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(54) Title: SIALYLATION OF GLYCOSPHINGOLIPIDS

(57) Abstract: The present invention relates to a method for the production of a sialylated glycosphingolipid, the method comprising the steps of: — providing a glycosphingolipid, a sialic acid donor, and an enzyme having a trans-sialidase activity, — mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, thereby producing said sialylated glycosphingolipid, and wherein the sialic acid donor is 3′-sialyllactose.

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DESCRIPTION

Sialylation of Glycosphingolipids

Field of the invention

The present invention relates to a novel and efficient method for the sialylation of glycosphingolipids.

Background

Glycosphingolipids (GSLs) are glycoconjugates wherein a glycan moiety is linked to the 1-hydroxyl group of a ceramide or a sphingoid base via a glycosidic linkage.

GSLs are involved in diverse biological processes and play important structural and functional roles such as cell–cell recognition, communication, and intercellular adhesion. Particularly, sialylated glycosphingolipids such as gangliosides are found in the brain, and can play roles in neurological diseases especially Alzheimer's, Parkinson's, and Huntington's diseases. Furthermore, certain gangliosides are found in the intestinal mucosa and can promote intestinal health, as well as act as anti-infective agents.

Sialylated glycosphingolipids such as gangliosides hold great potential as therapeutics, and as food ingredients however they are not readily available for fundamental and clinical research. In fact, they are characterized by a high structural complexity and their preparation represents a challenge.

Processes for the preparation of sialylated glycosphingolipids that are based on extraction from natural sources, chemical and/or enzymatic synthesis exist.

Sialylated glycosphingolipids may be extracted from animal brains, or animal epidermal tissues (EP 3095451 A1, US 5532141 A). However, extraction and isolation of sialylated glycosphingolipids from animal sources is a laborious and costly process, and typically yields the desired compounds in low amounts and with low purities. Furthermore, the obtained sphingolipids may be potentially unsafe due to the presence of hazardous biological contaminants.

Alternatively, sialylated glycosphingolipids may be obtained via chemical synthesis (J. A. Morales-Serna, Carbohydr. Res. 2007), wherein typically the glycan moiety is first synthesised and then coupled to a ceramide or a sphingoid base. Drawbacks connected to this approach are the control of stereo- and regiochemistry, the need for multiple protecting group manipulations, difficult purification and scale-up.

Enzymatic synthesis of sialylated glycosphingolipid has been described, wherein sialyltransferase enzymes are utilized to transfer sialic acid from CMP-sialic acid to a glycosphingolipid acceptor (Yu H. et al*, Org Biomol Chem.* 2018;16, 4076-4080; WO 9928491). Drawbacks connected to this approach comprise the use of expensive nucleotides donors and/or reagents, and low conversions.

Accordingly, there is a demand for the development of novel methodologies characterized by high technological feasibility and low costs, which enable the efficient and large-scale production of sialylated glycosphingolipids.

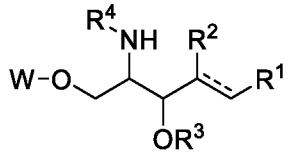
Summary of the invention

In a first aspect the present invention relates to a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, and an enzyme having a trans-sialidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, thereby producing said sialylated glycosphingolipid,

wherein

the glycosphingolipid is a compound of formula (1):



(1),

wherein

W is Galβ1-, or a glycosyl moiety carrying one or more terminal β-galactopyranosyl units,

R^1 is hydrogen, aryl, or a substituted or unsubstituted C_{1-50} alkyl, preferably a substituted or unsubstituted C_{1-17} alkyl, more preferably a substituted or unsubstituted C_{10-17} alkyl,

R^2 is hydrogen or $-OR^5$, wherein R^5 is selected from hydrogen, a substituted or unsubstituted C_{1-6} alkyl, or a substituted or unsubstituted C_{2-6} acyl, preferably R^5 is hydrogen,

the bond ⌐¬¬ may be a double or a single bond when R^2 is hydrogen, or is a single bond when R^2 is $-OR^5$,

R^3 is hydrogen, a substituted or unsubstituted C_{1-6} alkyl, or a substituted or unsubstituted C_{1-6} acyl, preferably hydrogen,

R^4 is selected from hydrogen, a substituted or unsubstituted aryl, a heteroalkyl, a substituted or unsubstituted C_{2-32} acyl,

and wherein the sialic acid donor is 3´-sialyllactose.

Brief Description of the Figures

Figure 1 shows a schematic diagram of a sialyltransferase cycle wherein CMP-Neu5Ac is generate/regenerated.

Figure 2 shows a schematic diagram of a sialyltransferase cycle wherein CMP-Neu5Ac is generated/regenerated, and ATP is regenerated.

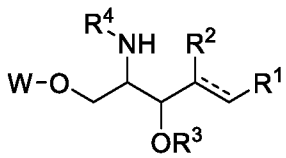
Detailed description of the invention

Surprisingly, the present inventors have found that sialylated glycosphingolipids can be produced in-vitro via a trans-sialidase catalysed sialylation of a glycosphingolipid acceptor using readily available and inexpensive 3´-sialyllactose as the sialic acid donor, and wherein the glycosphingolipid acceptor is preferably obtained via synthetic and/or biotechnological approaches. The trans-sialidase catalysed sialylation may be performed in the presence of a β-galactosidase and may include a nanofiltration step. Furthermore, the trans-sialidase catalysed sialylation may be followed by a further sialylation step catalysed by a sialyltransferase, wherein the expensive nucleotide donor is generated in-situ and regenerated during the sialyltransferase cycle. The method is characterized by high yields, high selectivity, and the desired product is obtained with high purity. Therefore, the method is suited for the large-scale production of sialylated glycosphingolipids such as gangliosides, the method comprising the following steps:

- providing a glycosphingolipid, a sialic acid donor, and an enzyme having a trans-sialidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, thereby producing said sialylated glycosphingolipid,

wherein

the glycosphingolipid is a compound of formula (1):



(1),

wherein

W is Galβ1-, or a glycosyl moiety carrying one or more terminal β-galactopyranosyl units,

R^1 is hydrogen, aryl, or a substituted or unsubstituted C_{1-50} alkyl, preferably a substituted or unsubstituted C_{1-17} alkyl, more preferably a substituted or unsubstituted C_{10-17} alkyl,

R^2 is hydrogen or $-OR^5$, wherein R^5 is selected from hydrogen, a substituted or unsubstituted C_{1-6} alkyl, or a substituted or unsubstituted C_{2-6} acyl, preferably R^5 is hydrogen,

the bond ⌐¬¬ may be a double or a single bond when R^2 is hydrogen, or is a single bond when R^2 is $-OR^5$,

R^3 is hydrogen, a substituted or unsubstituted C_{1-6} alkyl, or a substituted or unsubstituted C_{1-6} acyl, preferably hydrogen,

R^4 is selected from hydrogen, a substituted or unsubstituted aryl, a heteroalkyl, a substituted or unsubstituted C_{2-32} acyl,

and wherein the sialic acid donor is 3´-sialyllactose.

Non-limiting embodiments of different aspects of the invention are described below and illustrated by non-limiting examples.

The terms, definitions and embodiments described throughout the specification of the invention relate to all aspects and embodiments of the invention.

The term “a” grammatically is a singular, but it may as well mean the plural of e.g., the intended compound. For example, a skilled person would understand that in the expression “a glycosphingolipid”, the provision of not only one single glycosphingolipid, but of a variety of glycosphingolipids of the same type is meant.

As used herein, the term “alkyl” refers to an acyclic straight or branched hydrocarbyl group having 1-50 carbon atoms which may be saturated or contain one or more double and/or triple bonds (so, forming for example an alkenyl or an alkynyl), and/or which may be substituted or unsubstituted, as herein further described. Examples of “alkyl” include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, isobutyl, n-butyl, sec-butyl, tert-butyl, isopentyl, n-pentyl, neo-pentyl, n-hexyl, ethenyl, propenyl, 1-butenyl, 2-butenyl, isobutenyl,1-pentenyl, 2-pentenyl, 2-methyl-1-butenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, 1-hexenyl, 2-hexenyl, 3-hexenyl, methylpentenyl, dimethylbutenyl, ethynyl, propynyl, 1-butynyl, 2-butynyl, pentynyl, and hexynyl, each of which may be substituted or unsubstituted. Typically, the term alkyl refers to a straight saturated acyclic hydrocarbyl group having 1-31 carbons, which may be substituted or unsubstituted.

As used herein, the term “aryl” refers to an aromatic cyclic hydrocarbyl group having 5-14 ring carbon atoms, which may be mono- or polycyclic, which may contain fused rings, preferably 1 to 3 fused or unfused rings, and which may contain one or more heteroatoms, and/or which may be substituted or unsubstituted, as herein further described. Examples of “aryl” include, but are not limited to, phenyl,

naphtyl, anthracyl, phenantryl, pyrrolyl, imidazolyl, thiophenyl, furanyl, oxazolyl, thiazolyl, pyridinyl, pyrimidinyl, pyrazinyl, triazinyl, and benzofuranyl, each of which may be substitute or unsubstituted. Typically, the term "aryl" refers to a substituted or unsubstituted phenyl.

As used herein, the term "acyl" refers to a group derived by the removal of one or more hydroxyl group from an oxoacid, preferably from a carboxylic acid. The acyl group according to the present invention is typically a saturated or unsaturated C_{2-32} acyl, which may be substituted or unsubstituted.

As used herein, the term "substituted" means that the group in question is substituted with a group which typically modifies the general chemical characteristics of the group in question. The substituents can be used to modify characteristics of the molecule, such as molecule stability, molecule solubility and the ability of the molecule to form crystals. The person skilled in the art will be aware of other suitable substituents of a similar size and charge characteristics, which could be used as alternatives in a given situation.

In connection with the terms "alkyl", "aryl", and "acyl" the term substituted means that the group in question is substituted one or several times, preferably 1 to 3 times, with group(s) selected from hydroxy (which when bound to an unsaturated carbon atom may be present in the tautomeric keto form), oxo, C_{1-6}-alkoxy (i.e. C_{1-6}-alkyl-oxy), C_{2-6}-alkenyloxy, carboxy, oxo, C_{1-6}- alkoxycarbonyl, C_{1-6}-alkylcarbonyl, formyl, aryl, aryloxycarbonyl, aryloxy, arylamino, arylcarbonyl, heteroaryl, heteroarylamino, heteroaryloxycarbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C_{1-6}-alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)aminocarbonyl, amino-C_{1-6}-alkyl-aminocarbonyl, mono- and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkylcarbonylamino, cyano, guanidino, carbamido, C_{1-6}-alkyl-sulphonyl-amino, aryl-sulphonyl-amino, heteroaryl-sulphonyl-amino, C_{1-6}-alkanoyloxy, C_{1-6}-alkyl-sulphonyl, C_{1-6}-alkyl-sulphinyl, C_{1-6}-alkylsulphonyloxy, nitro, C_{1-6}-alkylthio, halogen, where any alkyl, alkoxy, and the like representing substituents may be substituted with hydroxy, C_{1-6}-alkoxy, C_{2-6}-alkenyloxy, carboxy, C_{1-6}-alkylcarbonylamino, halogen, C_{1-6}-alkylthio, C_{1-6}-alkyl-sulphonyl-amino, or guanidino.

In connection with the term "alkyl" the term "substituted" preferably means that the group in question is substituted one or several times, preferably 1 to 3 times, with group(s) selected from a hydroxyl group, an alkoxy group, an acyloxy group, an acylamido group, a thiol, a thioether or a phosphorus-containing functional group.

In connection with the term enzyme the term "functional analogue" refers to a protein wherein the amino acid sequence has a certain percent homology compared to the amino acid sequence of a reference protein (i.e. about 30% homology, preferably about 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher homology over a

specified region, for example over a region of at least about 25, 50, 75, 100, 150, 200, 250, 500, 1000, or more amino acids, up to the full length sequence, when compared and aligned for maximum correspondence over a comparison window or designated region) and maintains the same functional activity of the reference protein or polypeptide. The percent homology may be determined using e.g. a BLAST sequence comparison algorithm, or by manual alignment and visual inspection (see e.g. NCBI website http://www.ncbi.nlm.nih.gov/BLAST/ or the like). Such sequences may be termed "substantially identical". Typically, the term functional analogue refers to a mutant protein, a truncated variant of the protein, a recombinant protein, or to a fusion protein which maintains the same functional activity of the reference protein.

Amino acid sequences are herein typically defined by the commonly used one-letter code or by their three-letter code, as summarized in Table 1.

Table 1 Amino acid codes:

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

The skilled person will understand that in formulas showing a specific compound, like for example formulas (1), (2), and (3) unless the chemical formula expressly describes a carbon atom having a particular stereochemical configuration, the formula is intended to cover compounds where such a stereocenter has an R or an S configuration, or wherein a double bond has a cis or a trans configuration.

