthase activity as defined herein. Such an incubation advantageously allows the generation of a multiplicity of different HMOs. Generation of such a multiplicity of different HMOs is based on the use of enzymes with different activities provided in step c), but also on the use of diverse universal donors and acceptors according to steps a) and b), preferably as a mixture as already outlined in step d). Utilizing this approach, the method of the present invention advantageously allows variation of the possible number and type of oligosaccharides obtainable by the method in a simple and cost efficient manner. The use of enzymes furthermore allows the preparation of various HMOs to be carried out in a stereoselective manner. Generation of HMOs preferably occurs by transferring glycosyl moieties (eg, a sialyl moiety, fucosyl moiety, N-acetyllactosaminyl moiety, or lacto-N-biosyl moiety) by forming new bonds at desired positions of the molecule, etc., in a well defined manner to obtain a mixture of various human milk oligosaccharides or derivatives thereof.

Incubation according to step e) of the first aspest, including the preferred and more preferred embodiments, preferably occurs with a concentration of (each of the) enzymes in a concentration of 1 mU/l to 1,000 U/l, preferably 10 mU/l to 100 U/l, when the activity capable of forming 1 μmol of specific product for a defined protein starting from a defined educt is defined as 1 unit (U), e.g. for a glycotransferase the production of a glycose-containing complex carbohydrate at 37°C in 1 minute. The activity of each enzyme as defined herein may be assessed with repect to its naturally occurring or engineered substrate.

The incubation according to step e) of the first aspect, including the preferred and more preferred embodiments, may be carried out in a reaction medium, preferably an aqueous medium, comprising the mixture obtained according to step d) of the method of the present invention and optionally water; a buffer such as a phosphate buffer, a carbonate buffer, an acetate buffer, a borate buffer, a citrate buffer and a tris buffer, or combinations thereof; alcohol, such as methanol and ethanol; ester such as ethyl acetate; ketone such as acetone; amide such as acetamide; and the like.

Furthermore, the incubation according to step e) of the first aspect, including the preferred and more preferred embodiments, may be carried out in a reaction medium as defined above, wherein optionally a surfactant or an organic solvent may be added, if necessary. Any surfactant capable of accelerating the formation of a complex carbohydrate as defined

according to the present invention as a possible product of the invention can be used as the surfactant. Examples include nonionic surfactants such as polyoxyethylene octadecylamine (e.g., Nymeen S-215, manufactured by Nippon Oil & Fats); cationic surfactants, such as cetyltrimethylammonium bromide and alkyldimethyl benzylammoniumchloride (e.g., Cation F2-40E, manufactured by Nippon Oil & Fats); anionic surfactants such as lauroyl sarcosinate; tertiary amines such as alkyldimethylamine (e.g., Tertiary Amine FB, manufactured by Nippon Oil & Fats); and the like, which are used alone or as a mixture of two or more. The surfactant may be used generally in a concentration of 0.1 to 50 g/l. The organic solvent may include xylene, toluene, fatty acid alcohol, acetone, ethyl acetate, and the like, which may be used in a concentration of generally 0.1 to 50 ml/l.

The incubation according to step e) of the first aspect, including the preferred and more preferred embodiments, may be furthermore carried out in a reaction medium as defined above, preferably having a pH 3 to 10, pH 5 to 10, preferably pH 6 to 8.

The incubation according to step e) of the first aspect, including the preferred and more preferred embodiments, may be furthermore carried out at a temperature of about 0°C to about 100°C, preferably at a temperature of about 10 to about 50°C, e.g. at a temperature of about 20°C to about 50°C. In the reaction medium, inorganic salts, such as $MnCl_2$ and $MgCl_2$, may be added, if necessary.

The incubation according to step e) of the method of the present invention may be carried out in a bioreactor. The bioreactor is preferably suitable for either a continuous mode or a discontinuous mode.

The incubation according to step e) of the first aspect, including the preferred and more preferred embodiments, may be carried out in a continuous or discontinuous mode. If carried out in a continuous mode, the method preferably provides for a continuous flow of compounds and/or enzymes as necessary, preferably by continuously providing educts of the reaction to the reaction mixture and continuously removing products from the reaction mixture, while maintaining the concentration of all components, including enzymes at a predetermined level. The enzymes used in a continuous mode may be added either in free form or as bound or immobilized to a surface.

With regard to the first aspect of the present invention, the addition of donors and/or acceptors and/or enzymes may be repeated according to an optional step f). Particularly preferably, any

of steps a) to d) may be repeated, preferably with the mixture obtained according to step e). Such a mixture is preferably a mixture of at least one compound as defined herein for step a) and at least one compound as defined herein for step b) and at least one enzyme as defined herein according to step c). Such a stepwise proceeding may allow within multiple rounds the rational generation of a defined set of compounds in a controllable manner. It may also provide for a rational exclusion of specific components. To obtain such a variety of compounds, the compounds and/or enzymes may be added simultaneously or sequentially, more preferably compounds and/or enzymes may be added simultaneously in one step and/or sequentially in different steps.

Alternatively, a mixture with at least one compound as defined herein for step a) and at least one compound as defined herein for step b) and at least one enzyme as defined herein according to step c) may be incubated in one step without repetition of one or more steps. Such a proceeding may be preferable in certain circumstances, as it may lead to the largest variety of possible products.

With regard to the preferred and the more preferred embodiments of the first aspect, at least one more additional incubation cycle f) is needed when only one HMO or HMO derivative or precursor thereof is made after step e), as specified above. Particularly preferably, at least one of steps a) to c) shall be repeated, preferably with the mixture obtained according to step e). When repeating any of the steps a) to c), the at least one compound added according to step a) is preferably different to that/those provided in the first cycle, the at least one compound added according to step b) is preferably different to that/those provided in the first cycle, and the at least one enzyme added according to step c) is preferably different to that provided in the first cycle.

A person skilled in the art is able to explore and decide whether only one HMO or HMO derivative or precursor thereof is made after the first incubation step and whether at least one more incubation cycle is needed to achieve the goal. In a possible case, the selection of a particular donor, a particular acceptor and a particular enzyme leads to only one product, for example incubation of the system containing a sialic acid donor according to formula **6** (e.g. pNP sialic acid), an acceptor such as lactose or benzyl lactoside, and a 2-3-α-selective trans-sialidase enzyme results with high probability in the formation of one product, namely 3-sialyllactose (from lactose) or 3-sialyllactose benzyl glycoside (from benzyl lactoside). When

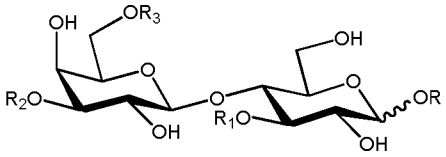
more than one donor and/or more than one acceptor and/or more than enzyme are used for generating an HMO, HMO derivative or HMO precursor in one incubation cycle, more than one product is expected to be formed. Similarly, when the glycosidase and/or glycosynthase enzyme used has lesser (regio)selectivity, more than one product can be expected, even if only one acceptor is provided, as the enzyme is able to transfer the glycosyl moiety to various parts of the acceptor. Moreover, according to a general rule the proportion of the donor and acceptor can have a huge impact on the product diversity: the higher the donor-acceptor ratio, the higher the chance of obtaining more than one product in a one donor-one acceptor system. The skilled person has the repertoire of detection and monitoring methods, both qualitative and quantitative (e.g. TLC, HPLC, GC, GC-MS, electrophoresis, etc.) to find out whether one or more products have been formed.

Furthermore, with regard to the preferred and the more preferred embodiment of the first aspect, the addition acceptors and/or donors and/or enzymes may be repeated according to an optional step g). Such a mixture is preferably a mixture of at least one compound as defined herein for step a), at least one compound as defined herein for step b) and at least one enzyme as defined herein according to step c). When repeating at least one of the steps a) to c), then step d) and step e), the at least one compound provided according to step a) is preferably different from that/those provided in the previous cycle; the at least one compound provided according to step b) is preferably different from that/those provided in the previous cycle, and the at least one enzyme provided according to step c) is preferably different from that/those provided in the previous cycle, Such a stepwise proceeding may allow within multiple rounds the rational generation of a defined set of compounds in a controllable manner. It may also provide for a rational exclusion of specific components. To obtain such a variety of compounds, the compounds and/or enzymes may be added simultaneously or sequentially, and preferably compounds and/or enzymes may be added simultaneously in one step and/or sequentially in different steps.

Alternatively, a mixture with at least one compound as defined herein for step a) and at least one compound as defined herein for step b) and at least one enzyme as defined herein according to step c) may be incubated in one step without repetition of one or more steps. Such a proceeding may be preferable in certain circumstances, as it may lead to the largest variety of possible products.

The method of the present invention as defined above preferably leads to generation of compounds on the basis of universal donors as provided in step a) and acceptors as provided in step b) upon adding enzymes according to step c) and incubating the mixture (step e)). An optional or compulsory repetition of these steps and optionally a hydrogenolysis reaction may be carried out as defined above. Preferably, the method of the present invention as described herein results in either single human milk oligosaccharides (HMOs) or derivatives or precursors thereof, or a diversified mixture comprising two or more human milk oligosaccharides (HMOs) or derivatives or precursors thereof, the single compounds of which may be defined according to

- compounds of general formula **1** and salts thereof:



general formula **1**

wherein

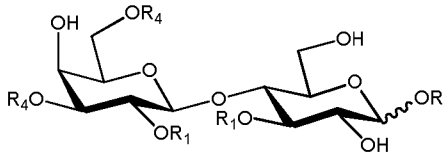
R is a group removable by hydrogenolysis,

R_1 is fucosyl or H,

R_2 is selected from N-acetyl-lactosaminyl and lacto-N-biosyl groups, wherein the N-acetyl lactosaminyl group may carry a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl groups; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_3 is H or N-acetyl-lactosaminyl group optionally substituted with a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl groups; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue;

- compounds of general formula **2** and salts thereof



general formula **2**

wherein

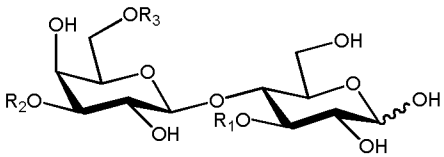
R is a group removable by hydrogenolysis,

R_1 independently of each other is fucosyl or H

R_4 independently of each other is sialyl or H,

with the proviso that at least one R_1 or R_4 is not H,

- compounds of general formula **3** and salts thereof



general formula **3**

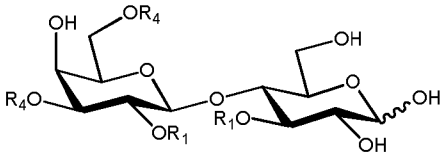
wherein

R_1 is fucosyl or H,

R_2 is selected from N-acetyl-lactosaminyl and lacto-N-biosyl groups, wherein the N-acetyl lactosaminyl group may carry a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl groups; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_3 is H or N-acetyl-lactosaminyl group optionally substituted with a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl groups; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue; and/or

- compounds of general formula **4** and salts thereof



general formula **4**

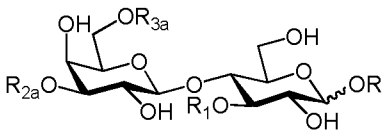
wherein

R_1 independently of each other is fucosyl or H

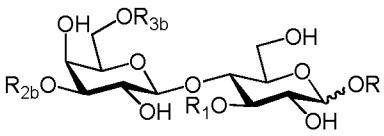
R_4 independently of each other is sialyl or H,

with the proviso that at least one R_1 or R_4 is not H.

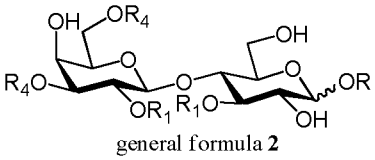
Even more preferably, the method of the present invention for generation of human milk oligosaccharides (HMOs) results in either single human milk oligosaccharide derivatives, or a mixture comprising two or more human milk oligosaccharide (HMO) derivatives as defined above, preferably after incubation step e) and/or a repetition of steps according to step f) or g), wherein compounds of formulae **1** and **2** are further characterized by general formulae **1a**, **1b** or **2** and salts thereof



general formula **1a**



general formula **1b**



general formula **2**

wherein

R, R_1 and R_4 are as defined above,

R_{2a} is N-acetyl-lactosaminyl group optionally substituted with a glycosyl residue comprising one N-acetyl-lactosaminyl and/or one lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_{3a} is H or N-acetyl-lactosaminyl group optionally substituted with a lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_{2b} is lacto-N-biosyl group optionally substituted with sialyl and/or fucosyl residue, and

R_{3b} is H or N-acetyl-lactosaminyl group optionally substituted with one or two N-acetyl-lactosaminyl and/or one lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue.

Particularly preferably, compounds obtained according to the method of the present invention for generation as defined above are characterized by their linkages and modifications. Preferably, the compounds obtained by the method of the present invention after incubation step e) and/or a repetition of steps according to step f) or g), preferably as defined according to general formulae **1a** and **1b**, are characterized in that:

- the N-acetyl-lactosaminyl group in the glycosyl residue of R_{2a} in general formula **1a** is attached to the another N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the lacto-N-biosyl group in the glycosyl residue of R_{2a} in general formula **1a** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the lacto-N-biosyl group in the glycosyl residue of R_{3a} in general formula **1a** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the N-acetyl-lactosaminyl group in the glycosyl residue of R_{3b} in general formula **1b** is attached to the another N-acetyl-lactosaminyl group with 1-3 or 1-6 interglycosidic linkage,
- the lacto-N-biosyl group in the glycosyl residue of R_{3b} in general formula **1b** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage.

Preferably, the compounds obtained by the method of the present invention, after incubation step e) and/or a repetition of steps according to step f) and g), are characterized in that general formula **1a** represents the R-glycosides of lacto-N-neotetraose, para-lacto-N-hexaose, para-lacto-N-neohexaose, lacto-N-neohexaose, para-lacto-N-octaose and lacto-N-neooctaose optionally substituted with one or more sialyl and/or fucosyl residue, and general formula **1b** represents the R-glycosides of lacto-N-tetraose, lacto-N-hexaose, lacto-N-octaose, iso-lacto-N-octaose, lacto-N-decaose and lacto-N-neodecaose optionally substituted with one or more sialyl and/or fucosyl residue.

Preferably, the compounds obtained by the method of the present invention, after incubation step e) and/or a repetition of steps according to step f) or g), are characterized in that:

- the fucosyl residue attached to the N-acetyl-lactosaminyl and/or the lacto-N-biosyl group is linked to
  - the galactose of the lacto-N-biosyl group with 1-2 interglycosidic linkage and/or

- the N-acetyl-glucosamine of the lacto-N-biosyl group with 1-4 interglycosidic linkage and/or
- the N-acetyl-glucosamine of the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,

- the sialyl residue attached to the N-acetyl-lactosaminyl and/or the lacto-N-biosyl group is linked to
  - the galactose of the lacto-N-biosyl group with 2-3 interglycosidic linkage and/or
  - the N-acetyl-glucosamine of the lacto-N-biosyl group with 2-6 interglycosidic linkage and/or
  - the galactose of the N-acetyl-lactosaminyl group with 2-6 interglycosidic linkage.

According to a further preferred aspect, compounds as obtained according to the method of the present invention, preferably compounds according to formulae **1** or **2** or of general subformulae **1a**, **1b** or **2** may be selected from the group of: R-glycosides of 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-3-fucosyllactose, lacto-N-tetraose, lacto-N-neotetraose, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LST-a, LST-b, LST-c, FLST-a, FLST-b, FLST-c, LNDFH-I, LNDFH-II, LNDFH-III, DS-LNT, FDS-LNT I and FDS-LNT II, or salts thereof. The core structures of these compounds are shown in Table 6 below. The R-glycosides may be alpha or beta-anomers. Preferably, said R-glycosides are the beta-anomers.

Abbreviation	Chemical Structure
2'-FL	Fuc(α1-2)Gal(β1-4)Glc
3-FL	Gal(β1-4)Glc \| Fuc(α1-3)
DFL	Fuc(α1-2)Gal(β1-4)Glc \| Fuc(α1-3)
3'-SL	Neu5Ac(α2-3)Gal(β1-4)Glc
FSL	Neu5Ac(α2-3)Gal(β1-4)Glc \| Fuc(α1-3)
LNT	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
LNFP I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc

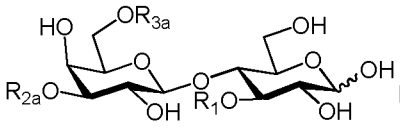
Abbreviation	Chemical Structure
LNFP II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)
LNFP III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
LNFP V	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
LNDFH I	Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)
LNDFH II	Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | | Fuc(α1-4) Fuc(α1-3)
LNDFH III	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | | Fuc(α1-3) Fuc(α1-3)
LSTa	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
LSTb	Neu5Ac(α2-6) | Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
LSTc	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc
F-LSTa	Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)
F-LSTb	Neu5Ac(α2-6) | Fuc(α1-2)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
F-LSTc	Neu5Ac(α2-6)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
DS-LNT	Neu5Ac(α2-6) | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc
FDS-LNT I	Neu5Ac(α2-6) | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-4)
FDS-LNT II	Neu5Ac(α2-6) | Neu5Ac(α2-3)Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)Glc | Fuc(α1-3)
LNnT	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc

Table 6: Core structures of R-glycosides of naturally occuring HMOs having a lactose, LNT or LNnT core

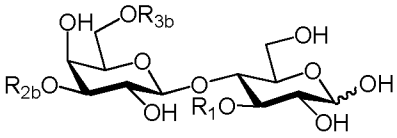
Most preferably, compounds as obtained according to the method of the present invention of generation of HMOs, preferably compounds according to formulae **1** or **2** or of general subformulae **1a**, **1b** or **2**, may be selected from compounds wherein R is benzyl, or salts thereof.

According to a further specific aspect of the method of the present invention the compounds obtained according to the method of the present invention, preferably compounds according to any of formulae **1** or **2** or of general subformulae **1a**, **1b** or **2** or of general subformulae **1a**, **1b** or **2**, are optionally subjected to a hydrogenolysis reaction subsequent to incubation according to step e) and/or a further repetition according to step f), resulting in the formation of HMOs characterized by general formulae **3** and **4**.

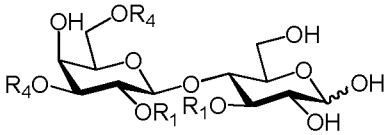
Likewise preferably, the method of the present invention for generation of human milk oligosaccharides (HMOs) results in human milk oligosaccharides as defined above, preferably after incubation step e) and/or a repetition of steps according to step f) or g), wherein compounds of formulae **3** and **4** are further characterized by general formulae **3a**, **3b** or **4** and salts thereof



general formula **3a**



general formula **3b**



general formula **4**

wherein R_1 and R_4 are as defined above,

R_{2a} is N-acetyl-lactosaminyl group optionally substituted with a glycosyl residue comprising one N-acetyl-lactosaminyl and/or one lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_{3a} is H or N-acetyl-lactosaminyl group optionally substituted with a lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_{2b} is lacto-N-biosyl group optionally substituted with sialyl and/or fucosyl residue,

R_{3b} is H or N-acetyl-lactosaminyl group optionally substituted with one or two N-acetyl-lactosaminyl and/or one lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue.

Particularly preferably, compounds obtained according to the method of the present invention for generation as defined above are characterized by their linkages and modifications. Preferably, the compounds obtained by the method of the present invention, after incubation step e) and/or a repetition of steps according to step f) or g), and as preferably defined according to general formulae **3a** or **3b**, are characterized in that:

- the N-acetyl-lactosaminyl group in the glycosyl residue of R_{2a} in general formula **3a** is attached to another N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the lacto-N-biosyl group in the glycosyl residue of R_{2a} in general formula **3a** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the lacto-N-biosyl group in the glycosyl residue of R_{3a} in general formula **3a** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the N-acetyl-lactosaminyl group in the glycosyl residue of R_{3b} in general formula **3b** is attached to another N-acetyl-lactosaminyl group with 1-3 or 1-6 interglycosidic linkage,
- the lacto-N-biosyl group in the glycosyl residue of R_{3b} in general formula **3b** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage.

Preferably, the compounds obtained by the method of the present invention after incubation step e) and/or a repetition of steps according to step f) or g) are characterized in that general formula **3a** represents lacto-N-neotetraose, para-lacto-N-hexaose, para-lacto-N-neohexaose, lacto-N-neohexaose, para-lacto-N-octaose and lacto-N-neooctaose optionally substituted with one or more sialyl and/or fucosyl residue, and general formula **3b** represents lacto-N-tetraose, lacto-N-hexaose, lacto-N-octaose, iso-lacto-N-octaose, lacto-N-decaose and lacto-N-neodecaose optionally substituted with one or more sialyl and/or fucosyl residue.

Preferably, the compounds obtained by the method of the present invention, after incubation step e) and/or a repetition of steps according to step f) or g), are characterized in that:

- the fucosyl residue attached to the N-acetyl-lactosaminyl and/or the lacto-N-biosyl group is linked to
  - the galactose of the lacto-N-biosyl group with 1-2 interglycosidic linkage and/or
  - the N-acetyl-glucosamine of the lacto-N-biosyl group with 1-4 interglycosidic linkage and/or
  - the N-acetyl-glucosamine of the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the sialyl residue attached to the N-acetyl-lactosaminyl and/or the lacto-N-biosyl group is linked to
  - the galactose of the lacto-N-biosyl group with 2-3 interglycosidic linkage and/or
  - the N-acetyl-glucosamine of the lacto-N-biosyl group with 2-6 interglycosidic linkage and/or
  - the galactose of the N-acetyl-lactosaminyl group with 2-6 interglycosidic linkage.

According to a further preferred aspect, compounds as obtained according to the method of the present invention of generation of HMOs, preferably compounds according to general subformulae **3a**, **3b** or **4** may be selected from the group of: 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-3-fucosyllactose, lacto-N-tetraose, lacto-N-neotetraose, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LST-a, LST-b, LST-c, FLST-a, FLST-b, FLST-c, LNDFH-I, LNDFH-II, LNDFH-III, DS-LNT, FDS-LNT I and FDS-LNT II, or salts thereof. The core structures of these compounds are shown in Table 6 above.

Likewise preferably, the compounds obtained by the method of the present invention, after incubation step e) and/or a repetition of steps according to step f) or g), are obtained depending on the activity of theat least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity as described above. Such an enzyme may be selected depending on the desired linkage or modification to be carried out during generation of HMOs using the method of the present invention.

Wild type or engineered fucosidases as defined above may be utilized herein, displaying transfucosidase activity and showing a α,1-2, α,1-3 and/or α,1-4 regioselectivity are targeted

in the present invention. Such wild type or engineered fucosidases preferably display transfucosidase activity and catalyze the transfer of the fucosyl residue to:

- the galactose of the lacto-N-biosyl group with α,1-2 interglycosidic linkage and/or
- the N-acetyl-glucosamine of the lacto-N-biosyl group with α,1-4 interglycosidic linkage and/or
- the N-acetyl-glucosamine of the N-acetyl-lactosaminyl group with α,1-3 interglycosidic linkage;

Such linkages are highly preferred in the context of the present method of the present invention and the compounds claimed herein, when using wild type or engineered fucosidases.

Additionally, wild type or engineered sialidases as defined above may be utilized herein, which display trans-sialidase activity and show a α,2-3 and/or α,2-6 regioselectivity. Such linkages are preferably targeted in the present invention. Such wild type or engineered sialidases preferably display trans-sialidase activity and catalyze the transfer of the sialyl residue to:

- the galactose of the lacto-N-biosyl group with 2-3 interglycosidic linkage and/or
- the N-acetyl-glucosamine of the lacto-N-biosyl group with 2-6 interglycosidic linkage and/or
- the galactose of the N-acetyl-lactosaminyl group with 2-6 interglycosidic linkage.

Such linkages are highly preferred in the context of the present method of the present invention and the compounds claimed herein, when using wild type or engineered sialidases.

Furthermore, wild type or engineered lacto-N-biosidases as defined above may be utilized herein, which display trans-lacto-N-biosidase activity and show a β,1-3 regioselectivity. Such linkages are preferably targeted in the present invention. Such wild type or engineered lacto-N-biosidases preferably display trans-lacto-N-biosidase activity and catalyze the transfer of the lacto-N-biosyl residue to an N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage are targeted in the present invention. Such linkages are highly preferred in the context of the present method of the present invention and the compounds claimed herein, when using wild type or engineered lacto-N-biosidases.

Finally, wild type or engineered glycosidases as defined above may be utilized herein, which display trans-N-acetyllactosaminidase activity and show a β,1-3 and/or β,1-6 regioselectivity are targeted in the present invention. Such wild type or engineered glycosidases preferably display trans-N-acetyllactosaminidase activity and catalysize the transfer of the N-acetyl-lactosaminyl residue to another N-acetyl-lactosaminyl group with 1-3 or 1-6 interglycosidic linkage. Such linkages are highly preferred in the context of the present method of the present invention and the compounds claimed herein, when using wild type or engineered N-acetyllactosaminidases.

According to another specific aspect of the method of the present invention the compounds obtained according to the method of the present invention, preferably compounds according to any of formulae **1**, **2**, **3** or **4** or of general subformulae **1a**, **1b** or **2** or of general subformulae **3a**, **3b** or **4** are optionally subjected to a purification reaction preferably via crystallization or precipitation.

According to a specific aspect of the method of the present invention the compounds obtained according to the method of the present invention, preferably compounds according to any of formulae **1**, **2**, **3** or **4** or of general subformulae **1a**, **1b** or **2** or of general subformulae **3a**, **3b** or **4** are optionally spray-dried.

According to a very particular aspect, the compounds obtained according to the present method of the present invention may be one or more naturally occurring HMO R-glycosides, preferably compounds according to formulae **1** or **2** or of general subformulae **1a**, **1b** or **2**, or one or more naturally occurring HMOs, preferably compounds according to formulae **3** or **4** or of general subformulae **3a**, **3b** or **4**. Naturally occuring HMOs are listed in TADASU URASHIMA et al, MILK OLIGOSACCHARIDES, Nova Biomedical Books, *New York*, 2011, ISBN: 978-1-61122-831-1, Table 4 in pp. 14-25.

Furthermore, according to a very particular aspect, the compounds obtained according to the present method of the present invention may be an HMO, wherein the HMO is derivatized with benzyl.

According to a further specific aspect of the method of the present invention the compounds obtained according to the method of the present invention, preferably single compounds or mixture of two or more compounds according to any of formulae **1** or **2** or of general subformulae **1a**, **1b** or **2** are optionally subjected to a hydrogenolysis reaction resulting in the

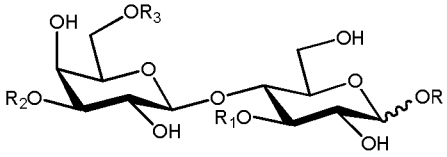
formation of single HMOs or mixture of two or more HMOs characterized by general formulae **3** and **4**.

According to an optional hydrogenolysis step, single compounds or mixture of two or more compounds according to any of formulae **1** or **2** or of general subformulae **1a**, **1b** or **2** after step e), f) or g) may be subjected to a hydrogenolysis reaction, e.g. as defined herein. In this context, such an optional hydrogenolysis step is preferably carried out to obtain the naturally occurring naked HMOs, e.g. as defined according to any of formulae as defined above, and to preferably remove possible protecting groups, such as benzyl groups.

Catalytic hydrogenolysis typically takes place in a protic solvent or in a mixture of protic solvents. A protic solvent may be selected from the group consisting of water, acetic acid or C_1-C_6 alcohols. A mixture of one or more protic solvents with one or more suitable aprotic organic solvents partially or fully miscible with the protic solvent(s) (such as THF, dioxane, ethyl acetate or acetone) may also be used. Water, one or more C_1-C_6 alcohols or a mixture of water and one or more C_1-C_6 alcohols are preferably used as the solvent system. Solutions containing the carbohydrate derivatives in any concentration or suspensions of the carbohydrate derivatives in the solvent(s) used are also applicable. The reaction mixture is stirred at a temperature in the range of 10-100 °C, preferably between 20-50 °C, in a hydrogen atmosphere of 1-50 bar absolute (100 to 5000 kPa) in the presence of a catalyst such as palladium, Raney nickel or any other appropriate metal catalyst, preferably palladium on charcoal or palladium black, until reaching the completion of the reaction. Transfer hydrogenolysis may also be performed, when the hydrogen is generated *in situ* from cyclohexene, cyclohexadiene, formic acid or ammonium formate. Addition of organic or inorganic bases or acids and/or basic and/or acidic ion exchange resins can also be used to improve the kinetics of the hydrogenolysis. The use of basic substances is especially preferred when halogen substituents are present on the substituted benzyl moieties of the precursors and/or the formation of mannosamine base is desirable. Preferred organic bases include, but are not limited to, triethylamine, diisopropyl ethylamine, ammonia, ammonium carbamate and diethylamine. An organic or an inorganic acid is favourably used as a co-solvent or additive in cases when mannosamine salts are the intended products. Preferred acids include, but are not limited to, formic acid, acetic acid, propionic acid, chloroacetic acid, dichloroacetic acid, trifluoroacetic acid, HCl and HBr. The conditions proposed above allow simple, convenient and delicate removal of the solvent(s) giving rise to a mixture or blend of pure HMOs.