The skilled person would understand that when speaking of position C-1, C-2, C-3, C-4, C-5 etc., reference is herein always made to the respective carbon atoms of glycosphingolipid such as those represented by formula (1), (2) and (3).

In the context of the present invention, the terms “about”, “around”, or “approximate” are applied interchangeably to a particular value (e.g. “a pH of about 4.5”, “a pH around 4.5”, or “a pH of approximate 4.5”), or to a range (e.g. “an amount from about 1% to about 99%”, “an amount from around 1% to around 99%”, or “an amount from approximate 1% to approximate 99%”), to indicate a deviation from 0.1% to 10% of that particular value or range.

The term “isolating”, in the context of the present invention, refers to a procedure or a step of the procedure that is applied to separate the desired compound from a mixture comprising said desired compound and other compounds. In this context, the other compounds of the mixture are regarded as contaminants. The term "isolation” and “isolation” may be used interchangeably.

The term “glycosphingolipid”, as used herein, refers to compounds that structurally consist of a glycosyl moiety and a sphingolipid moiety, or analogs thereof. The glycosyl moiety is typically linked to the sphingolipid moiety via a glycosidic bond between the anomeric carbon at the reducing end of the glycosyl moiety and the hydroxyl group at the C-1 position of the sphingolipid.

The glycosyl moiety of the glycosphingolipid according to the present invention may derive from a monosaccharide or from an oligosaccharide (more than one monosaccharide units), wherein the anomeric carbon of the monosaccharide or the anomeric carbon at the reducing end of the oligosaccharide is engaged in a glycosidic bond with another chemical entity, such as a sphingolipid, and the bond, if not further specified, may be an alpha or a beta glycosidic bond. A glycosyl moiety having more than one monosaccharide unit may represent a linear or a branched structure.

The monosaccharide unit is preferably any 5-9 carbon atom sugar, comprising aldoses (e.g. D-glucose, D-galactose, D-mannose, D-ribose, D-arabinose, L-arabinose, D-xylose, etc.), ketoses (e.g. D-fructose, D-sorbose, D-tagatose, etc.), deoxysugars (e.g. L-rhamnose, L-fucose, etc.), deoxy-aminosugars (e.g. N-acetylglucosamine, N-acetylmannosamine, N-acetylgalactosamine, etc.), uronic acids, ketoaldonic acids (e.g. sialic acid). The monosaccharide unit can form different cyclic structures such as pyranose (six-membered) cyclic structures or furanose (five-membered) cyclic structures. In some embodiments the glycosyl moiety derives from a monosaccharide, wherein the monosaccharide is a β-galactoside. In some embodiments, the glycosyl moiety derives from an oligosaccharide, wherein the oligosaccharide carries one or more terminal β-galactopyranosyl units.

The glycosyl moieties according to the present invention may be illustrated in the following style: Galβ1-4Glc1-, wherein the dash (-) represents the point of attachment of the glycosyl moiety and wherein the glycosyl moiety, may be linked via an alpha or a beta glycosidic bond, preferably a beta glycosidic bond.

The sphingolipid moiety of the glycosphingolipid of the present invention typically derives from an aliphatic amino alcohol such as a sphingoid base or a ceramide.

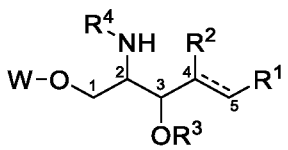
Sphingoid bases denote in the context of the present invention naturally occurring sphingoid bases, analogues thereof or derivatives thereof.

Naturally occurring sphingoid bases are D-*erythro*-sphingosine (S), 6-hydroxy-D-*erythro*-sphingosine (H), D-*ribo*-phytosphingosine (P) or DL-*erythro*-dihydrosphingosine (DS), wherein the number of sphingoid carbons may be expressed in parenthesis following the letters S, H, P, and DS.
The letters S, H, P, and DS refer to the shorthand nomenclature developed by Motta et al. (1993) Biochim Biophys Acta. 1182:147-151 and expanded by Rabionet (2014) Biochim Biophys Acta. 1841:422-434 and by Masukawa et al., *Journal of Lipid Research*, 2008, 49, 1466-1476. D-*Erythro*-dihydrosphingosine may also be represented by the letter G according to the INCI nomenclature.

Ceramides denote in the context of the present invention naturally occurring ceramides, analogues thereof or derivatives thereof. Preferred ceramides are those naturally occurring in humans. Naturally occurring human ceramides [CER] include, but are not limited to, CER[NS], CER[AS], CER[EOS], CER[NH], CER[AH], or CER[EOH], CER[NP], CER[AP], or CER[EOP], CER[NDS], CER[ADS], or CER[EODS]. The letters in brackets refer to the shorthand nomenclature developed by Motta et al. (1993) Biochim Biophys Acta. 1182:147-151 and expanded by Rabionet (2014) Biochim Biophys Acta. 1841:422-434 and Masukawa et al., *Journal of Lipid Research*, 2008, 49, 1466-1476. Particularly, the letters N, A, and EO represent non-hydroxy fatty acids (N), alpha-hydroxy fatty acids (A), and omega-linoleoyloxy fatty acids (EO), respectively, wherein the number of fatty acid carbons and unsaturations may be expressed in parentheses following the letters of N, A, E, and O. The letters, S, H, P, and DS represent D-*erythro*-sphingosine (S), 6-hydroxy-D-*erythro*-sphingosine (H), D-ribo-phytosphingosine (P), D-*erythro*-dihydrosphingosine (DS), respectively, wherein the number of sphingoid carbons may be expressed in parenthesis following the letters S, H, P, and DS. Ceramides, CER[NDS], CER[ADS], or CER[EODS], may also be referred to as CER[NG], CER[AG], or CER[EOG], respectively, wherein the letter G represents the INCI name for D-*erythro*-dihydrosphingosine.

Glycosphingolipids lacking the amide-linked fatty acyl group may also be referred to as lysosphingolipids.

Glycosphingolipid according to the present invention are typically represented by a glycosphingolipid of formula (1):



(1),

wherein

W is Galβ1-, or a glycosyl moiety carrying one or more terminal β-galactopyranosyl units,

R^1 is hydrogen, aryl, or a substituted or unsubstituted C_{1-50} alkyl, preferably a substituted or unsubstituted C_{1-17} alkyl, more preferably a substituted or unsubstituted C_{10-17} alkyl,

R^2 is hydrogen or $-OR^5$, wherein R^5 is selected from hydrogen, a substituted or unsubstituted C_{1-6} alkyl, or a substituted or unsubstituted C_{2-6} acyl, preferably R^5 is hydrogen,

the bond ⌐¬ may be a double or a single bond when R^2 is hydrogen, or is a single bond when R^2 is $-OR^5$,

R^3 is hydrogen, a substituted or unsubstituted C_{1-6} alkyl, or a substituted or unsubstituted C_{1-6} acyl, preferably hydrogen,

R^4 is selected from hydrogen, a substituted or unsubstituted aryl, a heteroalkyl, a substituted or unsubstituted C_{2-32} acyl,

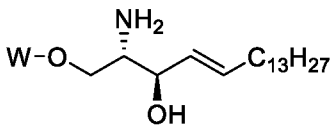
In some embodiments, for the glycosphingolipid of formula (1) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2, R^3 and R^4 are hydrogen, and the bond ⌐¬ is a double bond.

In some embodiments, for the glycosphingolipid of formula (1) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2 is OR^5, wherein R^5 is hydrogen, R^3 and R^4 are hydrogen, and the bond ⌐¬ is a single bond.

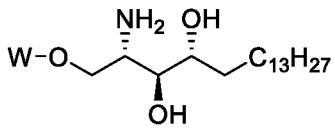
In some embodiments, for the glycosphingolipid of formula (1) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2, R^3 and R^4 are hydrogen, and the bond ⌐¬ is a single bond.

In some embodiments, for the glycosphingolipid of formula (1) R^1 is a C_{10}-C_{17} 1-hydroxyalkyl, R^2, R^3 and R^4 are hydrogen, and the bond ⌐¬ is a double bond.

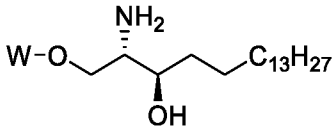
In some embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid selected from the group consisting of glycosphingolipids of formulas (3), (4), (5), and (6):



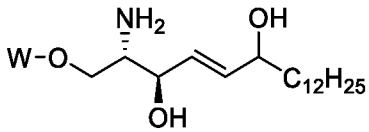
(3),



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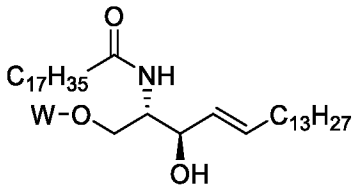
In some embodiments, for the glycosphingolipid of formula (1) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2 and R^3 are hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⌐⌐⌐ is a double bond.

In some embodiments, for the glycosphingolipid of formula (1) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2 is OR^5, wherein R^5 is hydrogen, R^3 is hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⌐⌐⌐ is a single bond.

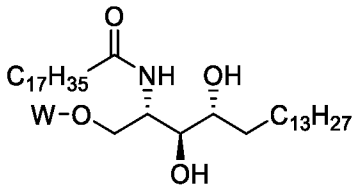
In some embodiments, for the glycosphingolipid of formula (1) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2 and R^3 are hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⌐⌐⌐ is a single bond.

In some embodiments, for the glycosphingolipid of formula (1) R^1 is a C_{10}-C_{17} 1-hydroxyalkyl, R^2, and R^3 are hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⌐⌐⌐ is a double bond.

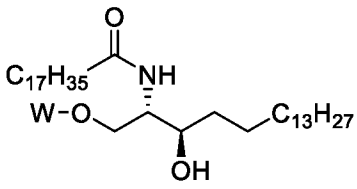
In some embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid selected from the group consisting of glycosphingolipids of formulas (7), (8), (9), and (10):



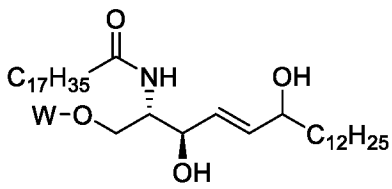
(7),



(8),



(9),



(10).

In some embodiments, W of the glycosphingolipid of formula (1), and of formulas (3)-(10) is a glycosyl moiety selected from the following glycosyl moieties, or salts thereof:

Galβ1-,
Galβ1-4Glc1-,

Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc1-,
Galβ1-3GalNAcβ1-4Galβ1-4Glc1-,
Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc1-,
Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3)Gal β1-4Glc1-,
Galβ1-3GlcNAcβ1-3Galβ1-4Glc1-,
Galβ1-4GlcNAcβ1-3Galβ1-4Glc1-
Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc1-,
Galβ1-4GlcNAcβ1-3Galβ1-4GlucNAcβ1-3Galβ1-4Glc1-,
Galβ1-4(Fucα1-3)Glc1-,
Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc1-,
Galβ1-3(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc1-,
Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc1-,
Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc-.

In some embodiments, W of the glycosphingolipid of formula (1), and of the glycosphingolipids of formulas (3)-(10) is a glycosyl moiety selected from the following glycosyl moieties, or salts thereof:

Galβ1-,
Galβ1-4Glc1-,
Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc1-.

In some embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid of formula (3), and wherein W of the glycosphingolipid of formula (3) is Galβ1-. Accordingly, in some embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid of formula (3), and wherein the glycosphingolipid of formula (3) is psychosine.

In some preferred embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid of formula (3), and wherein W of the glycosphingolipid of formula (3) is Galβ1-4Glcβ1-. Accordingly, in some preferred embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid of formula (3), and wherein the glycosphingolipid of formula (3) is lactosyl D-*erythro*-sphingosine.

In some embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid of formula (3), and wherein W of the glycosphingolipid of formula (3) is Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-. Accordingly, in some embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid of formula (3), and wherein the glycosphingolipid of formula (3) is *N*-lyso-GM1a.

In some embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid of formula (7), and wherein W of the glycosphingolipid of formula (7) is Galβ1-.

In some embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid of formula (7), and wherein W of the glycosphingolipid of formula (7) is Galβ1-4Glcβ1-.

In some preferred embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid of formula (7), and wherein W of the glycosphingolipid of formula (7) is Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-. Accordingly, in some preferred embodiments, the glycosphingolipid of formula (1) is a glycosphingolipid of formula (7), and wherein the glycosphingolipid of formula (7) is GM1a.

Glycosphingolipids according to our invention, may be produced by methods known to the skilled person. A method for the synthesis of glycosphingolipids is, for example, described in WO2023118378A1, or by Vaughan et al., *J. Am. Chem. Soc.* 2006, 128, 6300-6301wherein a glycosyl fluoride, such as for example lactosyl fluoride, is coupled to a sphingolipid using an endoglycoceramidase glycosynthases (EGCases) enzyme. Glycosphingolipids carrying complex oligosaccharide moieties may be produced via biotechnological methods such as that described in WO 2021170620 (A1).