Accordingly, a further aspect of the present invention relates to providing single compounds or a mixture of two or more compounds, which compounds are characterized by

- general formula **1** and salts thereof:



general formula **1**

wherein

R is a group removable by hydrogenolysis,

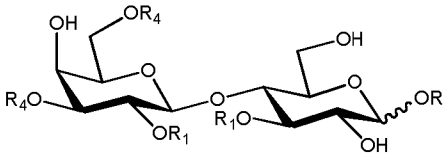
R_1 is fucosyl or H,

R_2 is selected from N-acetyl-lactosaminyl and lacto-N-biosyl groups, wherein the N-acetyl lactosaminyl group may carry a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl groups; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_3 is H or N-acetyl-lactosaminyl group optionally substituted with a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl groups; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

with the proviso that, if provided alone, the compound is not LNnT R-glycoside or LNT benzyl glycoside;

- or general formula **2** and salts thereof



general formula **2**

wherein

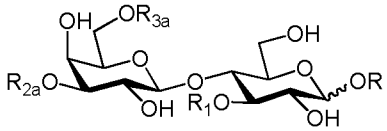
R is a group removable by hydrogenolysis,

R_1 independently of each other is fucosyl or H

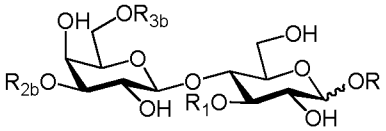
R_4 independently of each other is sialyl or H,

with the proviso that, that at least one of R_1 or R_4 is not H, and, if provided alone, the compound is not 3'-sialyllactose benzyl glycoside sodium salt or 6'-sialyllactose R-glycoside.

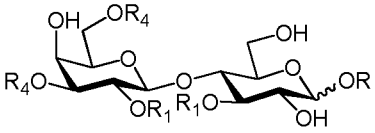
Even more preferably, the invention relates to either single human milk oligosaccharide derivatives, or a diversified mixture comprising two or more human milk oligosaccharide (HMOs) derivatives, wherein compounds of formulae **1** and **2** defined above are further characterized by general formulae **1a**, **1b** or **2** and salts thereof



general formula **1a**



general formula **1b**



general formula **2**

wherein

R, R_1 and R_4 are as defined above,

R_{2a} is N-acetyl-lactosaminyl group optionally substituted with a glycosyl residue comprising one N-acetyl-lactosaminyl and/or one lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_{3a} is H or N-acetyl-lactosaminyl group optionally substituted with a lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_{2b} is lacto-N-biosyl group optionally substituted with sialyl and/or fucosyl residue, and

R_{3b} is H or N-acetyl-lactosaminyl group optionally substituted with one or two N-acetyl-lactosaminyl and/or one lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue.

Particularly preferably, compounds defined above are characterized by their linkages and modifications. Preferably, the compounds defined according to general formulae **1a** and **1b,** are characterized in that:

- the N-acetyl-lactosaminyl group in the glycosyl residue of R_{2a} in general formula **1a** is attached to another N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,

- the lacto-N-biosyl group in the glycosyl residue of R_{2a} in general formula **1a** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the lacto-N-biosyl group in the glycosyl residue of R_{3a} in general formula **1a** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the N-acetyl-lactosaminyl group in the glycosyl residue of R_{3b} in general formula **1b** is attached to another N-acetyl-lactosaminyl group with 1-3 or 1-6 interglycosidic linkage,
- the lacto-N-biosyl group in the glycosyl residue of R_{3b} in general formula **1b** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage.

Preferably, the compounds characterized by general formula **1a** represent the R-glycosides of lacto-N-tetraose, para-lacto-N-hexaose, para-lacto-N-neohexaose, lacto-N-neohexaose, para-lacto-N-octaose and lacto-N-neooctaose optionally substituted with one or more sialyl and/or fucosyl residue, and general formula **1b** represent the R-glycosides of lacto-N-tetraose, lacto-N-hexaose, lacto-N-octaose, iso-lacto-N-octaose, lacto-N-decaose and lacto-N-neodecaose optionally substituted with one or more sialyl and/or fucosyl residue.

According to a further preferred aspect, compounds as defined above may be selected from the group of: R-glycosides of 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-3-fucosyllactose, lacto-N-tetraose, lacto-N-neotetraose, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LST-a, LST-b, LST-c, FLST-a, FLST-b, FLST-c, LNDFH-I, LNDFH-II, LNDFH-III, DS-LNT, FDS-LNT I and FDS-LNT II, or salts thereof. The R-glycosides may be alpha or beta-anomers. Preferably, said R-glycosides are the beta-anomers and more preferably R is benzyl.

According to a further embodiment, the method of the present invention further comprises addition of the compounds obtained in the incubation step or after the hydrogenolysis step to a consumable product, preferably as defined herein. The consumable product is preferably at least one of a pharmaceutical or nutritional formulation and preferably a liquid or a solid. According to another embodiment, the method may further comprise the addition of pharmaceutically acceptable carriers and/or the addition of prebiotics to the compounds obtained in the incubation step.

According to a second aspect, the present invention also provides a compound, particularly a mixture of HMOs, characterized and specified above, obtained or obtainable by the method of the present invention as described herein.

According to a further embodiment of the second aspect, the present invention provides a compound, preferably a mixture of compounds, more preferably a mixture of HMOs, obtained or obtainable by the method of the present invention as described herein. In this context, such a mixture of compounds obtained or obtainable by the method of the present invention as described herein is preferably to be understood as a mixture of at least 2 to 10, 2 to 10, 2 to 20, 2 to 20, 2 to 100, 2 to 200, or even more different compounds as generally defined above. Such compounds may be preferably selected without restriction from any of the compounds as defined according to any of formulae **1**, **2**, **3** or **4** or of any subformulae or selection as defined above.

According to a third aspect, the present invention also provides compounds which may be utilized in the present invention, e.g. as acceptor, and compounds which may be obtained during the method of the present invention of generation of HMOs.

The present invention also provides or utilizes salts of herein defined compounds. Such salts may be preferably selected from salts of the compounds according to general formulae **1-4** or subformulae thereof, which contain at least one sialyl residue, in salt form: an associated ion pair consisting of the negatively charged acid residue of sialylated oligosaccharides falling under general formulae **1-4** or subformulae thereof and one or more cations in any stoichiometric proportion. Cations, as used in the present context, are atoms or molecules with positive charge. The cation may be inorganic or organic. Preferred inorganic cations are ammonium ion, alkali metal, alkali earth metal and transition metal ions, more preferably Na^+, K^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+} and Cu^{2+}, most preferably K^+, Ca^{2+}, Mg^{2+}, Ba^{2+}, Fe^{2+} and Zn^{2+}. Basic organic compounds in positively charged form may be relevant organic cations. Such preferred positively charged counterparts are diethyl amine, triethyl amine, diisopropyl ethyl amine, ethanolamine, diethanolamine, triethanolamine, imidazole, piperidine, piperazine, morpholine, benzyl amine, ethylene diamine, meglumin, pyrrolidine, choline, tris-(hydroxymethyl)-methyl amine, N-(2-hydroxyethyl)-pyrrolidine, N-(2-hydroxyethyl)-piperidine, N-(2-hydroxyethyl)-piperazine, N-(2-hydroxyethyl)-morpholine, L-arginine, L-lysine, oligopeptides having L-arginine or L-lysine unit or oligopeptides having a

free amino group on the *N*-terminal, etc., all in protonated form. Such salt formations can be used to modify characteristics of the complex molecule as a whole, such as stability, compatibility to excipients, solubility and the ability to form crystals.

According to a particular embodiment of the third aspect, the compounds as defined herein may be subjected to a hydrogenolysis reaction, e.g. as defined herein, preferably, if R is not H, even more preferably, if R is benzyl. Such a hydrogenolysis reaction is preferably carried out as described above. Preferably, R may be as defined herein to be cleavable in a hydrogenolysis reaction, preferably as described above.

In a further embodiment of the third aspect, compounds or a mixture of compounds, more preferably a mixture of HMOs, obtained or obtainable by the method of the present invention as described herein or any further compound as defined herein, may be used for the preparation of a consumable product, preferably for the preparation of a pharmaceutical composition, a nutritional formulation or a food supplement. Such a compound or mixture of compounds, more preferably a mixture of HMOs, obtained or obtainable by the method of the present invention as described herein is particularly effective in the improvement and maturation of the immune system of neonatal infants, and has preventive effect against secondary infections following viral infections such as influenza. The use of compounds or a mixture of compounds, more preferably a mixture of HMOs, obtained or obtainable by the method of the present invention as described herein as prebiotic enhances the beneficial effects and efficiency of probiotics, such as *Lactobacillus* and *Bifidobacterium* species, in promoting the development of an early bifidogenic intestinal microbiota in infants, in reducing the risk of development or allergy and/or asthma in infants, and in preventing and treating pathogenic infections in such as diarrhoea in infants.

In a fourth aspect, the present invention provides a pharmaceutical composition, comprising compounds or a mixture of compounds, more preferably a mixture of HMOs, obtained or obtainable by the method of the present invention as described herein, and preferably a pharmaceutically acceptable carrier. "Pharmaceutically acceptable carriers" include but are not limited to additives, adjuvants, excipients and diluents (water, gelatine, talc, sugars, starch, gum arabic, vegetable gums, vegetable oils, polyalkylene glycols, flavouring agents, preservatives, stabilizers, emulsifying agents, lubricants, colorants, fillers, wetting agents, etc.). Suitable carriers are described in the most recent edition of Remington's Pharmaceutical

Sciences, a standard reference text in the field. The dosage form for administration includes, for example, tablets, powders, granules, pills, suspensions, emulsions, infusions, capsules, injections, liquids, elixirs, extracts and tinctures.

In a fifth aspect, nutritional formulations are provided such as foods or drinks, comprising compounds or a mixture of compounds, more preferably a mixture of HMOs, obtained or obtainable by the method of the present invention as described herein. The nutritional formulation may contain edible micronutrients, vitamins and minerals as well. The amounts of such ingredient may vary depending on whether the formulation is intended for use with normal, healthy infants, children, adults or subjects having specialized needs (e.g. suffering from metabolic disorders). Micronutrients include for example edible oils, fats or fatty acids (such as coconut oil, soy-bean oil, monoglycerides, diglycerides, palm olein, sunflower oil, fish oil, linoleic acid, linolenic acid etc.), carbohydrates (such as glucose, fructose, sucrose, maltodextrin, starch, hydrolyzed cornstarch, etc.) and proteins from casein, soy-bean, whey or skim milk, or hydrolysates of these proteins, but protein from other source (either intact or hydrolysed) may be used as well. Vitamins may be chosen from the group consisting of vitamin A, B1, B2, B5, B6, B12, C, D, E, H, K, folic acid, inositol and nicotinic acid. The nutritional formula may contain the following minerals and trace elements: Ca, P, K, Na, Cl, Mg, Mn, Fe, Cu, Zn, Se, Cr or I.

According to a general embodiment of the fifth aspect, a nutritional formulation as defined above may further contain one or more probiotics, e.g. lacto bacteriae, *Bifidobacterium* species, prebiotics such as fructooligosaccharides and galactooligosaccharides, proteins from casein, soy-bean, whey or skim milk, carbohydrates such as lactose, saccharose, maltodextrin, starch or mixtures thereof, lipids (e.g. palm olein, sunflower oil, safflower oil) and vitamins and minerals essential in a daily diet. Probiotics are preferably also contained in the nutritional formulation in an amount sufficient to achieve the desired effect in an individual, preferably in infants, children and/or adults.

In a preferred embodiment, the nutritional formulation as defined above is an infant formula. In the context of the present invention, the term "infant formula" preferably means a foodstuff intended for particular nutritional use by infants during the first 4-6 months or even 4 to 12 months of life and satisfying by itself the nutritional requirements of infants. It may contain one or more probiotic *Bifidobacterium* species, prebiotics such as fructooligosaccharides and

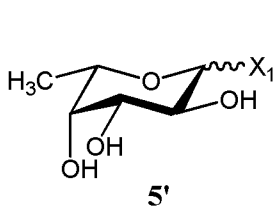
galactooligosaccharides, proteins from casein, soy-bean, whey or skim milk, carbohydrates such as lactose, saccharose, maltodextrin, starch or mixtures thereof, lipids (e.g. palm olein, sunflower oil, safflower oil) and vitamins and minerals essential in a daily diet.

In a sixth aspect, a food supplement may be provided. Such a food supplementcontains ingredients as defined for nutritional food above, e.g. compounds or a mixture of compounds, more preferably a mixture of HMOs, obtained or obtainable by the method of the present invention as described herein, vitamins, minerals, trace elements and other micronutritients, etc. The food supplement may be for example in the form of tablets, capsules, pastilles or a liquid. The supplement may contain conventional additives selected from but not limited to binders, coatings, emulsifiers, solubilising agents, encapsulating agents, film forming agents, adsorbents, carriers, fillers, dispersing agents, wetting agents, gellifying agents, gel forming agents, etc.

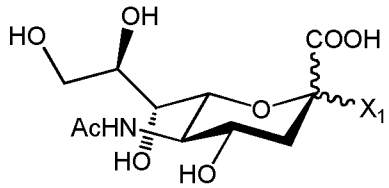
According to a preferred embodiment, the food supplement is a digestive health functional food as the administration of compounds or a mixture of compounds, more preferably a mixture of HMOs, obtained or obtainable by the method of the present invention as described herein, provides a beneficial effect on digestive health. A digestive health functional food is preferably a processed food used with the intention of enhancing and preserving digestive health by utilizing compounds or a mixture of compounds, more preferably a mixture of HMOs, obtained or obtainable by the method of the present invention as described herein, as physiologically functional ingredients or components in forms of tablet, capsule, powder, etc. Different terms such as dietary supplement, nutraceutical, designed food, or health product may also be used to refer to a digestive health functional food.

In a further aspect, compounds or a mixture of compounds, more preferably a mixture of HMOs, obtained or obtainable by the method of the present invention as described herein, may be used for the preparation of nutritional formulations including foods, drinks and feeds, preferably infant formulas, food supplements and digestive health functional foods, preferably any of these as described above. The nutritional formulation may be prepared in any usual manner.

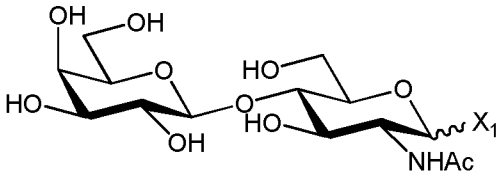
According to another aspect of the present invention is provided glycosyl donors used in the present invention which are characterized by general formulae **5'-8'**



5'



6'



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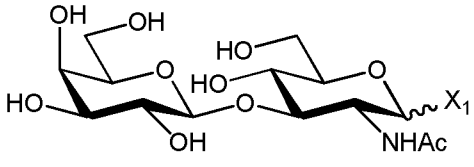
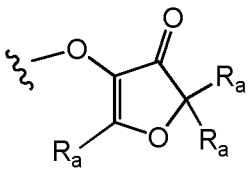
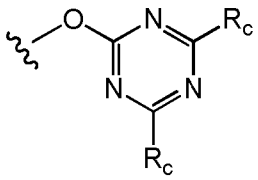


8'

wherein X_1 is group **A** or group **B**



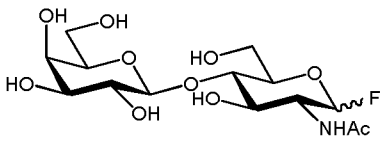
A

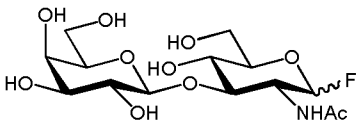


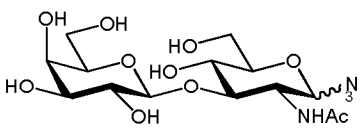
B

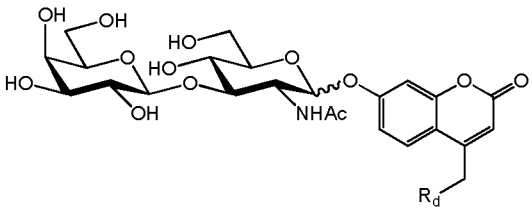
wherein R_a is independently H or alkyl, or two vicinal R_a groups represent a $=C(R_b)_2$ group, wherein R_b is independently H or alkyl, and R_c is independently selected from the group consisting of alkoxy, amino, alkylamino and dialkylamino,

and/or a compound of any of the following formulae









wherein R_d is selected from the group consisting of H, alkyl and $-C(=O)R_e$, and wherein R_e is OH, alkoxy, amino, alkylamino, dialkylamino, hydrazino, alkylhydrazino, dialkylhydrazino or trialkylhydrazino, or a salt of any of the aforementioned compounds.

In preferred compounds X_1 is group **A**, wherein R_a is independently H or C_{1-6} alkyl, preferably H or methyl. These compounds are especially advantageous donors in glycosylation/transglycosylation reactions because their water solubility is high, the leaving group after glycosylation can be detected easily by UV detection, and X_1 leaving group, being a natural aroma of fruits, causes no regulatory obstacles when using in the food industry.

In the present invention, if not otherwise indicated, different features of alternatives and embodiments may be combined with each other, where suitable.

Whilst the invention has been described with reference to a preferred embodiment, it will be appreciated that various modifications are possible within the scope of the invention.

In this specification, unless expressly otherwise indicated, the word 'or' is used in the sense of an operator that returns a true value when either or both of the stated conditions is met, as opposed to the operator 'exclusive or' which requires that only one of the conditions is met. The word 'comprising' is used in the sense of 'including' rather than in to mean 'consisting of'. All prior teachings acknowledged above are hereby incorporated by reference. No acknowledgement of any prior published document herein should be taken to be an admission or representation that the teaching thereof was common general knowledge in Australia or elsewhere at the date hereof.

EXPERIMENTAL

Example 1

General procedure for transglycosylation reactions:

A solution of appropriate glycosyl donor(s) such as derivatives of general formula **5** to **10** and glycosyl acceptor(s) (10 mM –1M) was incubated in an incubation buffer at a pH range from 5.0 to 9.0 with recombinant glycosidase, α-transglyccosidase or α-glycosynthase, such as α-fucosidase, α-transfucosidase, α-fucosynthase, α-sialidase, α-transsialidase, β-lacto-N-biosidase β-trans-lacto-N-biosidase, β-N-acetyllactosaminidase or β-trans-N-acetyllactosaminidase. The reaction mixture was stirred at a temperature in the range of from 20 to 70°C. Samples were taken at different times of the reaction, the reaction was stopped by the addition of 1M $NaHCO_3$-solution at pH=10 and products were analyzed by HPLC, or/and LC-MS, or/and LC-MS-MS. After completion, the enzyme was denatured and centrifuged.

The resulting solution was evaporated under reduced pressure. After lyophilisation, the dry residue was dissolved in water and products were purified by biogel chromatography (P-2 Biogel, 16x900 mm) with water or by reverse phase chromatography.

The following recombinant enzymes were used and tested in the transglycosylation reaction:

Transfucosidase P25 from *Thermotoga maritima* (see seq. ID 1) containing mutations G226S Y237H T264A L322P

Transfucosidase M3 from *Thermotoga maritima* (see seq. ID 1) containing mutations Y237H Y267F L322P.

Transsialidase from *Trypanosoma cruzi* (see seq. ID 15, 16)

Fucosidase Blon_2336 from *Bifidobacterium longum subsp. infantis* ATCC 15697 (see seq. ID 5)

These transglycosidases were produced in *E. coli* as reported in Osanjo et al. *Biochemistry* **46**, 1022 (2007), Sela et al. *Appl. Environ. Microbiol.* **78**, 795 (2012), Agustí et al. *Glycobiology* **14**, 659 (2004) and Neubacher et al. *Org. Biomol. Chem.* **3**, 1551 (2005). Purified transglycosidases were stored at -20°C to +4°C.

Example 2

Sialylation using p-nitrophenyl sialoside donor

General procedure: a solution of 2-*O*-(*p*-nitrophenyl)-α-D-sialoside (75 µmol) and the appropriate sialyl acceptor (35 µmol) in degassed incubation buffer (1.0 ml, 100 mM Tris/HCl, pH 7.5, 50 mg BSA, 0.02 % NaN_3) was incubated with recombinant transsialidase from *T. cruzi* (80 µl, 1.3 mg/ml) at 23°C for 24 h. The reaction was monitored by TLC (butanol/acetic acid/water 5:2:2). After completion, the enzyme was denatured and centrifuged before the supernatant is lyophilized. The dry residue was dissolved in water and purified by biogel chromatography (P-2 Biogel, 16x900 mm) with water or by reverse phase chromatography. The yields vary between 45-85 %.

4-Chlorobenzyl 3'-O-sialyl-β-lactoside

^{1}H-NMR (500 MHz, D_2O): δ [ppm] = 7.46-7.42 (m, 4H, $H_{(a/b)arom}$); 4.91 (d, 1H, CH_2a-Bn); 4.74 (d, 1H, CH_2b-Bn); 4.55-4.52 (m, 2H, H-1/H-1'); 4.11 (dd, 1H, H-3'); 2.76 (dd, 1H, H-3''$_{eq}$); 2.04 (s, 3H, $COCH_3$); 1.81 (dd, 1H, H-3''$_{ax}$).

$J_{(a,b)\text{-Bn}}$ = 11.8; $J_{2',3'}$ = 9.9; $J_{3',4'}$ = 2.9; $J_{3''ax,3''eq}$ = 12.4; $J_{3''ax,4''}$ = 12.1; $J_{3''eq,4''}$ = 4.5 Hz.

^{13}C-NMR (126 MHz, D_2O): δ [ppm] = 135.2, 133.5 (quart. C_{arom}); 130.2 ($CH_{a\text{-}arom}$); 128.6 ($CH_{b\text{-}arom}$); 102.6 (C-1'); 101.1 (C-1); 51.7 (C-5''); 39.6 (C-3''); 22.0 ($COCH_3$).

4-Methylbenzyl 3'-O-sialyl-β-lactoside

^{1}H-NMR (500 MHz, D_2O): δ [ppm] = 7.36 (d, 2H, H-a_{arom}); 7.28 (d, 2H, H-b_{arom}); 4.89 (d, 1H, CH_2a-Bn); 4.72 (d, 1H, CH_2b-Bn); 4.54-4.52 (m, 2H, H-1/H-1'); 4.12 (dd, 1H, H-3'); 2.76 (dd, 1H, H-3''$_{eq}$); 2.35 (s, 3H, CH_3-Tol); 2.04 (s, 3H, $COCH_3$), 1.81 (dd, 1H, H-3''$_{ax}$).

$J_{(a,b)arom}$ = 7.9; $J_{(a,b)\text{-Bn}}$ = 11.5; $J_{2',3'}$ = 9.9; $J_{3',4'}$ = 3.0; $J_{3''ax,3''eq}$ = 12.5; $J_{3'',4''}$ = 12.2; $J_{3''eq,4''}$ = 4.6 Hz.

^{13}C-NMR (126 MHz, D_2O): δ [ppm] = 138.8, 133.4 (quart. C_{arom}); 129.3 ($CH_{a\text{-}arom}$); 129.0 ($CH_{b\text{-}arom}$); 102.6 (C-1'); 100.9 (C-1); 99.8 (C-2''), 51.7 (C-5''); 39.6 (C-3''); 22.0 ($COCH_3$); 20.2 (CH_3-Tol).

2-Naphthylmethyl 3'-O-sialyl-β-lactoside

^{1}H-NMR (500 MHz, D_2O): δ [ppm] = 7.99-7.97 (m, 4H, H-arom); 7.62-7.59 (m, 3H, H-arom); 5.10 (d, 1H, CH_2a-Bn); 4.95 (d, 1H, CH_2b-Bn); 4.60 (d, 1H, H-1); 4.53 (d, 1H, H-1'); 4.12 (dd, 1H, H-3'); 2.77 (dd, 1H, H-3''$_{eq}$); 2.04 (s, 3H, $COCH_3$); 1.81 (dd, 1H, H-3''$_{ax}$).

$J_{(a,b)\text{-Bn}}$ = 11.9; $J_{1,2}$ = 8.0; $J_{1',2'}$ = 7.9; $J_{2',3'}$ = 9.9; $J_{3',4'}$ = 3.0; $J_{3''ax,3''eq}$ = 12.5; $J_{3''ax,\,4''}$ = 12.1; $J_{3''eq,\,4''}$ = 4.6 Hz.

^{13}C-NMR (126 MHz, D_2O): δ [ppm] = 128.3, 127.9, 127.7, 127.6, 126.6, 126.5 (CH_{arom}); 102.6 (C-1'); 101.1 (C-1); 51.7 (C-5''); 22.0 ($COCH_3$).

3-Phenylbenzyl 3'-O-sialyl-β-lactoside

^{1}H-NMR (400 MHz, D_2O): δ [ppm] = 7.75-7.44 (m, 9H, H-arom); 4.99 (d, 1H, CH_2a-Bn); 4.82 (d, 1H, CH_2b-Bn); 4.57 (d, 1H, H-1); 4.53 (d, 1H, H-1'); 4.13 (dd, 1H, H-3'); 2.78 (dd, 1H, H-3''$_{eq}$); 2.05 (s, 3H, $COCH_3$); 1.82 (dd, 1H, H-3''$_{ax}$).

$J_{(a,b)\text{-Bn}}$ = 11.8; $J_{1,2}$ = 8.0; $J_{1',2'}$ = 7.9; $J_{2',3'}$ = 9.9; $J_{3',4'}$ = 3.1; $J_{3''ax,3''eq}$ = 12.5; $J_{3''ax,4''}$ = 12.0; $J_{3''eq,4''}$ = 4.6 Hz.

^{13}C-NMR (100 MHz, D_2O): δ [ppm] = 140.9, 140.3, 137.5 (quart. C_{arom}); 129.4, 129.2, 127.9, 127.8, 127.1, 127.0, 126.9, (CH_{arom}); 102.7 (C-1'); 101.2 (C-1); 99.6 (C-2''); 51.8 (C-5''); 39.7 (C-3''); 22.1 ($COCH_3$).

Benzyl 3'''-O-sialyl-β-LNnT

^{1}H-NMR (400 MHz, D_2O): see Figure 3.

Example 3

Sialylation using p-nitrophenyl sialoside donor

General procedure: a solution of 2-*O*-(*p*-nitrophenyl)-α-D-sialoside (47-51 mM) and benzyl β-LNT (24-37 mM) in incubation buffer (0.5 ml, 100 mM Tris/HCl, pH 7.0) was incubated with recombinant transsialidase from *T. cruzi* (21-108 µg/ml) at 15 °C. Samples were taken after 3, 6 and 24 hours (50 µl each) and analyzed by HPLC. Formation of sialyl- β-LNT benzyl glycoside was detected with 65-95 % conversion.

Example 4

Sialylation using p-nitrophenyl sialoside donor

General procedure: a solution of 2-*O*-(*p*-nitrophenyl)-α-D-sialoside (50 mM) and 3-FL (25-50 mM) in incubation buffer (0.5 ml, 100 mM Tris/HCl, pH 7.0) was incubated with recombinant transsialidase from *T. cruzi* (21-108 µg/ml) at 15 °C. Samples were taken after 3, 6 and 24 hours (50 µl each) and qualitatively analyzed by TLC. Formation of sialyl-fucosyl-lactose was detected with medium conversion rate.

Example 5

Sialylation using p-nitrophenyl sialoside donor

General procedure: a solution of 2-*O*-(*p*-nitrophenyl)-α-D-sialoside (50 mM) and LNT (25-50 mM) in incubation buffer (0.5 ml, 100 mM Tris/HCl, pH 7.0) was incubated with recombinant transsialidase from *T. cruzi* (21-108 µg/ml) at 15 °C. Samples were taken after 3, 6 and 24 hours (50 µl each) and qualitatively analyzed by TLC. Formation of sialyl-LNT was detected with high conversion rate.