The term "sialic acid donor", as used herein, refers to a compound carrying a sialic acid unit that can be transferred to a suitable acceptor, such as a glycosphingolipid. Sialic acid donors, suitable for use in the context of the present invention, are typically α-sialylated compounds which can derive from natural sources or can be chemically synthesized. α-Sialylated compounds deriving from natural sources are for example 3'-sialyllactose, sialic acid rich protein, and colominic acid. Chemically synthesized α-sialylated compounds include but are not limited to p-nitrophenyl *N*-acetylneuraminic acid (Neu5AcαpNP), methylumbelliferyl *N*-acetylneuraminic acid (Neu5AcαMU) and derivatives thereof.

In a preferred embodiment, the sialic acid donor is 3'-sialyllactose.

The term "an enzyme having a trans-sialidase activity" may be interchangeably used with the term "trans-sialidase" and denotes, in the context of the present invention, an enzyme belonging to the glycoside hydrolase family 33 (GH33) which typically catalyses the reversible transfer of a glycosidically linked sialic acid from sialic acid donors such as for example oligosaccharides, glycoproteins, glycolipids, and colominic acid to acceptor molecules containing a terminal β-galactopyranosyl unit. In the absence of a suitable acceptor molecule, these enzymes may act as sialidases and transfer the glycosidically linked sialic acid to a water molecule. However, their hydrolytic activity is typically low.

The trans-sialidase in its wild-type form, may originate from parasitic euglenoids, such as *Trypanosoma cruzi*, *Trypanosoma congolense*, or *Trypanosome brucei*.

The trans-sialidase in its wildtype form, may originate from a microorganism having a vector, to which a gene encoding a wildtype trans-sialidase has been ligated, or introduced.

The trans-sialidase in its wildtype form may originate from any known trans-sialidase sequence or from any trans-sialidase sequence which has yet to be determined. Trans-sialidases yet to be determined can be identified using sequence databases and sequence alignment algorithms, for example, the publicly available GenBank database and the BLAST alignment algorithm.

In some embodiments, the enzyme having a trans-sialidase activity is a wildtype trans sialidase originating from, *Trypanosoma cruzi*, *Trypanosoma congolense*, or *Trypanosome brucei*, or a functional analogue thereof.

In some embodiments, the enzyme having trans-sialidase activity is a wild-type trans-sialidase originating from *Trypanosoma cruzi.* The amino acid sequence of the wild-type trans-sialidase originating from *Trypanosoma cruzi* can be found on *https://www.uniprot.org/*, accession: Q26966.

The trans-sialidase originating from *Trypanosoma cruzi* may also be referred to as TcTS.

In some embodiments, the enzyme having trans-sialidase activity is a mutant of the wild-type trans-sialidase originating from *Trypanosoma cruzi* (Q26966).

In some embodiments, the mutant trans-sialidase has at least five mutations at amino acid positions selected from the group consisting of the following positions (numbering corresponding to alignment of the amino acid sequence with the amino acid sequence of Q26966): S263T, R477H, V485L, E559V, N59F, S496K, V497G, E521K, D594G, I598D and H600R.

In some embodiments, the mutant trans-sialidase has mutations at the following amino acid positions (numbering corresponding to alignment of the amino acid sequence with the amino acid sequence of Q26966): S263T, R477H, V485L, E559V, N59F, S496K, V497G, E521K, D594G, I598D and H600R.

In some embodiments, the mutant trans-sialidase has mutations at the following amino acid positions (numbering corresponding to alignment of the amino acid sequence with the amino acid sequence of Q26966): N59F, S496K, V497G, E521K, D594G, I598D and H600R, as described in Amaya et al., *Structure* 2004, 12, 775–784.

In some embodiments, the mutant trans-sialidase has mutations at the following amino acid positions (numbering corresponding to alignment of the amino acid sequence with the amino acid sequence of Q26966): N59F, V497G, S496K, E521K, and E559V.

In some embodiments, the mutant trans-sialidase has mutations at the following amino acid positions (numbering corresponding to alignment of the amino acid sequence with the amino acid sequence of Q26966: S263T, R477H, V485L, E559V, and S496K.

In some embodiments, the mutant trans-sialidase has mutations at the following amino acid positions (numbering corresponding to alignment of the amino acid sequence with the amino acid sequence of Q26966): S496K, V497G, D594G, I598D, and H600R.

In some embodiments, the mutant trans-sialidase further comprises the N-terminal deletion of amino acid M1 (numbering corresponding to alignment of the amino acid sequence with the amino acid sequence of Q26966), and the insertion of the N-terminal 14 amino acids histidine tag MGGSHHHHHGMAS.

In some embodiments, the mutant trans-sialidase further comprises the C-terminal deletion of amino acids Δ636-642 (numbering corresponding to alignment of the amino acid sequence with amino the acid sequence Q26966).

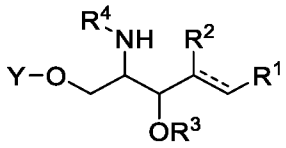
In some embodiments, the amino acids sequence of the mutant trans-sialidase corresponds to the amino acid sequence ID NO: 1MSO of the World Protein Data Bank (https://www.rcsb.org/structure/1MS0), wherein the reference amino acid sequence comprising the following mutations/modifications compared to the wild-type: S263T, R477H, V485L, E559V, N59F, S496K, V497G, E521K, D594G, I598D, H600R, *N*-terminal His-tag, C-terminal deletion of 7 amino acids Δ636-642.

In some embodiments, the amino acids sequence of the mutant trans-sialidase comprises or consists of an amino acid sequence of SEQ ID NO: 1, wherein the mutant comprising the following mutations/modifications compared to the wild-type amino acid sequence Q26966: S263T, R477H, V485L, E559V, S496K, *N*-terminal His-tag, deletion of 7 amino acids Δ636-642.

The mutant trans-sialidase, according to the present invention, may be produced by methods known to the skilled person. A method for the expression and purification of a mutant trans-sialidase is for example described in Paris et al., *Glycobiology* 2001, 11, 305-311, or in Buschiazzo et al., *Molecular Cell* 2002, 10, 757-768.

The method according to the present invention comprises the step of mixing the glycosphingolipid and the sialic acid donor in the presence of the enzyme having a trans-sialidase activity, thereby producing a sialylated glycosphingolipid.

Sialylated glycosphingolipid produced according to the method described above are typically represented by a sialylated glycosphingolipid of formula (2):



(2),

wherein

Y is a glycosyl moiety carrying at least one sialic acid unit,

R^1, R^2, R^3, R^4, and the bond ⎓ are as defined as for the glycosphingolipid of formula (1).

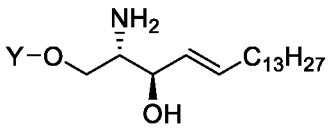
In some embodiments, for the sialylated glycosphingolipid of formula (2) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2, R^3 and R^4 are hydrogen, and the bond ⎓ is a double bond.

In some embodiments, for the sialylated glycosphingolipid of formula (2) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2 is OR^5, wherein R^5 is hydrogen, R^3 and R^4 are hydrogen, and the bond ⎓ is a single bond.

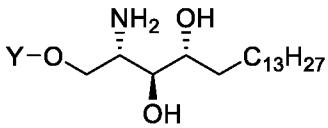
In some embodiments, for the sialylated glycosphingolipid of formula (2) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2, R^3 and R^4 are hydrogen, and the bond ⎓ is a single bond.

In some embodiments, for the sialylated glycosphingolipid of formula (2) R^1 is a C_{10}-C_{17} 1-hydroxyalkyl, R^2, R^3 and R^4 are hydrogen, and the bond ⎓ is a double bond.

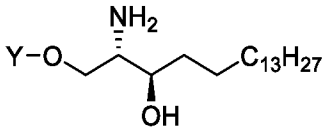
In some embodiments, the sialylated glycosphingolipid of formula (2) is a glycosphingolipid selected from the group consisting of glycosphingolipids of formulas (11), (12), (13), and (14):



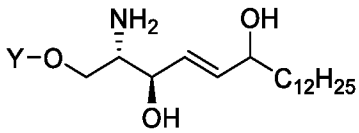
(11),



(12),



(13),



(14).

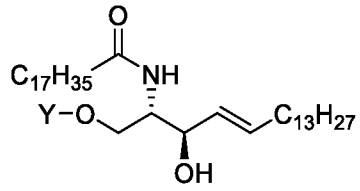
In some embodiments, for the sialylated glycosphingolipid of formula (2) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2 and R^3 are hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⎓ is a double bond.

In some embodiments, for the sialylated glycosphingolipid of formula (2) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2 is OR^5, wherein R^5 is hydrogen, R^3 is hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⎓ is a single bond.

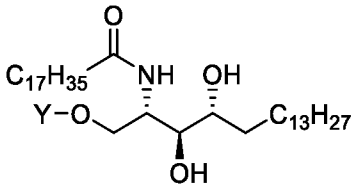
In some embodiments, for the sialylated glycosphingolipid of formula (2) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2, and R^3 are hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⎓ is a single bond.

In some embodiments, for the sialylated glycosphingolipid of formula (2) R^1 is a C_{10}-C_{17} 1-hydroxyalkyl, R^2, and R^3 are hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⎓ is a double bond.

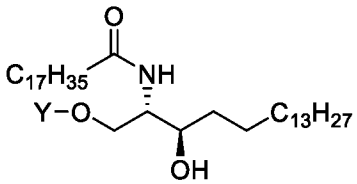
In some embodiments, the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid selected from the group consisting of sialylated glycosphingolipids of formulas (15), (16), (17), and (18):



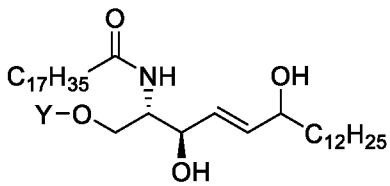
(15),



(16),



(17),



(18).

In some embodiments, Y of the sialylated glycosphingolipid of formula (2), and of formulas (11)-(18) is a glycosyl moiety selected from the following glycosyl moieties, or salts thereof:

Neu5Acα2-3Galβ1-,
Neu5Acα2-3Galβ1-4Glc1-,

Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc1-,
Neu5Acα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glc1-,
Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-3)Galβ1-4Glc1-,
Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3)Gal β1-4Glc1-,
Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc1-,
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc1-
Neu5Acα2-3Galβ1-3GlcNAcβ1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glc1-,
Neu5Acα2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlucNAcβ1-3Galβ1-4Glc1-,
Neu5Acα2-3Galβ1-4(Fucα1-3)Glc1-,
Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc1-,
Neu5Acα2-3Galβ1-3(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc1-,
Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)Glc1-,
Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GlcNAcβ1-3Galβ1-4Glc-.

In some embodiments, Y of the sialylated glycosphingolipid of formula (2), and of the sialylated glycosphingolipids of formulas (11)-(18) is a glycosyl moiety selected from the following glycosyl moieties, or salts thereof:

Neu5Acα2-3Galβ1-,
Neu5Acα2-3Galβ1-4Glc1-,
Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc1-.

In some embodiments, the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (11), and wherein Y of the sialylated glycosphingolipid of formula (11) is Neu5Acα2-3Galβ1-. Accordingly, in some embodiments, the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (11), and wherein the sialylated glycosphingolipid of formula (11) is *N*-lyso-GM4.

In some preferred embodiments, the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (11), and wherein Y of the sialylated glycosphingolipid of formula (11) is Neu5Acα2-3Galβ1-4Glcβ1-. Accordingly, in some preferred embodiments, the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (11), and wherein the sialylated glycosphingolipid of formula (11) is *N*-lyso-GM3.

In some embodiments, the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (11), and wherein Y of the sialylated glycosphingolipid of formula (11) is Neu5Acα2-3Galβ1-

3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-. Accordingly, in some embodiments the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (11), and wherein the sialylated glycosphingolipid of formula (11) is *N*-lyso-GD1a.

In some embodiments, the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (15), and wherein Y of the sialylated glycosphingolipid of formula (15) is Neu5Acα2-3Galβ1-. Accordingly, in some embodiments the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (15), and wherein the sialylated glycosphingolipid of formula (15) is GM4.

In some embodiments, the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (15), and wherein Y of the sialylated glycosphingolipid of formula (15) is Neu5Acα2-3Galβ1-4Glcβ1-. Accordingly, in some embodiments, the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (15), and wherein the sialylated glycosphingolipid of formula (15) is GM3.

In some preferred embodiments, the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (15), and wherein Y of the sialylated glycosphingolipid of formula (15) is Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-. Accordingly, in some preferred embodiments, the sialylated glycosphingolipid of formula (2) is a sialylated glycosphingolipid of formula (15), and wherein the sialylated glycosphingolipid of formula (15) is GD1a.

In some embodiments, the method further comprising a step of adding an enzyme having β-galactosidase activity.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, thereby producing said sialylated glycosphingolipid, and sequentially,
- adding the enzyme having β-galactosidase activity,

and wherein the sialic acid donor is 3´-sialyllactose.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, and an enzyme having β-galactosidase activity,

– mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, and said enzyme having β-galactosidase activity thereby producing said sialylated glycosphingolipid,

and wherein the sialic acid donor is 3´-sialyllactose.

The term "an enzyme having a β-galactosidase activity" may be interchangeably used with the term "β-galactosidase" and denotes, in the context of the present invention, an enzyme belonging to the glycoside hydrolase family 35 (GH35) which typically catalyses the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides.

In the context of the present invention a β-galactosidase may also be referred to as lactase.

The β-galactosidase in its wild-type form, may originate from microorganisms such as bacteria, yeasts, ascomycete, actinomycetes, hyphomycetes, basidiomycotina, and the like.

The β-galactosidase in its wild-type form, may originate from *Aspergillus oryzae*.

The β-galactosidase in its wildtype form, may originate from a microorganism having a vector, to which a gene encoding a wildtype β-galactosidase has been ligated, or introduced.

The β-galactosidase in its wildtype form may originate from any known β-galactosidase sequence or from any β-galactosidase sequence which has yet to be determined. β-Galactosidase yet to be determined can be identified using sequence databases and sequence alignment algorithms, for example, the publicly available GenBank database and the BLAST alignment algorithm.

In some embodiments, the enzyme having β-galactosidase activity is a wild-type β-galactosidase originating from *Aspergillus orizyae*, or a functional analogue thereof. The amino acid sequence of the wild-type β-galactosidase originating from *Aspergillus orizyae* can be found on https://www.uniprot.org/, accession: Q2UCU3.

In some preferred embodiments, the enzyme having β-galactosidase activity is a truncated variant of the wild-type β-galactosidase originating from *Aspergillus orizyae* (Q2UCU3).

The truncated variant of the β-galactosidase, according to the present invention, can be purchased from established manufacturers, e.g. Calza Clemente, or produced by methods known to the skilled person such as that described in M.M. Maksimainen et al., International Journal of Biological Macromolecules 2013, 60, 109–115.

The step of adding an enzyme having a β-galactosidase activity may advantageously be used to hydrolyze the lactose formed during the sialyltransferase catalyzed sialylation of the glycosphingolipid of formula (1), or of formulas (3)-(10). Depending on the sialic acid donor utilized, the trans-sialidase catalyzed sialylation may be a reversible process. When the sialic acid donor is 3'-sialyllactose, lactose is

formed during the transfer and may act as an acceptor for the trans-sialidase leading to an equilibrium. When using a β-galactosidase the lactose formed during the transfer can be hydrolyzed into galactose and glucose which typically do not act as acceptors for the trans-sialidase leading the reaction towards completion.

In some embodiments, the enzyme having β-galactosidase activity is added after a certain conversion of 3'-sialyllactose is reached. Preferably, the step of adding the enzyme having a β-galactosidase activity is performed when a conversion of at least about 50% of 3'-sialyllactose is reached, preferably when a conversion of at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, or 85% of 3'-sialyllactose is reached. The conversion of 3'-sialyllactose can be determined by standard techniques known to the skilled person. Typically, the conversion of 3'-sialyllactose is determined by HPLC and may be given in mol.% or *wt.*%.

In some embodiments, the sialylated glycosphingolipid, according to the present invention, is produced in the presence of a cyclodextrin.
The use of a cyclodextrin provides several advantages such as high yields and eliminates the need for the use of a detergent or organic solvent to increase accessibility to the glycosyl moiety of the glycosphingolipid. However, detergents or organic solvents can also be used in the method of the invention.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, and a cyclodextrin,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase and of said cyclodextrin, thereby producing said sialylated glycosphingolipid,

and wherein the sialic acid donor is 3´-sialyllactose.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, a cyclodextrin, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity and of said cyclodextrin, thereby producing said sialylated glycosphingolipid, and sequentially,
- adding the enzyme having β-galactosidase activity,

and wherein the sialic acid donor is 3´-sialyllactose.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, a cyclodextrin, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, of said enzyme having β-galactosidase activity and of said cyclodextrin, thereby producing said sialylated glycosphingolipid,

and wherein the sialic acid donor is 3´-sialyllactose.

The term "cyclodextrin", in the context of the present invention, refers to a cyclic oligosaccharide consisting of a macrocyclic ring of monosaccharide subunits (e.g., glucose). Cyclodextrins typically contain 6-, 7- or 8-monosaccharide subunits and may be referred to as α-cyclodextrins, β-cyclodextrins, and γ-cyclodextrins, respectively. The cyclodextrin may be modified such that some or all of the primary or secondary hydroxyl groups of the macrocycle, or both, may be alkylated or acylated. Methods of modifying these alcohols are well known to the person skilled in the art and many derivatives are commercially available. Thus, some or all of the hydroxyl groups of the cyclodextrin may be substituted with an $-OR^6$ group and/or an $O-C(=O)-R^7$ group, wherein R^6 and R^7 are independently selected from a saturated or unsaturated C_{1-6} alkyl, a saturated or unsaturated C_{1-6} heteroalkyl, a saturated or unsaturated cycloalkyl, a saturated or unsaturated heterocycloalkyl, an aryl, or a heteroaryl, each of which may be substituted or unsubstituted. In some embodiments, R^6 and R^7 are independently selected from the group consisting of 2-hydroxyethyl, 2-hydroxypropyl, and sulfobutylether.
In some embodiments, the cyclodextrin is α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, or derivatives thereof.

In some embodiments, the cyclodextrin is selected from the group consisting of β-cyclodextrin, hydroxypropyl-β-cyclodextrin, randomly methylated β-cyclodextrin, or sulfobutylether-β-cyclodextrin. In some preferred embodiments, the cyclodextrin is β-cyclodextrin.

The cyclodextrin is typically used in an amount between about 0.1 equivalents to about 1 equivalent based on the amount of the glycosphingolipid. In some preferred embodiments the cyclodextrin is used in an amount between about 0.1 equivalents to about 0.5 equivalents based on the amount of the glycosphingolipid. Accordingly, in some preferred embodiments, the cyclodextrin is used in an amount of about 0.1, 0.2, 0.3, 0.4, or 0.5 equivalents based on the amount of the glycosphingolipid.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, wherein the method described herein further comprises a nanofiltration step.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, thereby producing said sialylated glycosphingolipid, and sequentially
- adding the enzyme having β-galactosidase,
- nanofiltration of the reaction mixture,

and wherein the sialic acid donor is 3´-sialyllactose.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity and of said enzyme having β-galactosidase, thereby producing said sialylated glycosphingolipid,
- nanofiltration of the reaction mixture,

and wherein the sialic acid donor is 3´-sialyllactose.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, a cyclodextrin, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity and of said cyclodextrin, thereby producing said sialylated glycosphingolipid, and sequentially
- adding the enzyme having a β-galactosidase activity,
- nanofiltration of the reaction mixture,

and wherein the sialic acid donor is 3´-sialyllactose.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, an enzyme having β-galactosidase activity, and a cyclodextrin,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, of said enzyme having β-galactosidase, and of said cyclodextrin, thereby producing said sialylated glycosphingolipid,
- nanofiltration of the reaction mixture,

and wherein the sialic acid donor is 3´-sialyllactose.

The nanofiltration (NF) step may be used to concentrate the mixture containing the sialylated glycosphingolipid, to remove ions, mainly monovalent ions, and/or to remove organic materials having a molecular weight lower than that of the sialylated glycosphingolipid, such as monosaccharides. In a preferred embodiment, the nanofiltration step is used to remove galactose and glucose from the mixture containing the sialylated glycosphingolipid.

Typically, the nanofiltration membrane has a molecular weight cut-off (MWCO) that ensures the retention of the sialylated glycosphingolipid of interest. As an example, a nanofiltration membrane having a MWCO of about 200-500 Da, is suitable for retaining the sialylated glycosphingolipid. In this regard the sialylated glycosphingolipid is accumulated in the NF retentate (NFR). Nanofiltration can be combined with diafiltration (DF) with water in order to remove permeable molecules more effectively, e.g. until the conductivity of the permeate shows no or very low presence of salts.

The NF step according to the present invention is conducted, with or without the optional DF step, at a constant temperature, preferably between about 15-45 °C, more preferably between about 20-35 °C. The NF step, with or without diafiltration, is continued until reaching the desired concentration of the sialylated glycosphingolipid in the NFR. Other technical parameters like setting in the flux and pressure is a matter of routine skills.

The sialylation method according to the present invention comprises a step of mixing a glycosphingolipid with a sialic acid donor in the presence of an enzyme having trans-sialidase activity. In some embodiments, the sialylation method according to the present invention further comprises a step of adding a β-galactosidase to the mixture of the sialic acid donor, the glycosphingolipid, and the trans-sialidase.
In some embodiments, the sialylation is performed in the presence of a cyclodextrin.

The enzyme(s), substrates and in some embodiments the cyclodextrin may be added in any order, and it is appreciated that the order of combining the reactants may be adjusted as needed.

For example, the sialic acid donor may be added to a solution of the glycosphingolipid, followed by the addition of the trans-sialidase. As another example, the sialic acid donor may be added to a solution of the glycosphingolipid, followed by the addition of the trans-sialidase and β-galactosidase.

The sialic acid donor, the glycosphingolipid, the trans-sialidase, and the β-galactosidase, as well as any other component used during the sialylation reaction may be added to the reaction mixture either as a solid or dissolved in a solvent, and in any quantities and manner effective for the intended result of the process.

The temperature at which the above process is carried out can range from just above freezing to the temperature at which the most sensitive enzyme denatures. That temperature range is preferably at about 0 °C to about 45 °C, and more preferably at about 20 °C to 37 °C.

The glycosphingolipid and the sialic acid donor are reacted in the presence of the enzyme(s) and in some embodiments in the presence of a cyclodextrin , for a period of time sufficient to obtain the desired high yield of the desired sialylated glycosphingolipid.
Typically, the reaction is allowed to proceed for between about 1 to about 24 hours, preferably between about 5 to about 10 hours. In some embodiments, reaction is allowed to proceed for about 5, 6, 7, 8, 9, or 10 hours.

The glycosphingolipid, the enzyme(s), and in some embodiments the cyclodextrin, may be combined by admixture in an aqueous reaction medium. The medium generally has a pH value of about 5 to about 7.5. The selection of the medium is based on the ability of the medium to maintain the pH value at the desired level. Accordingly, in some embodiments the medium is buffered to a pH value of about 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5. In some preferred embodiments, the medium is buffered to a pH value of about 5.5 to 6.5. Accordingly, in some preferred embodiments, the medium is buffered to a pH value of about 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5.

Suitable buffers include, but are not limited to, MES, Bis-Tris, ADA, ACES, PIPES, MOPSO, MOPS, HEPES, PBS, sodium acetate buffer, sodium citrate buffer. Preferably, sodium acetate buffer. If a buffer is not used, the pH of the medium should be maintained at about 5 to about 7.5 by the use of a base or an acid. A suitable base is NaOH, a suitable acid is HCl.

The sialylated glycosphingolipid produced by the above processes can be used without purification. However, the sialylated glycosphingolipid may be purified via an isolation step.

Accordingly, in some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, wherein the method further comprises a step of isolating the sialylated glycosphingolipid from the rection mixture.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, thereby producing said sialylated glycosphingolipid, and sequentially
- adding the enzyme having β-galactosidase activity,
- nanofiltration of the mixture of the preceding steps,
- Isolating the sialylated glycosphingolipid,

and wherein the sialic acid donor is 3´-sialyllactose.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, and of said enzyme having β-galactosidase activity, thereby producing said sialylated glycosphingolipid,
- nanofiltration of the mixture of the preceding steps,
- Isolating the sialylated glycosphingolipid produced in the preceding steps,

and wherein the sialic acid donor is 3´-sialyllactose.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, a cyclodextrin, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, said enzyme having β-galactosidase activity and said cyclodextrin thereby producing said sialylated glycosphingolipid,
- nanofiltration of the mixture of the preceding steps,
- Isolating the sialylated glycosphingolipid produced in preceding steps.

and wherein the sialic acid donor is 3´-sialyllactose.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, a cyclodextrin, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, and of said cyclodextrin thereby producing said sialylated glycosphingolipid, and sequentially
- adding the enzyme having β-galactosidase activity,
- nanofiltration of the mixture of the preceding steps,
- Isolating the sialylated glycosphingolipid produced in preceding steps.

and wherein the sialic acid donor is 3´-sialyllactose.