Example 6

Fucosylation using the following transfucosidases:

P25 from *Thermotoga Maritima* containing mutations G226S Y237H T264A L322P;

M3 from *Thermotoga Maritima* containing mutations Y237H Y267F L322P.

General procedure: a solution of fucosyl donor and acceptor in degassed incubation buffer (0.5 ml, 50 mM citrate-phosphate, 145 mM NaCl, pH 5.5) was incubated with transfucosidase at 60 °C (except for fucosyl fluoride as donor, where the incubation was carried out at 35 °C). Sample was taken after 21 hours and the conversion determined by HPLC.

Results:

P25 mutant:

200 mM 2,5-dimethyl-4-oxo-3-furanyl α-L-fucopyranoside, 200 mM lactose, conversion: 6 % 2'-FL

500 mM 2,5-dimethyl-4-oxo-3-furanyl α-L-fucopyranoside, 500 mM lactose, conversion: 9 % 2'-FL

500 mM 2,5-dimethyl-4-oxo-3-furanyl α-L-fucopyranoside, 1000 mM lactose, conversion: 22 % 2'-FL

200 mM 2,5-dimethyl-4-oxo-3-furanyl α-L-fucopyranoside, 200 mM LNT, conversion: 13 % fucosylated LNT (position of fucosylation not determined)

500 mM 2,5-dimethyl-4-oxo-3-furanyl α-L-fucopyranoside, 500 mM LNT, conversion: 10 % fucosylated LNT (position of fucosylation not determined)

M3 mutant:

200 mM 2,5-dimethyl-4-oxo-3-furanyl α-L-fucopyranoside, 200 mM lactose, conversion: 19 % 2'-FL

500 mM 2,5-dimethyl-4-oxo-3-furanyl α-L-fucopyranoside, 1000 mM lactose, conversion: 35 % 2'-FL

200 mM 2,5-dimethyl-4-oxo-3-furanyl α-L-fucopyranoside, 200 mM LNT, conversion: 19 % fucosylated LNT (position of fucosylation not determined)

500 mM 2,5-dimethyl-4-oxo-3-furanyl α-L-fucopyranoside, 500 mM LNT, conversion: 20 % fucosylated LNT (position of fucosylation not determined)

200 mM fucosyl fluoride, 200 mM LNT, conversion: 10 % fucosylated LNT (position of fucosylation not determined).

Example 7

Sialylation of multiple acceptors using p-nitrophenyl sialoside donor

Protocol: A solution of *p*NP-α-NeuAc as donor (150 mM) with 3-fucosyllactose, lacto-N-tetraose and lacto-N-neotetraose as acceptors (50 mM each) was incubated in incubation buffer Tris-HCl (100mM) at a pH 7.0 with recombinant transsialidase from *Trypanosoma cruzi.* The reaction mixture was stirred at a temperature of 30°C for 24 h. Products were analyzed by HPLC and LC-MS using reference standards (for SFL, LSTa, LSTd).

Products detected: 3'-sialyl-3-fucosyllactose, LSTa: Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc, sialylated lacto-N-neotetraose (LSTd): Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc, disialylated Lacto-N-tetraose and/or disialylated lacto-N-neotetraose, correct molecular mass was confirmed by LC-MS (1290 $[M+H]^+$, 1307 $[M+NH_4]^+$, 1328 $[M+K]^+$).

Example 8

Sialylation of multiple acceptors using p-nitrophenyl sialoside as donor

Protocol: A solution of *p*NP-α-NeuAc as donor (100 mM) with lacto-N-tetraose-β-OBn and lacto-N-neotetraose-β-OBn as acceptors (50 mM each) was incubated in incubation buffer Tris-HCl (100mM) at a pH 7.0 with recombinant transsialidase from *Trypanosoma cruzi.* The reaction mixture was stirred at a temperature of 30°C for 30 min. Products were analyzed by HPLC and LC-MS.

Example 9

Glycosylation of LNT using multiple donors and enzymes

Protocol: In a first cycle, a solution of α-L-fucosyl fluoride as donor (200 mM) with lacto-N-tetraose as acceptor (200 mM) was incubated in incubation buffer $KHPO_4$ (100mM) at pH 7.0 with the recombinant transfucosidase M3 from *Thermotoga maritima.* The reaction mixture was stirred at a temperature of 30°C for 24 hours.

In a second cycle, the pH of the resulting reaction mixture was adjusted to 7.0 and the solution was incubated at 30 °C for an additional 30 min after adding 200 mM of α-L-fucosyl

fluoride and the recombinant fucosidase Blon_2336 from *Bifidobacterium longum subsp. infantis* ATCC 15697.

In a third cycle, the pH of the resulting reaction mixture was adjusted to 7.0 and the solution was incubated at 30 °C for an additional 24 h after adding 50 mM of *p*NP-α-NeuAc and the recombinant transsialidase from *Trypanosoma cruzi*. The reaction was stopped by addition of 1M $NaHCO_3$-solution at pH=10 and products were analyzed by HPLC, LC-MS and LC-MS-MS.

LC-MS conditions:

Instrument: AB Sciex API 2000 tandem MS

Ionization mode: electrospray in positive mode

Scan type: Q1MS

Sample insertion mode: HPLC

Column: TSK Gel amide 80 (Tosoh, 3µm,150x4.6mm)

Eluent: 10 mM ammonium formate buffer pH=6 – acetonitrile: 30%/70%

Flow rate: 1 ml/min

Injected volume: 5 µl

Result:

Retention time (min)	Molecular mass (Dalton)	Mass (Dalton) of the main fragment ions of the MH^+ in MS/MS
12.2	998 (sialyl LNT)	819, 657, 546, 454, 366, 274, 204, 186, 168
16.3	707	
19.7	853 (fucosyl LNT I)	512, 366, 204, 186, 138
25.7	853 (fucosyl LNT II)	512, 366, 350, 204, 186, 138

Example 10

Glycosylation of LNnT using multiple donors and enzymes

Protocol: In a first cycle, a solution of α-L-fucosyl fluoride as donor (200 mM) with lacto-N-tetraose as acceptor (200 mM) was incubated in incubation buffer $KHPO_4$ (100mM) at pH 7.0 with the recombinant transfucosidase M3 from *Thermotoga maritima*. The reaction mixture was stirred at a temperature of 30°C for 24 hours.

In a second cycle, the pH of the resulting reaction mixture was adjusted to 7.0 and the solution was incubated at 30 °C for an additional 30 min after adding 200 mM of α-L-fucosyl fluoride and the recombinant fucosidase Blon_2336 from *Bifidobacterium longum subsp. infantis* ATCC 15697.

In a third cycle, the pH of the resulting reaction mixture was adjusted to 7.0 and the solution was incubated at 30 °C for an additional 24 h after adding 50 mM of *p*NP-α-NeuAc and the recombinant transsialidase from *Trypanosoma cruzi*. The reaction was stopped by addition of 1M $NaHCO_3$-solution at pH=10 and products were analyzed by HPLC, LC-MS and LC-MS-MS.

Results (HPLC condition: see Example 9):

Retention time (min)	Molecular mass (Dalton)	Mass (Dalton) of the main fragment ions of the MH^+ in MS/MS
11.2	998 (sialyl LNnT)	657, 546, 454, 366, 292, 274, 204, 138
16.9	707	
25.1	853 (fucosyl LNnT)	512, 366, 204, 186, 138

Example 11

Fucosylation reactions

General procedure: A solution of the appropriate fucosyl donor (such as *p*-nitrophenyl α-L-fucopyranoside, α-L-fucosyl fluoride, 2,5-dimethyl-3-oxo-(2H)-furan-4-yl α-L-fucopyranoside or 2'-O-fucosyllactose) and acceptor (10–500 mmol, donor acceptor ratio is 5:1 to 1:5) was incubated in degassed incubation buffer at a pH range from 5.0 to 9.0) with recombinant α-fucosidase, α-transfucosidase or α-fucosynthase. The reaction mixture was stirred for 24 hours at a temperature range from 20 to 70°C. Samples were taken at different times of the reaction, the reaction was stopped by the addition of 1M $NaHCO_3$-solution at pH=10 and analyzed by TLC and/or HPLC. After completion, the enzyme was denatured and centrifuged. The resulting solution was evaporated under reduced pressure. After lyophilisation, the dry residue was dissolved in water and purified by biogel chromatography (P-2 Biogel, 16x900 mm) with water or by reverse phase chromatography. The yields vary between 2.5-85 %.

Recombinant enzymes used and tested in this set of transfucosylation reactions:

P25 from *Thermotoga Maritima* (see seq. ID 1) containing mutations G226S Y237H T264A L322P;

M3 from *Thermotoga Maritima* (see seq. ID 1) containing mutations Y237H Y267F L322P.

These transfucosidases were produced in *E. coli* as reported in G. Osanjo et al. *Biochemistry* **46**, 1022 (2007). Purified transfucosidases P25 and M3 are stored at -20°C or +4°C, respectively.

Optimal buffer for the enzymes: 50 mM sodium citrate/phosphate buffer and 150 mM NaCl, pH=5.5.

Optimum temperature: 60 °C (except for α-L-fucosyl fluoride acceptor, where the temperature is 35 °C).

LC-MS conditions:

Instrument: AB Sciex API 2000 tandem MS

Ionization mode: electrospray in positive mode

Scan type: Q1MS

Sample insertion mode: HPLC

Column: Phenomenex HILIC 250 x 4.6 mm

Flow: isocratic (water-acetonitrile 22:78)

Flow rate: 1 ml/min

Injected volume: 5 µl

a) acceptor: Galβ1-4Glcβ1-*O*-Bn

donor: *p*-nitrophenyl α-L-fucopyranoside, α-L-fucosyl fluoride or 2,5-dimethyl-3-oxo-(2H)-furan-4-yl α-L-fucopyranoside

product: Fucα1-2Galβ1-4Glcβ1-*O*-Bn (identified by comparison to the standard sample made by chemical means from 2'-O-fucosyllactose)

Characteristic ^{1}H NMR peaks (DMSO-d_6) δ: 7.41-7.25 (m, 5 H, aromatic), 5.20 (d, 1 H, $J_{1'',2''}$= 2 Hz, H-1''), 4.82 and 4.59 (ABq, 2H, J_{gem}= 12.3 Hz, -CH_2Ph), 4.32 (d, 1

H, $J_{1',2'}$= 7.31 Hz, H-1′), 4.28 (d, 1 H, $J_{1,2}$= 7.79 Hz, H-1), 1.05 (d, 1 H, $J_{5'',6''}$= 6.43 Hz, H-6′′).

^{13}C NMR (DMSO-d_6) δ: 137.97, 128.15, 128.15, 127.58, 127.58 and 127.43 (aromatic), 101.86, 100.94 and 100.20 (C-1, C-1′and C-1′′), 77.68, 76.78, 75.36, 75.33, 74.70, 73.79, 73.45, 71.60, 69.72, 69.69, 68.74, 68.20, 66.38, 60.23 and 59.81 (C-2, C-3, C-4, C-5, C-6, C-2′, C-3′, C-4′, C-5′, C-6′, C-2′′, C-3′′, C-4′′, C-5′′ and $\underline{C}H_2$Ph), 16.47 (C-6′′).

b) acceptor: Galβ1-4Glcβ1-*O*-CH_2-(4-NO_2-Ph)

donor: *p*-nitrophenyl α-L-fucopyranoside or 2,5-dimethyl-3-oxo-(2H)-furan-4-yl α-L-fucopyranoside

product: Fucα1-2Galβ1-4Glcβ1-*O*-CH_2-(4-NO_2-Ph) (identified by comparison to the standard sample made by chemical means from 2'-O-fucosyllactose)

Characteristic ^{1}H NMR peaks (DMSO-d_6) δ: 8.20 and 7.68 (each d, 4 H, aromatic), 5.04 (d, 1 H, $J_{1'',2''}$= 2 Hz, H-1′′), 4.97 and 4.76 (ABq, 2H, J_{gem}= 12.3 Hz, -CH_2Ph), 4.40 (d, 1 H, $J_{1',2'}$= 9.53 Hz, H-1′), 4.32 (d, 1 H, $J_{1,2}$= 8.04 Hz, H-1), 1.04 (d, 1 H, $J_{5'',6''}$= 6.43 Hz, H-6′′).

^{13}C NMR (DMSO-d_6) δ: 162.38, 147.68, 127.95, 127.95, 123.33 and 123.33 (aromatic), 102.18, 100.95 and 100.18 (C-1, C-1′and C-1′′), 77.62, 76.74, 75.36, 75.36, 74.61, 73.78, 73.45, 71.59, 69.67, 68.74, 68.67, 68.21, 66.38, 60.22 and 59.77 (C-2, C-3, C-4, C-5, C-6, C-2′, C-3′, C-4′, C-5′, C-6′, C-2′′, C-3′′, C-4′′, C-5′′ and $\underline{C}H_2$Ph), 16.45 (C-6′′).

c) acceptor: Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-*O*-Bn

donor: α-L-fucosyl fluoride, 2'-O-fucosyllactose or 2,5-dimethyl-3-oxo-(2H)-furan-4-yl α-L-fucopyranoside

product: monofucosylated Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-*O*-Bn, correct molecular mass was confirmed by LC-MS (944 $[M+H]^+$, 961 $[M+NH_4]^+$, 966 $[M+Na]^+$)

d) acceptor: Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-*O*-Bn

donor: α-L-fucosyl fluoride, 2'-O-fucosyllactose or 2,5-dimethyl-3-oxo-(2H)-furan-4-yl α-L-fucopyranoside

product: monofucosylated Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-*O*-Bn, correct molecular mass was confirmed by LC-MS (944 $[M+H]^+$, 961 $[M+NH_4]^+$, 966 $[M+Na]^+$)

Example 12

Manufacture of acceptor benzyl/substituted benzyl glycosides

A) Benzyl/substituted benzyl lactosides

A1) General procedure: lactose (5 g, 14.6 mmol) and $TsOH \cdot H_2O$ (0.2 g, 1.05 mmol) were added in one portion to a mixture of DMF (20 ml) and benzaldehyde dimethyl acetal (5.5 ml, 35.4 mmol, 2.4 eq.) at room temperature. The reaction mixture was vigorously stirred at 70 °C under exclusion of humidity for 1 hour. After cooling triethyl amine (0.15 ml) was added then the volatile components (MeOH, triethyl amine, remaining benzaldehyde dimethyl acetal) were removed in vacuo. To the reaction mixture the benzyl bromide derivative (1.5 eq.) – predissolved in 5-10 ml of DMF, if the reagent is a solid – was added and the mixture was cooled to 0 °C for 20 min. Still under cooling NaH (0.8 g of a 55 % dispersion in mineral oil, 1.3 eq.) was added in one portion, and the mixture was stirred under cooling until the hydrogen formation stopped then at room temperature for 2-3 hours. Methanol (2 ml) was added carefully and the reaction was stirred for a further 5 min. The reaction mixture was portioned between 100 ml of DCM and 100 ml of water and extracted. The water layer was back-extracted twice with 100 ml of DCM. The combined organic phases were evaporated; the residue was dissolved in 100 ml of acetonitrile and extracted with 100 ml of hexane. The acetonitrile was distilled off and the residue was taken up in isopropanol (10 ml) and isopropyl ether (50 ml) at 50 °C. The clear solution was cooled to -20 °C for between 2-12 hours. The crystals obtained were filtered off and washed twice with TBME and dried. Recrystallization can be carried out from a mixture of TBME (~50 ml) and ethanol (~20 ml).