The step of isolating the sialylated glycosphingolipid may be performed by standard methods known to the skilled person, such as for example extraction with organic solvents, chromatography and/or ion exchange chromatography.

A preferred method of isolation involves diafiltration (DF) of the reaction mixture, wherein the DF is used to remove ions, mainly monovalent ions, and/or to remove organic materials such as protein and organic molecules. In a preferred embodiment, the diafiltration is performed using a membrane having a MWCO of about 100-300 kDa, preferably of about 200-300 kDa is utilized. In some embodiments, the diafiltration membrane having a MWCO of about 100 - 150 kDa, 150-200 kDa, 200-250 kDa, or 250-300 kDa.

It is noted that, even though a MWCO of about 100-300 kDa is well above the molecular weight of the sialylated glycosphingolipid, the sialylated glycosphingolipid is accumulated in the DF retentate (DFR). It has been described that ganglioside GM1, can form micellar aggregates in aqueous solutions having molecular weights between about 250 kDa and 450 kDa, wherein the size of the micellar aggregate will depend on the length of the fatty acid chain in the constituent molecule (D.B. Gammak, Biochem J 1963, 88, 373). This property may render gangliosides, such as GM1 not permeable through ultrafiltration membranes having a MWCO higher than that of GM1.
However, surprisingly, the present inventors have found that also *N*-lyso forms of gangliosides, as well as glycosylated sphingoid bases, which lack a fatty acid chain are not permeable through diafiltration membranes having a MWCO higher than that of the lysosphingolipid. Accordingly, without being bound by theory, micellar aggregation may surprisingly occur independently from the presence of a fatty acid chain in the molecule. The person skilled in the art will understand that the DF step, according to the

method of the present invention, results in the removal of any contaminant present in the aqueous media which is permeable through the diafiltration membrane.

The DF step, according to the present invention, is conducted at a constant temperature, preferably between about 15-45 °C, more preferably between about 20-35 °C. The DF step is continued until reaching the desired concentration of the sialylated glycosphingolipid in the DFR. Other technical parameters like setting in the flux and pressure is a matter of routine skills.
The DF step may optionally be followed by a concentration step.

In some embodiments, the method further comprising a step of concentrating the DFR wherein the step of concentrating the DFR.
The concentration of the DFR is typically performed using the same membrane used during the diafiltration step, and for a period of time required to reduce the volume of the DFR to the desired final volume.

The DFR enriched with the sialylated glycosphingolipid, is spray dried or spray granulated in a subsequent step.

In some preferred embodiments, the DFR comprising the glycosphingolipid of formula (1), is spray dried.

The spray drying step, according to the present invention, is conducted with a fast-rotating disk or a nozzle which generates small particles. The particles can then fall, under gravity, towards the bottom of a spray drying tower. Here, a fluid bed may be provided, which can use hot air to effect drying (suitably at around 80° C to around 95° C). Here, agglomeration can take place, and the particles can stick together. Following this, the agglomerated (granular) particles are subjected to drying, for example on a belt drying bed or on a sub-fluidized bed.

Another technique is to use a fluidized bed agglomeration. Here, powder can be fluidized in a gas flow. In the particle bed a fluid is sprayed with water that wets the powder and enhances the agglomeration. This combination of spray-drying in combination with a fluid bed after dryer is suited for the agglomeration of many different types of solutions.

Drying can occur under air or under an inert gas, such as nitrogen. With fluidized and sub-fluidized bed drying, the temperature in the bed can be adjusted to pre-set values. These values can range widely, for example, from 35° to 120°C, such as 50 to 90°C, e. g. from 60 to 80°C.

The spray-drying of the DFR retentate will result in the production of a spray-dried powder comprising a sialylated glycosphingolipids.

The spray-dried powder, obtained following the method of the present invention, will typically have a median particles diameter between about 15 µm and about 30 µm.

The Span of the particles will typically be less than about 3, preferably less than about 2.
The Span of the particle is a dimensionless parameter indicative of the uniformity of the particle size distribution and it is defined as: [D(0.9) – D(0.1)] / D(0.5), wherein D(0.9), D(0.1), and D(0.5) represent the cutoff size below which 10%, 50%, and 90% (by volume) of particles are distributed, respectively. Generally, a low Span (i.e., less than 3) is characteristic of a narrow particle size distribution, resulting in improved flow characteristics of the spray-dried powder.

The spray-dried powder, obtained following the method of the present invention, will typically have a specific volume of less than about 4 mL/g, preferably less than about 3 ml/g.
Spray-dried powders with such low specific volumes (i.e., less than 4 ml/g) are generally preferred as they have improved flow characteristics.

The spray-dried powder, obtained following the method of the present invention, will typically have a glycosphingolipid content of at least about 65 *wt.*%, usually of at least about 70 wt.%, preferably of at least about 75 wt.%, more preferably of at least about 85 wt.%.

In some embodiments, the spray-dried powder comprising at least about 70 *wt.*% of *N*-lyso-GM3, or at least about 75 *wt.*% of *N*-lyso-GM3, or at least about 80 *wt.*% of *N*-lyso-GM3.

In some embodiments, the spray-dried powder comprising about 75-80 wt.% of *N*-lyso-GM3, and wherein the spray dried-powder further comprising about 7-9 wt.% of lactosyl D-*erythro*-sphingosine, and about 0.1-1.0 wt.% of glucosyl D-*erythro*-sphingosine.

In some embodiments, the sialylated glycosphingolipid produced by the method described above can be utilized as acceptor substrate for a second enzymatic sialylation step. Typically, the second enzymatic sialylation step is carried out as part of a sialyltransferase cycle, which comprises a CMP-sialic acid recycling system wherein several enzymes are utilized to generate/regenerate CMP-sialic acid from sialic acid and CMP. CMP-sialic acid is a relatively expensive sugar nucleotide, therefore the *in situ* generation and regeneration of the sialic acid donor is of economic advantage and enables the scale up of the process.

The sialyltransferase cycle described in the present invention, typically comprises sialic acid, cytidine monophosphate (CMP), a nucleoside triphosphate, and at least five enzymes, wherein the at least five enzymes comprise at least one enzyme having sialyltransferase activity, at least one enzyme having a *N*-acylneuraminate citydyltransferase activity, at least one enzyme having inorganic diphosphatase activity, and at least two enzymes having kinase activity.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, thereby producing said sialylated glycosphingolipid, and sequentially
- adding the enzyme having β-galactosidase activity,
- nanofiltration of the mixture of the preceding steps,
- isolating the sialylated glycosphingolipid produced in the preceding steps, and
- reacting the produced sialylated glycosphingolipid, with a mixture comprising sialic acid, cytidine monophosphate (CMP), a nucleoside triphosphate, and at least five enzymes, wherein the at least five enzymes comprise at least one enzyme having sialyltransferase activity, at least one enzyme having a *N*-acylneuraminate citydyltransferase activity, at least one enzyme having inorganic diphosphatase activity, and at least two enzymes having kinase activity.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, and said enzyme having β-galactosidase activity thereby producing said sialylated glycosphingolipid, and
- nanofiltration of the mixture of the preceding steps,
- isolating the sialylated glycosphingolipid produced in the preceding steps, and
- reacting the produced sialylated glycosphingolipid, with a mixture comprising sialic acid, cytidine monophosphate (CMP), a nucleoside triphosphate, and at least five enzymes, wherein the at least five enzymes comprise at least one enzyme having sialyltransferase activity, at least one enzyme having a *N*-acylneuraminate citydyltransferase activity, at least one enzyme having inorganic diphosphatase activity, and at least two enzymes having kinase activity.

The term “sialic acid” refers to any member of a family of nine-carbon carboxylated sugars. The most common member of the sialic acid family is *N*-acetyl-neuraminic acid (often abbreviated as Neu5Ac, NeuAc, or NANA). A second member of the family is *N*-glycolyl-neuraminic acid (Neu5Gc or NeuGc), in which the *N*-acetyl group of Neu5Ac is hydroxylated. A third sialic acid family member is 2-keto-3-deoxy-nonulosonic acid (KDN). Also included are 9-substituted sialic acids such as a 9-O-C_1-C_6 acyl-Neu5Ac like

9-O-lactyl-Neu5Ac or 9-O-acetyl-Neu5Ac, 9-deoxy-9-fluoro-Neu5Ac and 9-azido-9-deoxy-Neu5Ac. Preferably, in the context of the present invention, the term sialic acid refers to *N*-acetyl-neuraminic acid (Neu5Ac). *N*-Acetyl-neuraminic acid can be synthesized by methods known to skilled person such as for example the method described in US2011165626 (A1).

Nucleoside triphosphates suitable for use in the context of the present invention are adenosine-5'-triphosphate (ATP), uridine-5´-triphosphate (UTP), guanosine-5´-triphosphate (GTP), inosine triphosphate (ITP) and thymidine-5´-triphosphate (TTP). A preferred nucleoside triphosphate is adenosine-5'-triphosphate (ATP).

In some preferred embodiments, the nucleoside triphosphate is adenosine 5'-triphosphate (ATP), and the at least five enzymes comprise the following enzyme:

- an enzyme having sialyltransferase activity,
- an enzyme having cytidine monophosphate kinase activity,
- an enzyme having nucleoside diphosphate kinase activity,
- an enzyme having N-acylneuraminate cytidyltransferase activity, and
- an enzyme having inorganic diphosphatase activity.

Accordingly in some preferred embodiments, the sialyltransferase cycle comprises *N*-acetyl-neuraminic acid (Neu5Ac), cytidine monophosphate (CMP), adenosine 5'-triphosphate (ATP), an enzyme having cytidine monophosphate kinase activity (CMK) (for the phosphorylation of CMP), an enzyme having nucleoside diphosphate kinase activity (NDK) (for the phosphorylation of CDP), an enzyme having N-acylneuraminate cytidyltransferase activity (CSS) (for the transfer of CMP from CTP to Neu5Ac), an enzyme having sialyltransferase activity (for the transfer of Neu5Ac from CMP-Neu5Ac to the acceptor substrate), and an enzyme having inorganic diphosphatase activity (PPase) (to degrade the inorganic pyrophosphate (PPi) formed as by-product during the cycle). The sialyltransferase cycle described in this preferred embodiment is depicted in Figure 1.

In some embodiments, the sialyltransferase cycle further comprises the regeneration of ATP, wherein ATP is regenerated by using a source of phosphate and an enzyme having kinase activity.

Sources of phosphate that can be used for the regeneration of ATP include but are not limited to polyphosphate, phosphoenol pyruvate, and acetyl phosphate. The selection of a particular kinase for use in the regeneration of ATP depends upon the phosphate sourced employed.

In some embodiments ATP is regenerated by using polyphosphate as the source of phosphate and an enzyme having polyphosphatase kinase activity.

Accordingly, in some embodiments the sialyltransferase cycle comprises *N*-acetyl-neuraminic acid (Neu5Ac), cytidine monophosphate (CMP), adenosine 5'-triphosphate (ATP), polyphosphate, an enzyme having cytidine monophosphate kinase activity (CMK) (for the phosphorylation of CMP), an enzyme having nucleoside diphosphate kinase activity (NDK) (for the phosphorylation of CDP), an enzyme having N-acylneuraminate cytidyltransferase activity (CSS) (for the transfer of CMP from CTP to Neu5Ac), an enzyme having sialyltransferase activity (for the transfer of Neu5Ac from CMP-Neu5Ac to the acceptor substrate), an enzyme having polyphosphatase kinase activity (PPK) (for the phosphorylation of ADP), and an enzyme having inorganic diphosphatase activity (PPase) (to degrade the inorganic pyrophosphate (PPi) formed as by-product during the cycle). The sialyltransferase cycle described in this embodiment is depicted in Figure 2.

The term "an enzyme having a sialyltransferase activity" may be interchangeably used with the term "sialyltransferase" and denotes, in the context of the present invention, an enzyme belonging to the glycosyltransferase family 29 (GT29), or to the glycosyltransferase family 42 (GT42) which typically catalyzes the transfer of sialic acid from CMP-sialic acid to a saccharide acceptor.

Suitable sialyl transferases for use in the context of the present invention are sialyltransferases capable of catalyzing the addition of a sialic acid residue to the *O*-8 of an α-2-3-linked sialic acid residue of a saccharide acceptor.

The sialyltransferase, in its wild-type form, may originate from microorganisms such as bacteria, yeasts, ascomycete, actinomycetes, hyphomycetes, basidiomycotina, and the like, or mammals.

The sialyltransferase in its wild-type form, may originate from *Campilobacter jejuni*.

The sialyltransferase in its wildtype form, may originate from a microorganism having a vector, to which a gene encoding a wildtype sialyltransferase has been ligated, or introduced.

The sialyltransferase in its wildtype form may originate from any known sialyltransferase sequence or from any sialyltransferase sequence which has yet to be determined. Sialyltransferase yet to be determined can be identified using sequence databases and sequence alignment algorithms, for example, the publicly available GenBank database and the BLAST alignment algorithm.

In some embodiments, the enzyme having sialyltransferase activity is a wild-type α-2,3/α-2,8-sialyltransferase originating from *Campylobacter jejuni*, strain OX=197, or a functional analogue thereof. The amino acid sequence of the wild-type α-2,3/α-2,8-sialyltransferase originating from *Campylobacter jejuni*, strain OX=19 corresponds to the amino acid sequence having accession No: Q9LAK3 (https://www.ncbi.nlm.nih.gov/protein).

In some embodiments, the enzyme having sialyltransferase activity is a mutant of the wild-type α-2,3/α-2,8-sialyltransferase originating from *Campylobacter jejuni*, strain OX=197 (Q9LAK3).

In some embodiments, the mutant α-2,3/α-2,8-sialyltransferase has the mutation I53G (the numbering corresponding to alignment of the mutant amino acid sequence with the amino acid sequence of Q9LAK3), as described in Gilbert et al., *Biological Chemistry* 2002, 277, 327–337.

In some embodiments, the mutant α-2,3/α-2,8-sialyltransferase is a mutant derived from the wild-type CST-II Q9LAK3, wherein the mutant comprising the following mutations/modifications compared to the wild-type: *N*-terminal histidine tag MGHHHHHH.

In some embodiments, the mutant α-2,3/α-2,8-sialyltransferase is a mutant derived from the wild-type CST-II Q9LAK3, wherein the mutant comprising the following mutations/modifications compared to the wild-type: I53G and *N*-terminal histidine tag MGHHHHHH.

The α-2,3/α-2,8-sialyltransferase originating from *Campylobacter jejuni* may also be referred to as CST-II.

The term “an enzyme having cytidine monophosphate kinase activity” may be interchangeably used with the term “CMP kinase” or “CMK” and denotes, in the context of the present invention, an enzyme that catalyzes the phosphorylation of CMP (or dCMP), using ATP as the preferred phosphoryl donor.

In some embodiments, the enzyme having cytidine monophosphate kinase activity is a wild-type CMP kinase originating from *Mycobacterium tuberculosis*, or a functional analogue thereof. The amino acid sequence of the wild-type CMP kinase originating from *Mycobacterium tuberculosis* corresponds to the amino acid sequence having accession No: WP_129368399 (//www.ncbi.nlm.nih.gov/genbank/)

In some embodiments, the enzyme CMP kinase activity is a recombinant CMP kinase derived from the wild-type CMP kinase originating from *Mycobacterium tuberculosis*, wherein the recombinant CMP kinase comprising the following modifications compared to the wild-type: *N*-terminal histidine tag MGHHHHHH.

The CMP kinase originating from *Mycobacterium tuberculosis* may also be referred to as *Mt*CMK.

The term an enzyme having “nucleoside diphosphate kinase activity” may be interchangeably used with the term “nucleoside-diphosphate kinase” or “NDK” and denotes, in the context of the present invention, an enzyme that catalyzes the phosphorylation of a nucleoside diphosphate.

In some preferred embodiments, the enzyme having nucleoside diphosphate kinase activity is a wild-type nucleoside-diphosphate kinase originating from *Mycobacterium tuberculosis complex*, or a functional analogue thereof. The amino acid sequence of the wild-type nucleoside-diphosphate kinase originating from *Mycobacterium tuberculosis complex* corresponds to amino acid sequence having accession No: WP_003412592 (https://www.ncbi.nlm.nih.gov/genbank).

In some embodiments, the enzyme having NDK activity is a recombinant NDK derived from the wild-type NDK originating from *Mycobacterium tuberculosis complex*, wherein the recombinant NDK comprising the following modifications compared to the wild-type: *N*-terminal histidine tag MGHHHHHH.

The nucleoside-diphosphate kinase originating from *Mycobacterium tuberculosis complex* may also be referred to as *Mt*NDK.

The term "an enzyme having *N*-acylneuraminate cytidyltransferase activity" may be interchangeably used with the term "*N*-acylneuraminate cytidylyltransferase" or "CSS" and denotes, in the context of the present invention, an enzyme that catalyzes the transfer of CMP from CTP to *N*-acetyl-neuraminic acid (Neu5Ac).

In some embodiments, the enzyme having *N*-acylneuraminate cytidyltransferase activity is a wild-type *N*-acylneuraminate cytidylyltransferase originating from *Neisseria meningitidis* or a functional analogue thereof. The amino acid sequence of the wild-type *N*-acylneuraminate cytidylyltransferase originating from *Neisseria meningitidis* corresponds to the amino acid sequence having accession No: WP_061726245 (https://www.ncbi.nlm.nih.gov/genbank/)

In some embodiments, the enzyme having CSS activity is a recombinant CSS derived from the wild-type CSS originating from *Neisseria meningitidis*, wherein the recombinant CSS comprising the following modifications compared to the wild-type: *N*-terminal histidine tag MGHHHHHH.

The *N*-acylneuraminate cytidyltransferase originating from *Neisseria meningitidis* may also be referred to as *Nm*CSS.

The term "an enzyme having inorganic diphosphatase activity" may be interchangeably used with the term "inorganic diphosphatase" or "PPase" and denotes, in the context of the present invention, an enzyme that catalyses the hydrolysis of pyrophosphate (Ppi).

In some embodiments, the enzyme having inorganic diphosphatase activity is a wild-type inorganic diphosphatase originating from *Escherichia coli*, or a functional analogue thereof. The amino acid sequence of the wild-type inorganic diphosphatase originating from *Escherichia coli* corresponds to the amino acid sequence having accession No: WP_073849715 (https://www.ncbi.nlm.nih.gov/genbank/).

In some embodiments, the enzyme having PPase activity is a recombinant PPase derived from the wild-type PPase originating from *Escherichia coli*, wherein the recombinant PPase comprising the following modifications compared to the wild-type: *N*-terminal histidine tag MGHHHHHH.

The inorganic diphosphatase originating from *Escherichia coli* may also be referred to as *Ec*PPase.

The term "an enzyme having polyphosphate kinase activity" may be interchangeably used with the term "polyphosphate kinase" or "PPK" and denotes, in the context of the present invention, an enzyme that catalyses the phosphorylation of ADP.

In some preferred embodiments, the enzyme having polyphosphate kinase activity is a wild-type polyphosphate kinase originating from *Meiothermus ruber* strain DSM 1279, or a functional analogue thereof. The amino acid sequence of the wild-type polyphosphate kinase originating from *Meiothermus ruber* strain DSM 1279 corresponds to the amino sequence having accession No: ADD29239 (https://www.ncbi.nlm.nih.gov/genbank/).

In some embodiments, the enzyme having PPK activity is a recombinant PPK derived from the wild-type PPK originating from *Meiothermus ruber strain DSM 1279*, wherein the recombinant PPK comprising the modifications compared to the wild-type: *N*-terminal histidine tag MGHHHHHH.

The polyphosphate kinase originating from *Meiothermus ruber* may also be referred to as *Mr*PPK.

The mutated or recombinant variants and the wild-type enzymes used during the sialyltransferase cycle, can be purchased from established manufacturers, or produced by methods known to the skilled person. For instance, the mutant of the wild-type α-2,3/α-2,8-sialyltransferase originating from *Campylobacter jejuni* can be produced by the method described in Gilbert et al., *Biological Chemistry* 2002, 277, 327–337. The wild-type enzymes can be produced by the method described in the examples below.

For the sialyltransferase cycles, the concentrations or amounts of the various reactants used in the processes depend upon numerous factors including reaction conditions such as temperature and pH value, and the choice and amount of acceptor saccharides to be sialylated. Because the sialylation process permits regeneration of activating nucleotides, activated donor sugars and scavenging of produced PPi in the presence of catalytic amounts of the enzymes, the process is limited by the concentrations or amounts of the stoichiometric substances. The upper limit for the concentrations of reactants that can be used in accordance with the method of the present invention is determined by the solubility of such reactants. Preferably, the concentrations of activating nucleotides, phosphate donor, the donor sugar and enzymes are selected such that glycosylation proceeds until the acceptor is consumed.

The second enzymatic sialylation step can also include other ingredients that facilitate sialyltransferase activity. These ingredients can include a divalent cation (e.g., Mg^{+2} or Mn^{+2}), materials necessary for ATP regeneration, phosphate ions etc. The reaction medium may also comprise solubilizing detergents (e.g., Triton or SDS) and organic solvents such as methanol or ethanol, or a cyclodextrin.

In a preferred embodiment the reaction medium comprises a cyclodextrin.

In some embodiments, the cyclodextrin is selected from the group consisting of β-cyclodextrin, hydroxypropyl-β-cyclodextrin, randomly methylated β-cyclodextrin, or sulfobutylether-β-cyclodextrin. In some preferred embodiments, the cyclodextrin is β-cyclodextrin.

The cyclodextrin is typically used in an amount between about 0.1 equivalents to about 1 equivalent based on the amount of the glycosphingolipid acceptor. In some preferred embodiments the cyclodextrin is used in an amount between about 0.1 equivalents to about 0.5 equivalents based on the amount of the glycosphingolipid acceptor. Accordingly, in some preferred embodiments, the cyclodextrin is used in an amount of about 0.1, 0.2, 0.3, 0.4, or 0.5 equivalents based on the amount of the glycosphingolipid acceptor.

The use of a cyclodextrin provides several advantages such as high yields and eliminates the need for the use of a detergent or organic solvent to increase accessibility to the glycosyl moiety of the glycosphingolipid. However, detergents or organic solvents can also be used in the method of the invention.

In optimized reactions, the above ingredients can be combined by admixture in an aqueous reaction medium (solution) which has a pH value of about 6 to about 8.5. The medium is devoid of chelators that bind enzyme cofactors such as Mg^{+2} or Mn^{+2}. The selection of a medium is based on the ability of the medium to maintain pH value at the desired level. Thus, in some embodiments, the medium is buffered to a pH value at about 6.5 to about 8.5. If a buffer is not used, the pH of the medium should be maintained at about 6.5 to 8.0, preferably about 7.3 to 8.0, by the addition of base. A suitable base is NaOH. Accordingly, in some preferred embodiments the pH is buffered or kept at a value of about 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0

The temperature at which the above process is carried out can range from just above freezing to the temperature at which the most sensitive enzyme denaturates. That temperature range is preferably at about 0 °C to about 45 °C, and more preferably at about 20 °C to 37 °C.

The reaction mixture so formed is maintained for a period of time sufficient for the sialyltransferase to sialylate a high percentage of the acceptors. Typically, the reaction will often be allowed to proceed for about 8 to about 240 hours, preferably between about 24 and 48 hours.

The *N*-acetyl-neuraminic acid (Neu5Ac), the cytidine monophosphate (CMP), the adenosine 5'-triphosphate (ATP), polyphosphate, all enzymes required for the sialyltransferase cycle, as well as any other component used during the cycle may be added to the reaction mixture either as a solid or dissolved in a solvent, and in any quantities and manner effective for the intended result of the process.

In the context of the present invention, all enzymes' amounts, or concentrations are expressed in activity Units, which is a measure of the initial rate of catalysis. One activity Unit catalyses the formation of 1 µmol of product per minute at a given pH and temperature.

The enzymes can be utilized free in solution or can be bound to a support such as a polymer.

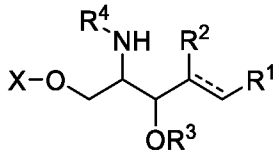
The enzymes may be provided as purified proteins, as cell-free extract, or as lysate.

In some embodiments, the enzymes are provided as purified proteins, with a purity of about 50% to about 95%.

In some embodiments, the enzymes are provided as cell-free extract, wherein the cell-free extract contains from about 5 wt% to about 70 wt% of the enzyme. Preferably, the cell-free extract contains from about 20 wt% to about 70 wt% of the enzyme.

The enzymes are usually present in a catalytic amount. The catalytic amount of a particular enzyme varies according to the concentration of that enzyme's substrate as well as to the reaction conditions such as temperature, time, and pH value. Means for determining the catalytic amount for a given enzyme under preselected substrate concentrations and reaction conditions are well known to those skilled in the art.

The step of subjecting a sialylated glycosphingolipid such as a sialylated glycosphingolipid of formula (2) or of formula (11)-(18) to a second enzymatic sialylation step as that described in the embodiments above, results in the formation of a sialylated glycosphingolipid of formula (19):



(19),

wherein

X is a glycosyl moiety carrying at least two sialic acid units,

R^1, R^2, R^3, R^4, and the bond ⌐⌐⌐ are as defined as for the glycosphingolipid of formula (1).