4-Chlorobenzyl 4',6'-O-benzylidene-β-lactoside

Yield: 1.71 g

4-Methylbenzyl 4',6'-O-benzylidene-β-lactoside

Yield: 3.20 g

3-Phenylbenzyl 4',6'-O-benzylidene-β-lactoside

Yield: 2.70 g

2-Naphthylmethyl 4',6'-O-benzylidene-β-lactoside

Yield: 1.77 g

B1) To a mixture of one of the above benzylidene acetals (500 mg) in methanol (10 ml) and water (0.5 ml) TFA was added at room temperature and the reaction mixture was stirred for 2-4 hours under exclusion of humidity then evaporated. The remaining material was co-evaporated with ethanol 3-4 times giving a crude solid, which, after drying, can be recrystallized from a mixture of methanol (~10-35 ml) and water (~0-2 mL).

4-Chlorobenzyl β-lactoside

Yield: 333 mg

^{13}C-NMR (75.1 MHz, D_2O): δ = 135.25, 133.67, 130.30, 128.70, 103.00, 101.13, 78.39, 75.44, 74.89, 74.49, 72.88, 72.58, 71.03, 70.83, 68.62, 61.11, 60.13.

4-Methylbenzyl β-lactoside

Yield: 439 mg

^{13}C-NMR (75.1 MHz, D_2O): δ = 138.91, 133.50, 129.37, 129.07, 103.01, 100.96, 78.43, 75.44, 74.87, 74.52, 72.90, 72.59, 71.47, 71.03, 68.63, 61.11, 60.17, 20.34.

3-Phenylbenzyl β-lactoside

Yield: 438 mg

^{13}C-NMR (75.1 MHz, d_6-DMSO/d_4-MeOH/D_2O 8:1:1): δ = 140.29, 140.24, 138.88, 129.13, 129.02, 127.66, 126.88, 126.83, 126.03, 125.90, 103.95, 102.03, 80.76, 75.65, 75.07, 75.00, 73.34, 73.28, 70.66, 69.81, 68.27, 60.56.

2-Naphthylmethyl β-lactoside

Yield: 378 mg

^{13}C-NMR (75.1 MHz, D_2O/d_6-DMSO): δ = 134.96, 133.24, 133.12, 128.59, 128.31, 128.08, 127.46, 126.98, 126.90, 126.79, 103.26, 101.59, 78.89, 75.62, 75.09, 74.81, 73.14, 72.81, 71.33, 71.14, 68.75, 61.22, 60.39.

B) Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-*O*-Bn (1-*O*-benzyl-β-LNT) can be prepared according to A. Malleron et al. *Carbohydr. Res.* **341**, 29 (2006).

C) Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-*O*-Bn (1-*O*-benzyl-β-LNnT) can be prepared according to WO 2011/100980.

Example 13.

Manufacture of 2,5-dimethyl-3-oxo-(2H)-furan-4-yl α-L-fucopyranoside

a) The solution of 2,3,4-tri-O-(4-methoxybenzyl)-L-fucopyranose trichloroacetimidate (α/β mixture, prepared from 85 mmol of 2,3,4-tri-O-(4-methoxybenzyl)-L-fucopyranose and trichloroacetonitrile in the presence of NaH in quantitative yield) in diethyl ether (100 ml) was added to a mixture of 2,5-dimethyl-4-hydroxy-3-oxo-(2H)-furan (85 mmol) and TMS-triflate (1.2 ml) in diethyl ether (200 ml) at -14 °C. After 3 hours the cooling bath was removed and the stirring continued for 1 hour. The reaction mixture was diluted with ethyl acetate and extracted with sat. $NaHCO_3$-solution (3 x 150 ml) and brine (150 ml). The organic phase was dried over Na_2SO_4 and evaporated. The resulting syrup was purified by column chromatography yielding 23.27 g of 2,5-dimethyl-3-oxo-(2H)-furan-4-yl 2,3,4-tri-O-(4-methoxybenzyl)-α-L-fucopyranoside as a thick yellow syrup in a 1:1 mixture of diastereoisomers. Selected NMR chemical shifts in $CDCl_3$: anomeric protons: 5.32 and 5.57 ppm, $J_{1,2}$= 3.4 Hz; anomeric carbons: 113.81 and 113.95 Hz.

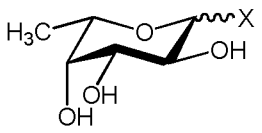
b) A suspension of the above product and Pd on charcoal (12.5 g) in ethyl acetate (1 l) was stirred at room temperature for 6 hours under H_2. The catalyst was filtered off and washed with ethyl acetate. The combined filtrate was evaporated to a solid mass which was suspended in ethyl acetate and allowed to stand at 5 °C for overnight. The crystalline material was filtered off and dried. The mother liquor was subjected to column chromatography. The combined products gave 5.50 g of 2,5-dimethyl-3-oxo-(2H)-furan-4-yl α-L-fucopyranoside as white solid in 1:1 mixture of diastereoisomers. Selected NMR chemical shifts in DMSO-d_6: anomeric protons: 5.07 and 5.52 ppm,

$J_{1,2}$= 2.6 Hz; anomeric carbons: 100.19 and 101.03 Hz. Purity by GC (after silylation): 98.9 %.

CLAIMS

1. A method for making one or more human milk oligosaccharides (HMOs) or derivatives or precursors thereof, the method comprising the steps of

a) providing at least one donor selected from the group consisting of compounds of any of formulae **5** to **10**



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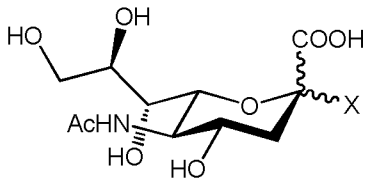

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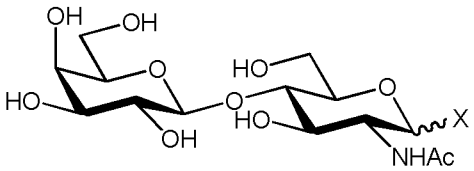

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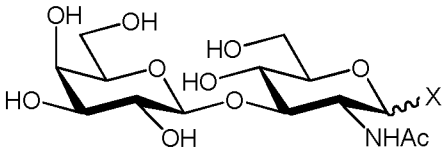

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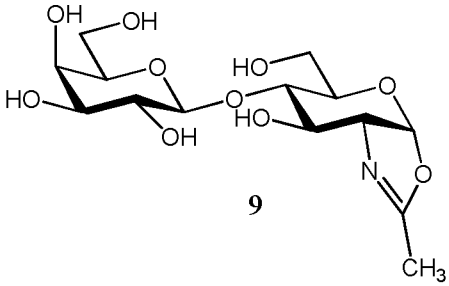

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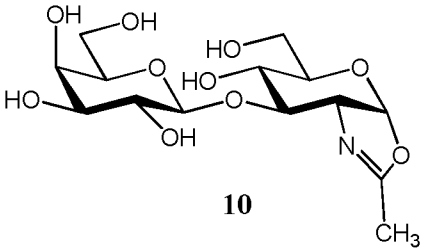
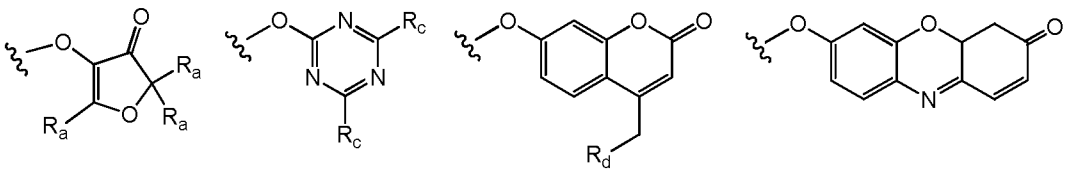


10

and salts thereof,

wherein X is selected from the group consisting of azide, fluoro, optionally substituted phenoxy, optionally substituted pyridinyloxy, group **A**, group **B**, group **C** and group **D**



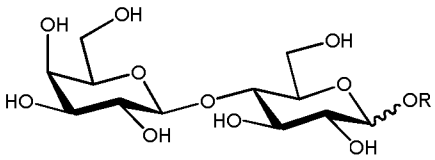
A **B** **C** **D**

wherein R_a is independently H or alkyl, or two vicinal R_a groups represent a $=C(R_b)_2$ group, wherein R_b is independently H or alkyl, R_c is independently selected from the group consisting of alkoxy, amino, alkylamino and dialkylamino, R_d is selected from

the group consisting of H, alkyl and –C(=O)R_e, wherein R_e is OH, alkoxy, amino, alkylamino, dialkylamino, hydrazino, alkylhydrazino, dialkylhydrazino or trialkylhydrazino.

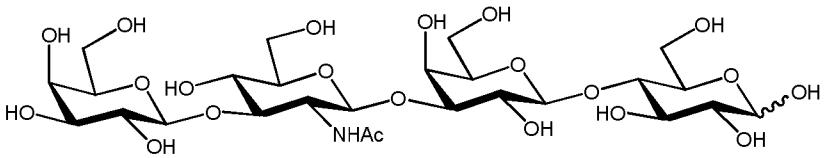
b) providing at least one acceptor selected from the group consisting of:

- lactose
- lactose derivatives of the following formula:

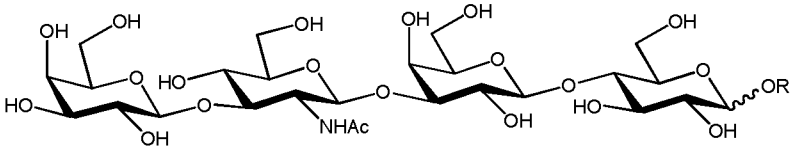


wherein R is a group removable by hydrogenolysis,

- LNT:

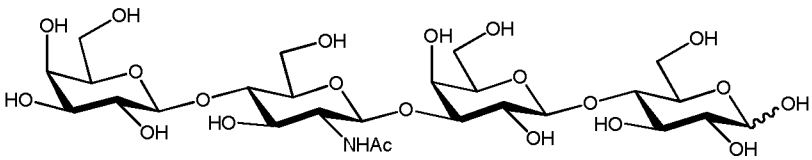


- LNT derivatives of the formula:

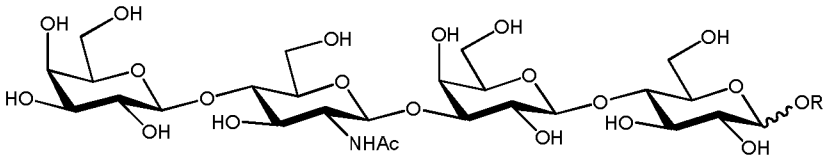


wherein R is a group removable by hydrogenolysis, or salts thereof

- LNnT:



- LNnT derivatives of the formula:



wherein R is a group removable by hydrogenolysis, or salts thereof

c) providing at least one enzyme comprising a transglycosidase activity and/or a glycosynthase activity;

d) preparing a mixture of the at least one donor, at least one acceptor and at least one enzyme provided in steps a), b) and c);

e) incubating the mixture prepared according to step d);

f) repeating at least one of the steps a) to c), then repeating step d) and step e) in the case where:

f-a) a single donor of formula **5** is provided in step a), a single acceptor is provided according to step b), the enzyme comprising a transglycosidase activity and/or a glycosynthase activity added in step c) is an enzyme having a trans-fucosidase and/or fucosynthase activity, and a single HMO or derivative or precursor thereof is made as a result of step e); or

f-b) a single donor of formula **6** is provided in step a) donor, a single acceptor is provided according to step b), the enzyme comprising a transglycosidase activity and/or a glycosynthase activity added in step c) is an enzyme having a trans-sialidase activity, and a single HMO or derivative or precursor thereof is made as a result of step e); or

f-c) a single donor of formula **8** is provided in step a), a single acceptor is provided according to step b), the enzyme comprising a transglycosidase activity and/or a glycosynthase activity added in step c) is an enzyme having a trans-lacto-N-biosidase activity, and a single HMO or derivative or precursor thereof is made as a result of step e);

g) optionally: repeating at least one of the steps a) to c), then repeating step d) and step e) with the mixture obtained according to step e) or f);

h) optionally subjecting the mixture obtained as a result of step e), f) or g) to a hydrogenolysis reaction.

2. The method according to claim 1, wherein step f) comprises:

repeating at least one of the steps a) to c), then repeating step d) and step e) in the case where: a single donor is provided in step a), a single acceptor is provided according to step b), a single enzyme comprising a transglycosidase activity and/or a glycosynthase activity added in step c), and a single HMO or derivative or precursor thereof is made as a result of step e).

3. The method according to claim 1, wherein step f) comprises:
repeating at least one of the steps a) to c), then repeating step d) and step e) in the case where a single HMO or derivative or precursor thereof is made as a result of step e).

4. The method according to any one of claims 1 to 3, wherein two, three, four, five, one to five, three to ten, five to ten or even more different donors are provided in step a).

5. The method according to any one of claims 1 to 4, wherein two, three, four, five, one to five, three to ten, five to ten or even more different acceptors are provided in step b).

6. The method according to any one of claims 1 to 5, wherein two, three, four, five, one to five, three to ten, five to ten or even more different enzymes are provided in step c).

7. The method according to any one of claims 1 to 6, wherein the compounds or enzymes are added simultaneously or sequentially.