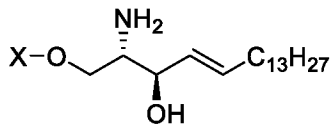
In some embodiments, for the sialylated glycosphingolipid of formula (19) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2, R^3 and R^4 are hydrogen, and the bond ⌐⌐⌐ is a double bond.

In some embodiments, for the sialylated glycosphingolipid of formula (19) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2 is OR^5, wherein R^5 is hydrogen, R^3 and R^4 are hydrogen, and the bond ⌐¬ is a single bond.

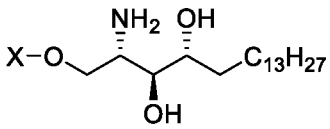
In some embodiments, for the sialylated glycosphingolipid of formula (19) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2, R^3 and R^4 are hydrogen, and the bond ⌐¬ is a single bond.

In some embodiments, for the sialylated glycosphingolipid of formula (19) R^1 is a C_{10}-C_{17} 1-hydroxyalkyl, R^2, R^3 and R^4 are hydrogen, and the bond ⌐¬ is a double bond.

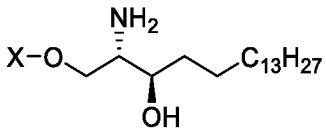
In some embodiments, the sialylated glycosphingolipid of formula (19) is a sialylated glycosphingolipid selected from the group consisting of sialylated glycosphingolipids of formulas (20), (21), (22), and (23):



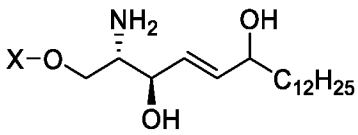
(20),



(21),



(22),



(23).

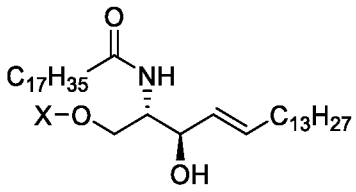
In some embodiments, for the sialylated glycosphingolipid of formula (19) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2 and R^3 are hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⌐¬ is a double bond.

In some embodiments, for the sialylated glycosphingolipid of formula (19) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2 is OR^5, wherein R^5 is hydrogen, R^3 is hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⌐¬ is a single bond.

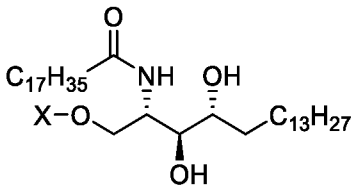
In some embodiments, for the sialylated glycosphingolipid of formula (19) R^1 is a saturated unsubstituted C_{10}-C_{17} alkyl, R^2, and R^3 are hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⌐¬ is a single bond.

In some embodiments, for the sialylated glycosphingolipid of formula (19) R^1 is a C_{10}-C_{17} 1-hydroxyalkyl, R^2, and R^3 are hydrogen, R^4 is a substituted or unsubstituted C_{16-32} acyl, and the bond ⌐¬ is a double bond.

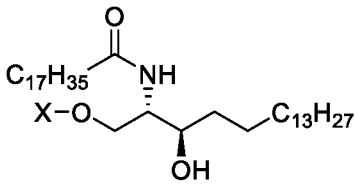
In some embodiments, the sialylated glycosphingolipid of formula (19) is a sialylated glycosphingolipid selected from the group consisting of sialylated glycosphingolipids of formulas (24), (25), (26), and (27):



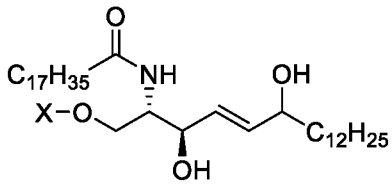
(24),



(25),



(26),



(27).

In some embodiments, X of the sialylated glycosphingolipid of formula (19), and of formulas (20)-(27) is a glycosyl moiety selected from the following glycosyl moieties, or salts thereof:

Neu5Acα2-8Neu5Acα2-3Galβ1-4Glc1-,
Neu5Acα2-8Neu5Acα2-3Galβ1-3GalNAcβ1-4Gal β1-4Glcβ1-,
Neu5Acα2-8Neu5Acα2-3Galβ1-3GalNAcβ1-4Gal β1-4Glc1-

In some embodiments, X of the sialylated glycosphingolipid of formula (19), and of the sialylated glycosphingolipids of formulas (20)-(27) is Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ1-.

In some embodiments, the sialylated glycosphingolipid of formula (19) is a sialylated glycosphingolipid of formula (20), and wherein X of the sialylated glycosphingolipid of formula (20) is Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ1-. Accordingly, in some embodiments, the sialylated glycosphingolipid of formula (19) is a sialylated glycosphingolipid of formula (20), and wherein the sialylated glycosphingolipid of formula (20) is *N*-lyso-GD3.

In some embodiments, the sialylated glycosphingolipid of formula (19) is a sialylated glycosphingolipid of formula (24), and wherein X of the sialylated glycosphingolipid of formula (24) is Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ1-. Accordingly, in some embodiments, the sialylated glycosphingolipid of

formula (19) is a sialylated glycosphingolipid of formula (24), and wherein the sialylated glycosphingolipid of formula (24) is GD3.

The sialylated glycosphingolipid obtained after the second enzymatic sialylation step may be isolated from the reaction mixture. The isolation may be performed by standard methods known to the skilled person, such as for example extraction with organic solvents, chromatography and/or ion exchange chromatography.

In some embodiments, the present invention describes a method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

- providing a glycosphingolipid, a sialic acid donor, an enzyme having a trans-sialidase activity, and an enzyme having β-galactosidase activity,
- mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, thereby producing said sialylated glycosphingolipid, and sequentially
- adding the enzyme having β-galactosidase activity,
- nanofiltration of the mixture of the preceding steps,
- isolating the sialylated glycosphingolipid produced in the preceding steps,
- reacting the produced sialylated glycosphingolipid, with a mixture comprising sialic acid, cytidine monophosphate (CMP), a nucleoside triphosphate, and at least five enzymes, wherein the at least five enzymes comprise at least one enzyme having sialyltransferase activity, at least one enzyme having a *N*-acylneuraminate citydyltransferase activity, at least one enzyme having inorganic diphosphatase activity, and at least two enzymes having kinase activity, and
- isolating the sialylated glycosphingolipid produced in the preceding step.

A preferred method of isolation involves diafiltration (DF) of the reaction mixture, wherein the DF is performed as described in the embodiments above.

In some embodiments, the isolation step is followed by a step of spray-drying or spray-granulating the sialylated glycosphingolipid, wherein the spray-drying or spray-granulating is performed as described in the embodiments above.

In some embodiments, the glycosphingolipids or sialylated glycosphingolipids according to the present invention may be produced or utilized in the form of salts, preferably in the form of pharmaceutical acceptable salts.

In some embodiments, the salts may be formed from the following acids: hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, polyphosphoric acid, acetic acid, camphor sulfonic acid, p-toluene sulfonic acid, methane sulfonic acid, trifluoromethanesulfonic acid, perchloric acid.

Examples

The working examples below describe non-limiting embodiments of the invention and are given only to illustrate the invention.

General methods and materials

LCMS analysis was performed with a Shimadzu ECO 2020 LC system coupled with a Shimadzu LCMS-2020 system equipped with a Merck Ascentis Express RP-Amide column (15cm x 4.6mm, 2.7 µm). HPLC analyses were performed on a Dionex Ultimate 3000 HPLC system coupled with a Corona Veo Charged Aerosol Detector using an Accucore aQ (150 mm x 4.6 mm, 2.6 µm) column.

Mutant TcTS was expressed from E. coli strains following methods described in Paris et al., *Glycobiology* 2001, 11, 305-311, or in Buschiazzo et al., *Molecular Cell* 2002, 10, 757-768, or following the general method described in Example 11.

Mutant CST-II (wild-type Q9LAK3, mutations/modifications: I53G and *N*-terminal histidine tag MGHHHHHH) was expressed from E. coli strains following methods described in Gilbert et al., *Biological Chemistry* 2002, 277, 327–337, or by following the general method described in Example 11.

Recombinant *Mt*CMK (wild-type: WP_129368399, modification: N-terminal histidine tag MGHHHHHH), recombinant *Mt*NDK (wild-type: WP_003412592, modification: N-terminal histidine tag MGHHHHHH), recombinant *Mr*PPK (wild-type: ADD29239, modification: N-terminal histidine tag MGHHHHHH), recombinant *Nm*CSS (wild-type: WP_061726245, modification: N-terminal histidine tag MGHHHHHH) , and recombinant *Ec*PPase (wild-type: WP_073849715, modification: N-terminal histidine tag MGHHHHHH) were expressed from *E. coli* strains following the general method described in Example 11.

Example 1. General procedure for the sialylation of glycosphingolipids with 2,3-trans-sialidase

The 2,3-trans-sialidase catalyzed sialylation reaction was performed in an aqueous solution at a pH between about 6.5 to about 7.0. A typical reaction mixture contained the glycosphingolipid acceptor (1 eq.), 3'-sialyllactose (1.5-2.0 eq.), the 2,3-transialidase (TcTS, 0.4 g/L) and the β-galactosidase (0.5 g/L). During the course of the reaction nanofiltration was applied. The nanofiltration of the reaction mixture was performed applying 300-500 Da membranes, a pressure of 15-20 bar and a temperature of about 30-40 °C for about 6-8 hours. The NF retentate (NFR) was heated at a temperature between about 60-95 °C for about 10-60 minutes, and then diafiltered as described in Example 7.

Example 2. Production of α-N-acetylneuraminosyl-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1′)-D-erythro-sphingosine (*N*-lyso-GM3)

N-lyso-GM3 was produced using lactosylsphingosine as the glycosphingolipid acceptor following the general procedure described in Example 1.

LC/MS: Rt = 4.38 min; ESI-MS calculated for [$C_{41}H_{74}N_2O_{20}$]: 914, found: 915 $[M+H]^+$, 913 $[M-H]^-$.

Example 3. Production of α-*N*-acetylneuraminosyl-(2→3)-*O*-β-D-galactopyranosyl-(1→3)-*O*-2-(acetylamino)-2-deoxy-β-D-galactopyranosyl-(1→4)-*O*-[α-*N*-acetylneuraminosyl-(2→3)]-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1´)-D-erythro-sphingosine (N-lyso-GD1a)

N-lyso-GD1a was produced using *N*-lyso-GM1a as the glycosphingolipid acceptor following the general procedure described in example 1.

LC/MS: Rt = 7.8 min; ESI-MS calculated for [$C_{66}H_{114}N_4O_{38}$]: 1571, found 1572 $[M+H]^+$, 1570 $[M-H]^-$.

Example 4. Production of α-*N*-acetylneuraminosyl-(2→3)-*O*-β-D-galactopyranosyl-(1→3)-*O*-2-(acetylamino)-2-deoxy-β-D-galactopyranosyl-(1→4)-*O*-[α-*N*-acetylneuraminosyl-(2→3)]-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1´)-N-stearoyl-D-erythro-sphingosine (GD1a)

GD1a was produced using GM1a as the glycosphingolipid acceptor following the general procedure described in Example 1.

LC/MS: Rt = 12.5 min; ESI-MS calculated for [$C_{84}H_{148}N_4O_{39}$]: 1837, found 1836 $[M-H]^-$.

Example 5. General procedure for the sialylation of glycosphingolipids with sialyltransferase cycle

5.1. Sialyltransferase cycle with CMP-Sialic acid generation/regeneration

The sialyltransferase cycle was performed in an aqueous solution at a pH between about 6.5 to about 7.5, and a temperature of about 37 °C. A typical reaction mixture contained the sialylated glycosphingolipid acceptor (1 eq.), N-acetylneuraminic acid (Neu5Ac, 2.5 eq.), β-cyclodextrin (0.5 eq.), ATP (3.5 eq.), CMP (0.27 eq.), $MgCl_2$ (20 mM), and the following enzymes: mutant CST-II (5g/L), recombinant *Mt*CMK, (12g/L), recombinant *Mt*NDK (6g/L), recombinant *Nm*CSS (1g/L), recombinant *Ec*PPase (2.5 µL/mL). The sialylation cycle was monitored by LCMS (for methods and condition see Example10).

5.2 Sialyltransferase cycle with CMP-Sialic acid generation/regeneration and ATP regeneration

The sialyltransferase cycle was performed in an aqueous solution at a pH between about 7.0 to about 8.0, and a temperature of about 37 °C. A typical reaction mixture contained the sialylated glycosphingolipid acceptor (1 eq.), N-acetylneuraminic acid (Neu5Ac, 1.5 eq.), β-cyclodextrin (0.5 eq.), ATP (between about 0.1 eq. to about 0.5 eq.), polyphosphate (2.5 eq.), CMP (0.27 eq.), $MgCl_2$ (20 mM), and the following enzymes: mutant CST-II (3g/L), recombinant *Mt*CMK (2.5g/L), recombinant *Mt*NDK(

2.5g/L, recombinant *Nm*CSS (0.5g/L), recombinant *Ec*PPase (40 to 60 mg/mL), recombinant *Mr*PPK, (40 to 60 mg/mL). The sialylation cycle was monitored by LCMS (for methods and condition see Example10).