8. The method according to any one of claims 1 to 7, wherein

- the donors are selected dependent on the activity of the selected transglycosidase(s) from compounds according to formulae **5** to **8**, wherein X is optionally substituted 4-nitrophenoxy, group **A**, group **B**, group **C**, or from compounds **9** and **10**; and/or
- the donors are selected dependent on the activity of the selected glycosynthase(s) from compounds according to formulae **5** to **8**, wherein X is azide or fluoro.

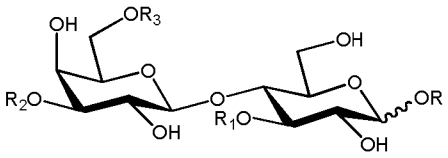
9. The method according to any one of claims 1 to 8, wherein the universal donors are selected from compounds according to formulae **5** to **8**, wherein X is 4-nitrophenoxy, 2,4-dinitrophenoxy, 2-chloro-4-nitrophenoxy, 2,5-dimethyl-3-oxo-(2H)-furan-4-yloxy, 2-ethyl-5-methyl-3-oxo-(2H)-furan-4-yloxy, 5-ethyl-2-methyl-3-oxo-(2H)-furan-4-

yloxy, 4,6-dimethoxy-1,3,5-triazin-2-yloxy, 4,6-diethoxy-1,3,5-triazin-2-yloxy, or 4-methylumbelliferyloxy.

10. The method according to any one of claims 1 to 9, wherein the at least one enzyme comprising transglycosidase activity and/or glycosynthase activity is an enzyme comprising a trans-fucosidase, trans-sialidase, trans-lacto-N-biosidase and/or trans-N-acetyllactosaminidase activity, preferably an α-trans-fucosidase, α-trans-sialidase, β-trans-lacto-N-biosidase and/or β-trans-N-acetyllactosaminidase activity.

11. The method according to any one of claims 1 to 10, wherein incubation leads to a human milk oligosaccharide or derivative or precursor thereof or mixture of human milk oligosaccharides or derivatives or precursors thereof, the single compound of which as defined according to

- compounds of general formula **1** and salts thereof



general formula **1**

wherein

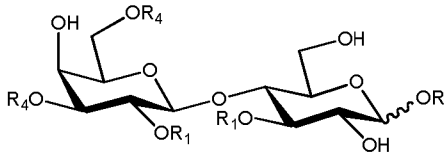
R is a group removable by hydrogenolysis,

R_1 is fucosyl or H,

R_2 is selected from N-acetyl-lactosaminyl and lacto-N-biosyl groups, wherein the N-acetyl lactosaminyl group may carry a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl groups; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_3 is H or N-acetyl-lactosaminyl group optionally substituted with a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl groups; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue;

- compounds of general formula **2** and salts thereof



general formula **2**

wherein

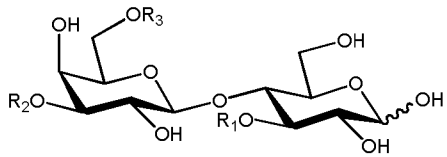
R is a group removable by hydrogenolysis,

R_1 independently of each other is fucosyl or H

R_4 independently of each other is sialyl or H,

with the proviso that at least one R_1 or R_4 is not H;

- compounds of general formula **3** and salts thereof



general formula **3**

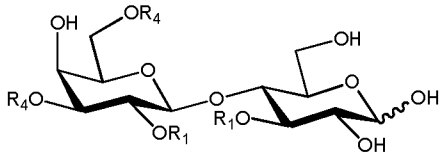
wherein

R_1 is fucosyl or H,

R_2 is selected from N-acetyl-lactosaminyl and lacto-N-biosyl groups, wherein the N-acetyl lactosaminyl group may carry a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl groups; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_3 is H or N-acetyl-lactosaminyl group optionally substituted with a glycosyl residue comprising one or more N-acetyl-lactosaminyl and/or one or more lacto-N-biosyl groups; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue; and/or

- compounds of general formula **4** and salts thereof



general formula **4**

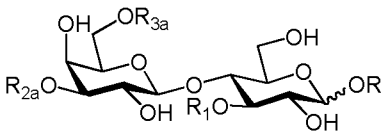
wherein

R_1 independently of each other is fucosyl or H

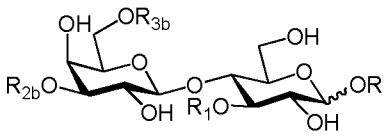
R_4 independently of each other is sialyl or H

with the proviso that at least one R_1 or R_4 is not H.

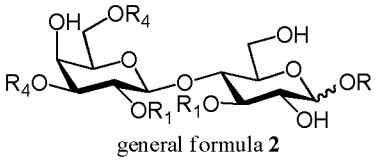
12. The method according to claim 11, wherein compounds of formulae **1** and **2** are further characterized by general formulae **1a**, **1b** or **2** or salts thereof



general formula **1a**

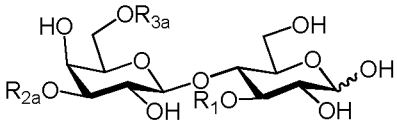


general formula **1b**

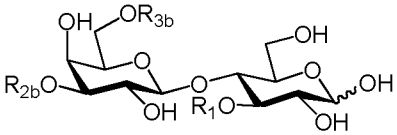


general formula **2**

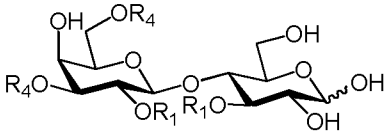
and compounds of formulae **3** and **4** are further characterized by general formulae **3a**, **3b** or **4** or salts thereof



general formula **3a**



general formula **3b**



general formula **4**

wherein

R, R_1 and R_4 are as defined in claim 11,

R_{2a} is N-acetyl-lactosaminyl group optionally substituted with a glycosyl residue comprising one N-acetyl-lactosaminyl and/or one lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_{3a} is H or N-acetyl-lactosaminyl group optionally substituted with a lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue,

R_{2b} is lacto-N-biosyl group optionally substituted with sialyl and/or fucosyl residue, and

R_{3b} is H or N-acetyl-lactosaminyl group optionally substituted with one or two N-acetyl-lactosaminyl and/or one lacto-N-biosyl group; any N-acetyl-lactosaminyl and lacto-N-biosyl group can be substituted with one or more sialyl and/or fucosyl residue.

13. The method according to claim 12, wherein

- the N-acetyl-lactosaminyl group in the glycosyl residue of R_{2a} in general formula **1a** or **3a** is attached to another N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the lacto-N-biosyl group in the glycosyl residue of R_{2a} in general formula **1a** or **3a** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the lacto-N-biosyl group in the glycosyl residue of R_{3a} in general formula **1a** or **3a** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the N-acetyl-lactosaminyl group in the glycosyl residue of R_{3b} in general formula **1b** or **3b** is attached to the another N-acetyl-lactosaminyl group with 1-3 or 1-6 interglycosidic linkage,
- the lacto-N-biosyl group in the glycosyl residue of R_{3b} in general formula **1b** or **3b** is attached to the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage.

14. The method according to any one of claims 11 to 13, wherein general formula **1a** represents the R-glycosides of lacto-N-neotetraose, para-lacto-N-hexaose, para-lacto-N-neohexaose, lacto-N-neohexaose, para-lacto-N-octaose and lacto-N-neooctaose optionally substituted with one or more sialyl and/or fucosyl residue, general formula **1b** represents the R-glycosides of lacto-N-tetraose, lacto-N-hexaose, lacto-N-octaose, iso-lacto-N-octaose, lacto-N-decaose and lacto-N-neodecaose optionally substituted with one or more sialyl and/or fucosyl residue, general formula **3a** represents lacto-N-neotetraose, para-lacto-N-hexaose, para-lacto-N-neohexaose, lacto-N-neohexaose, para-lacto-N-octaose and lacto-N-neooctaose optionally substituted with one or more sialyl and/or fucosyl residue, and general formula **3b** represents lacto-N-tetraose, lacto-N-hexaose, lacto-N-octaose, iso-lacto-N-octaose, lacto-N-decaose and lacto-N-neodecaose optionally substituted with one or more sialyl and/or fucosyl residue.

15. The method according to any one of claims 11 to 14, wherein

- the fucosyl residue attached to the N-acetyl-lactosaminyl and/or the lacto-N-biosyl group is linked to
  - the galactose of the lacto-N-biosyl group with 1-2 interglycosidic linkage and/or
  - the N-acetyl-glucosamine of the lacto-N-biosyl group with 1-4 interglycosidic linkage and/or
  - the N-acetyl-glucosamine of the N-acetyl-lactosaminyl group with 1-3 interglycosidic linkage,
- the sialyl residue attached to the N-acetyl-lactosaminyl and/or the lacto-N-biosyl group is linked to
  - the galactose of the lacto-N-biosyl group with 2-3 interglycosidic linkage and/or
  - the N-acetyl-glucosamine of the lacto-N-biosyl group with 2-6 interglycosidic linkage and/or
  - the galactose of the N-acetyl-lactosaminyl group with 2-6 interglycosidic linkage.

16. The method according to any one of claims 11 to 15, wherein the compounds are selected from the group consisting of: R-glycosides of 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-3-fucosyllactose, lacto-N-tetraose, lacto-N-neotetraose, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LST-a, LST-b, LST-c, FLST-a, FLST-b, FLST-c, LNDFH-I, LNDFH-II, LNDFH-III, DS-LNT, FDS-LNT I and FDS-LNT II, and salts thereof.

17. The method according to any one of claims 11 to 16, wherein said R-glycoside is a beta-anomer, and preferably wherein R is benzyl.

18. The method according to any one of claims 11 to 15, wherein the compounds are selected from the group consisting of: 2'-fucosyllactose, 3-fucosyllactose, 2',3-difucosyllactose, 3'-sialyllactose, 6'-sialyllactose, 3'-sialyl-3-fucosyllactose, lacto-N-tetraose, lacto-N-neotetraose, LNFP-I, LNFP-II, LNFP-III, LNFP-V, LST-a, LST-b,

LST-c, FLST-a, FLST-b, FLST-c, LNDFH-I, LNDFH-II, LNDFH-III, DS-LNT, FDS-LNT I and FDS-LNT II, and salts thereof.

19. The method according to any one of claims 1 to 17, wherein the compounds obtained in the incubation step are subjected to a hydrogenolysis reaction.

20. The method according to any one of claims 1 to 19, wherein the compounds obtained in the incubation or hydrogenolysis step are subsequently purified, preferably via crystallization or precipitation.

21. The method according to any one of claims 1 to 20, further comprising a step of spray-drying the compounds obtained in step e), f), g) or h).

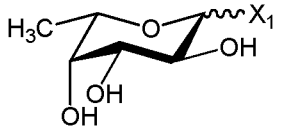
22. The method of any one of claims 1 to 21, further comprising addition of the compounds obtained in the incubation or hydrogenolysis step to a consumable product, wherein the consumable product is preferably at least one of a pharmaceutical or nutritional formulation.

23. The method of claim 22, further comprising the addition of pharmaceutically acceptable carriers and/or the addition of prebiotics to the compounds obtained in the incubation or hydrogenolysis step.

24. Use of human milk oligosaccharides, derivatives or precursors obtained or obtainable by a method according to any one of claims 1 to 21 for the preparation of a consumer product, a pharmaceutical composition, a nutritional formulation or a food supplement.

25. Use according to claim 24, wherein the nutritional formulation is an infant formula.

26. A compound of general formulae **5'-8'**



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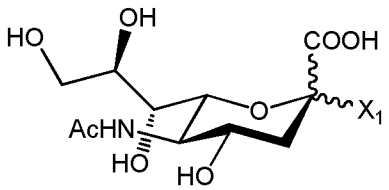

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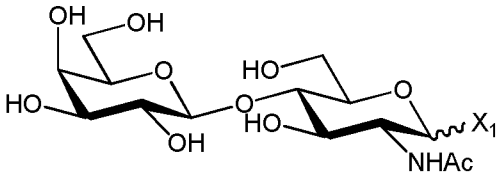

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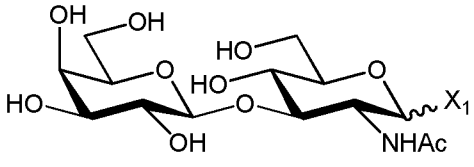
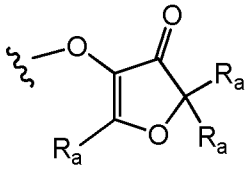
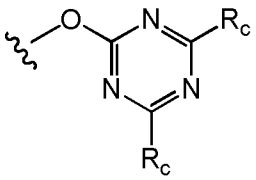


8'

wherein X_1 is group **A** or group **B**



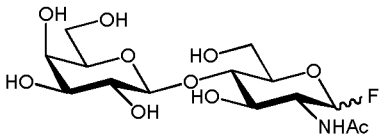
A

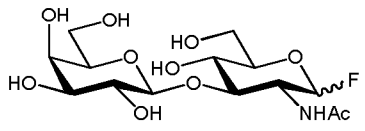


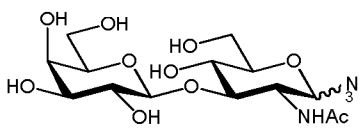
B

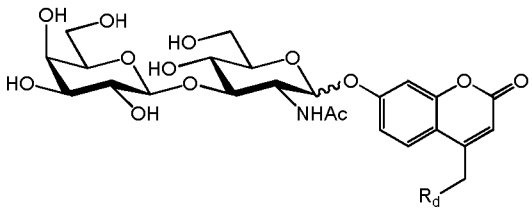
wherein R_a is independently H or alkyl, or two vicinal R_a groups represent a $=C(R_b)_2$ group, wherein R_b is independently H or alkyl, and R_c is independently selected from the group consisting of alkoxy, amino, alkylamino and dialkylamino,

and/or a compound of any of the following formulae









wherein R_d is selected from the group consisting of H, alkyl and $-C(=O)R_e$, and wherein R_e is OH, alkoxy, amino, alkylamino, dialkylamino, hydrazino, alkylhydrazino, dialkylhydrazino or trialkylhydrazino,

or a salt of any of the aforementioned compounds.

5

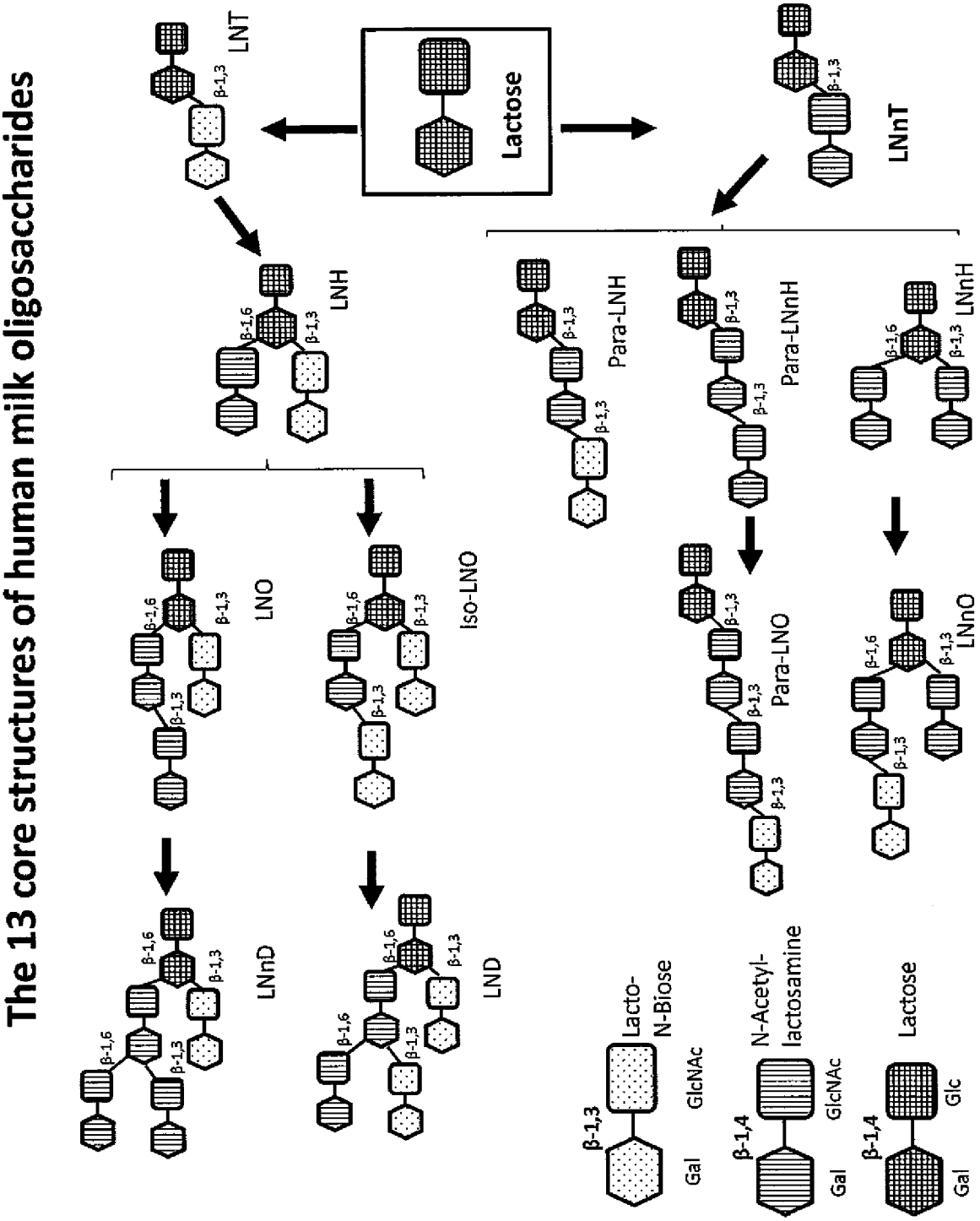

The 13 core structures of human milk oligosaccharides
Lactose
LNT
LNnT
LNH
Para-LNH
Para-LNnH
LNnH
LNO
Iso-LNO
Para-LNO
LNnO
LNnD
LND
Lacto-N-Biose
N-Acetyl-lactosamine
Lactose
Gal
GlcNAc
Glc
β-1,3
β-1,4
β-1,6

Figure 1.

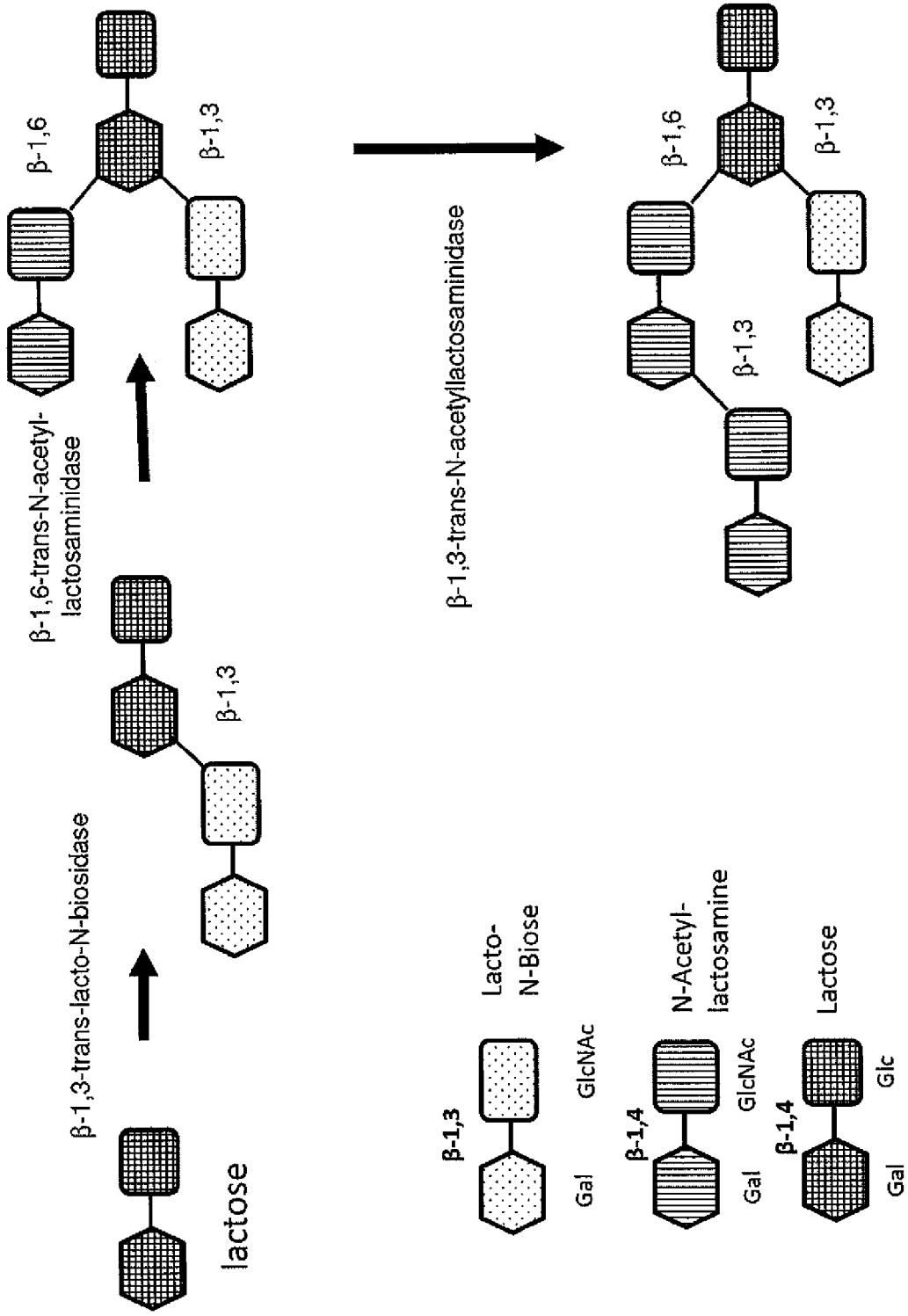

β-1,6
β-1,3
β-1,6-trans-N-acetyl-lactosaminidase
β-1,3
β-1,3-trans-lacto-N-biosidase
lactose
β-1,3-trans-N-acetyllactosaminidase
β-1,6
β-1,3
β-1,3
Lacto-N-Biose
Gal
GlcNAc
β-1,4
N-Acetyl-lactosamine
Gal
GlcNAc
β-1,4
Lactose
Gal
Glc

Figure 2.

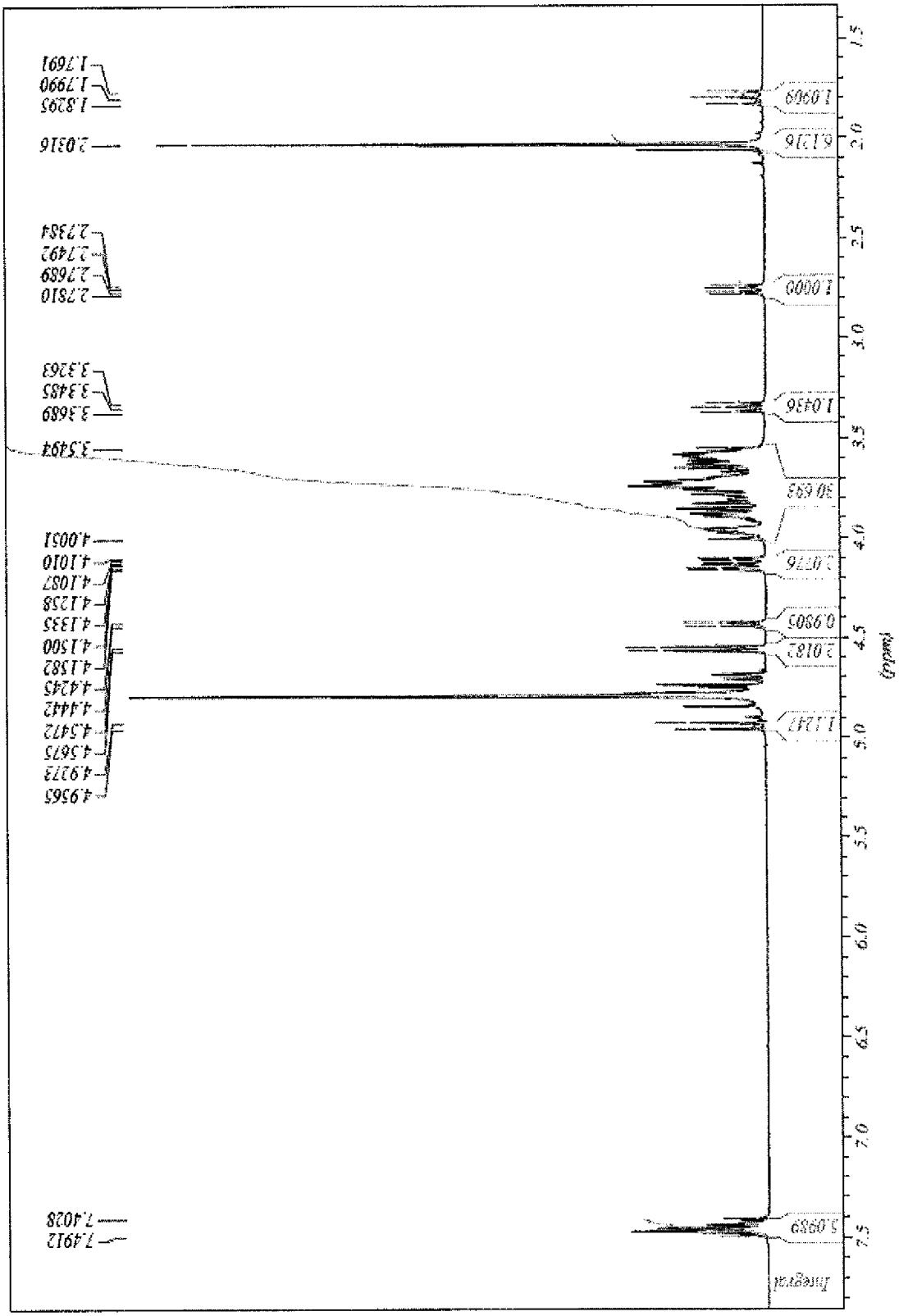


Figure 3. The 400 MHz ^{1}H-NMR spectrum of benzyl 3'''-*O*-sialyl-β-LNnT

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2012/052400

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet

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)

IPC: C07H, C12P

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)

EPO-Internal, PAJ, WPI data, BIOSIS, CHEM ABS Data, MEDLINE, reaxys

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Akihiro Imamura et al, "Di-tert-butylsilylene-directed alpha-selective synthesis of 4-methylumbelliferyl T-antigen", Organic Letters, 2005, 7(20),4415-4418; abstract; Figure 1 --	26
A	Björn Neubacher et al, "Preparation of sialyted oligosaccharides employing recombinant trans-sialidase from Trypanosoma cruzi, Org. Biomol. Chem.,2005,3,1551-1556; abstract; Scheme 1, compound 2 --	1-25
A	WO 9632492 A1 (CYTEL CORP), 17 October 1996 (1996-10-17); claims --	1-26

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"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)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"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
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09-08-2012	10-08-2012
Name and mailing address of the ISA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Carolina Gomez Lagerlöf Telephone No. + 46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2012/052400

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Wei Li et al, "Synthesis of oligosaccharides with lactose and N-acetylglucosamine as substrates by using Î-d-galactosidase from Bacillus circulans", European Food Research and Technology ,2010,231(1),55-63; whole document -- --------	1-26

Continuation of: second sheet

International Patent Classification (IPC)

C12P 19/18 (2006.01)
C07H 5/04 (2006.01)
C07H 17/02 (2006.01)
C07H 17/04 (2006.01)
C12P 19/44 (2006.01)

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Paper copies can be ordered at a cost of 50 SEK per copy from PRV InterPat (telephone number 08-782 28 85).

Cited literature, if any, will be enclosed in paper form.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2012/052400

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 24-25
because they relate to subject matter not required to be searched by this Authority, namely:

The scope of the claims 24-25, in as far as the expressions " derivatives or precursors obtained" are concerned, is so unclear (Article 6 PCT) that a meaningful International Search is impossible with regard to these expressions.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

WO	9632492	A1	17/10/1996	AT	222294	T 15/08/2002
				AU	5445396	A 30/10/1996
				DE	69623005	T2 03/04/2003
				DK	820520	T3 02/12/2002
				EP	0820520	A1 28/01/1998
				ES	2180757	T3 16/02/2003
				JP	H11503328	A 26/03/1999