Example 6. Production of α-N-acetylneuraminosyl-(2→8)-*O*-α-N-acetylneuraminosyl-(2→3)-*O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→1´)-D-erythro-sphingosine (*N*-lyso-GD3)

N-lyso-GD3 was produced using N-lyso-GM3 as the sialylated glycosphingolipid acceptor following the general procedure described in example 5.

LC/MS: Rt = 4.74 min; ESI-MS calculated for [$C_{52}H_{91}N_3O_{28}$]: 1205, found 1206 $[M+H]^+$, 1204 $[M-H]^-$.

Example 7. Isolation of Sialylated glycosphingolipids

Sialylated glycosphingolipids produced as described in Examples 1-6 were isolated from the reaction mixture via diafiltration (DF). The DF was performed by applying 250 kDa spiral-wound membranes having a membrane area of about 0.668 m^2, a flow rate of about 10 l/h, a transmembrane pressure of about 8-10 bar, a temperature between about 20-25 °C, and around 2-10 DF volumes relative to the volume of the feed solution. During diafiltration, a high flux of about 15.3-18.1 l/m^2 h was maintained. The DF retentate (DFR), containing the sialylated glycosphingolipid, was spray-dried on a Mobile Minor ® (GEA) spray drier under the following conditions:

Inlet flow rate: 45-50 g/min
Atomizer speed: 20,000 rpm
Inlet temperature: 160 °C
Outlet temperature: 85 °C

Following this procedure, a spry-dried powder comprising about 70-90 *wt.*% of one or more sialylated glycosphingolipids was obtained.

Example 8. Particle size analysis and water content of the spray-dried powder

The mean particle diameter, as well as the D(0.1), D(0.5), and D(0.9) values were measured by laser diffraction particle size analysis using a Malvern Mastersizer 3000 (Malvern Instruments). The spray-dried powder obtained in Example 7 was dispersed in cyclohexane including 0.1 % soy lecithin. The samples were sonicated before size measurement to disperse the aggregated particles.

The water content of the spray-dried powder obtained in Example 2 was determined by thermogravimetry (TG) and differential scanning calorimetry (DSC), or via Karl Fisher titration. TG and DSC measurements were performed on a Setaram LabsysEvo (Setaram). The spray-dried powder typically contains between about 2-3 *wt.*% of water.

Example 9. Spray-dried powder comprising *N*-lyso-GM3

A DFR comprising *N*-lyso-GM3, obtained following the procedure of Example 7, was spray-dried under the condition of Example 8 to afford a spray-dried powder having the following characteristics:

Glycosphingolipid Content			Water Content	Bulk Specific Volume	Mean Particle Diameter	Span
Compound	*wt.*%	Rt (min)	2.5 *wt.*%	2.5 mL/g	18.0 µm	1.4
N-lyso-GM3	78	10.1				
Lactosyl sphingosine	8	7.9				
Glucosyl sphingosine	1	8.7				

Example 10: LC/MS and HPLC Analysis

LC/MS Analysis

Samples (50 µL) were taken from reaction mixtures of examples 5.1 and 5.2 , mixed with DMSO (950 µL) and subjected to centrifugation (16.000 rpm, 5 min). For analysis of N-lyso-GD1a and GD1a the eluent consisted of solvent D (2 mM ammonium formate, 0.2 % v/v formic acid, 75% v/v MeOH, 25% v/v ACN) – solvent C (2 mM ammonium formate, 0.2 % v/v formic acid in water), for analysis of N-lysoGM3 and GD3 the eluent consisted of solvent D (2 mM ammonium formate, 0.2 % v/v formic acid, 75% v/v MeOH, 25% v/v ACN) – solvent C (2 mM formic acid in filtered water), and the following gradients were applied: N-lyso GM3 70-95% (D in C), *N*-lyso-GD1a 60-85% (D in C), GD1a 60-85% (D in C), N-lyso-GD3 70-98% (D in C).

HPLC Analysis

The glycosphingolipid content of spray-dried powders comprising *N*-lyso-GM3 was determined under the following conditions:

HPLC eluent profile: solvent A: 1 L water + 0.5 mL formic acid + 4 mmol ammonium formate, and solvent B: 1 L MeOH + 1 L acetonitrile + 4 mL formic acid + 4 mmol ammonium formate.

A gradient of 50-100% B in A was applied over 13 min, followed by an isocratic of 100% B for 18 min, followed by an isocratic of 50% B in A for 40 min. The glycosphingolipid content of the powder was quantified via peak area analysis using external standards.

Example 11. Production of Enzymes

Genes encoding the enzymes are usually ordered as codon-optimized synthetic genes for optimal expression in E. coli. The synthetic constructs contain overhangs with BsaI restriction sites for golden gate cloning into a pET28a-based expression vector (carrying introduced BsaI restriction sites and a fluorescent drop-out cassette). The gene is expressed under a lactose-inducible T7 promoter.

Overview of the SEQ ID NOs of the present invention.

SEQ ID NO:	Description
1	Amino acid sequence of the wild-type *Trypanosoma cruzi* trans-sialidase Q26966, with the difference of having the following mutations/modifications: *N*-terminal His-tag, deletion of 7 amino acids Δ636-642, S263T, R477H, V485L, E559V, S496K mutations.
2	nucleic acid sequence encoding SEQ ID NO: 1, codon-optimized for expression in *E. coli*
3	N-terminal histidine tag MGHHHHHH

Sequence List

SEQ ID NO: 1:

MGGSHHHHHHGMASLAPGSSRVELFKRQSSKVPFEKDGKVTERVVHSFRLPALVNVDGVMVAIADARYETSNDNSLI
DTVAKYSVDDGETWETQIAIKNSRASSVSRVVDPTVIVKGNKLYVLVGSYNSSRSYWTSHGDARDWDILLAVGEVTKST
AGGKITASIKWGSPVSLKEFFPAEMEGMHTNQFLGGAGVAIVASNGNLVYPVQVTNKKKQVFSKIFYSEDEGKTWKFG
KGRSAFGCSEPVALEWEGKLIINTRVDYRRRLVYESSDMGNTWLEAVGTLSRVWGPSPKSNQPGSQSSFTAVTIEGMR
VMLFTHPLNFKGRWLRDRLNLWLTDNQRIYNVGQVSIGDENSAYSSVLYKDDKLYCLHEINSNEVYSLVFARLVGELRII
KSVLQSWKNWDSHLSSICTPADPAASSSERGCGPAVTTVGLVGFLSHSATKTEWEDAYRCVNASTANAERVPNGLKFA
GVGGGALWPVSQQGQNQRYHFANHAFTLVASVTIHEVPKVASPLLGASLDSSGGKKLLGLSYDERHQWQPIYGSTPV
TPTGSWEMGKRYHVVLTMANKIGSVYIDGEPLEGSGQTVVPDERTPDISHFYVGGYKRSDMPTISHVTVNNVLLYNRQ
LNAEEIRTLFLSQDLIGTEAHMD

SEQ ID NO: 2:

ATGGGGGGTTCTCATCATCATCATCATCATGGTATGGCTAGCCTGGCACCCGGATCGAGCCGAGTTGAGCTGTTTA
AGCGGCAAAGCTCGAAGGTGCCATTTGAAAAGGACGGCAAAGTCACCGAGCGGGTTGTCCACTCGTTCCGCCTCC
CCGCCCTTGTTAATGTGGACGGGGTGATGGTTGCCATCGCGGACGCTCGCTACGAAACATCCAATGACAACTCCCT
CATTGATACGGTGGCGAAGTACAGCGTGGACGATGGGGAGACGTGGGAAACCCAAATTGCCATCAAGAACAGTC
GTGCATCGTCTGTTTCTCGTGTGGTGGATCCCACAGTGATTGTGAAGGGCAACAAGCTTTACGTCCTGGTTGGAAG
CTACAACAGTTCGCGTAGCTACTGGACGTCGCATGGTGATGCGCGTGACTGGGATATTCTGCTTGCCGTTGGTGA
GGTCACGAAGTCCACTGCGGGCGGCAAGATAACTGCGAGTATCAAATGGGGGAGCCCCGTGTCACTGAAGGAAT
TTTTTCCGGCGGAAATGGAAGGAATGCACACAAATCAATTTCTTGGCGGTGCAGGTGTTGCCATTGTGGCGTCCA
ACGGGAATCTTGTGTACCCTGTGCAGGTTACGAACAAAAAGAAGCAAGTTTTTTCCAAGATCTTCTACTCGGAAGA
CGAGGGCAAGACGTGGAAGTTTGGGAAGGGTCGCAGCGCTTTTGGCTGCTCTGAACCTGTGGCCCTTGAGTGGG
AGGGGAAGCTCATCATAAACACTCGAGTTGACTATCGCCGCCGTCTGGTGTACGAGTCCAGTGACATGGGGAATA
CGTGGCTGGAGGCTGTCGGCACGCTCTCACGTGTGTGGGGCCCCTCACCAAAATCGAACCAGCCCGGCAGTCAGA
GCAGCTTCACTGCCGTGACCATCGAGGGAATGCGTGTTATGCTCTTCACACACCCGCTGAATTTTAAGGGACGTTG
GCTGCGCGACCGACTGAACCTCTGGCTGACGGATAACCAGCGCATTTATAACGTTGGGCAAGTATCCATTGGTGA
TGAAAATTCCGCCTACAGCTCCGTCCTGTACAAGGATGATAAGCTGTACTGTTTGCATGAGATCAACAGTAACGAG
GTGTACAGCCTTGTTTTTGCGCGCCTGGTTGGCGAGCTACGGATCATTAAATCAGTGCTGCAGTCCTGGAAGAATT
GGGACAGCCACCTGTCCAGCATTTGCACCCCTGCTGATCCAGCCGCTTCGTCGTCAGAGCGTGGTTGTGGTCCCGC
TGTCACCACGGTTGGTCTTGTTGGCTTTTTGTCGCACAGTGCCACCAAAACCGAATGGGAGGATGCGTACCGCTGC
GTGAACGCAAGCACGGCAAATGCGGAGCGGGTTCCGAACGGTTTGAAGTTTGCGGGGGTTGGCGGAGGGGCGC
TTTGGCCGGTGAGCCAGCAGGGGCAGAATCAACGGTATCACTTTGCAAACCACGCGTTCACCCTGGTGGCGTCGG
TGACGATTCACGAGGTTCCGAAAGTCGCGAGTCCTTTGCTGGGTGCGAGCCTGGACTCTTCTGGTGGCAAAAAAC
TCCTGGGGCTCTCGTACGACGAGCGTCACCAGTGGCAGCCAATATACGGATCAACGCCGGTGACGCCGACCGGAT
CGTGGGAGATGGGTAAGCGTTACCACGTGGTTCTTACGATGGCGAATAAAATTGGCTCCGTGTACATTGATGGAG
AACCTCTGGAGGGTTCAGGGCAGACCGTTGTGCCAGACGAGCGGACGCCTGACATCTCCCACTTCTACGTTGGCG

GGTATAAACGTAGTGATATGCCAACCATCAGCCACGTGACGGTGAATAATGTTCTTCTTTACAACCGTCAGCTGAA
TGCCGAGGAGATCCGCACCTTGTTCTTGAGCCAGGACCTGATTGGCACGGAAGCACACATGGACTAA

SEQ ID NO: 3: MGHHHHHH

The disclosure should not be seen in any way restricted to the embodiments described and a person with ordinary skill in the art will foresee many possibilities to modifications thereof.

The above described embodiments are combinable.

The following claims further set out particular embodiments of the disclosure.

CLAIMS

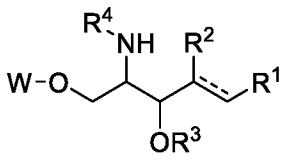
1. Method for the production of a sialylated glycosphingolipid, the method comprising the steps of:

   – providing a glycosphingolipid, a sialic acid donor, and an enzyme having a trans-sialidase activity,

   – mixing said glycosphingolipid with said sialic acid donor in the presence of said enzyme having trans-sialidase activity, thereby producing said sialylated glycosphingolipid,

   wherein

   the glycosphingolipid is a compound of formula (1):



(1),

wherein

W is Galβ1-, or a glycosyl moiety carrying one or more terminal β-galactopyranosyl units,
R^1 is hydrogen, aryl, or a substituted or unsubstituted C_{1-50} alkyl, preferably a substituted or unsubstituted C_{1-17} alkyl, more preferably a substituted or unsubstituted C_{10-17} alkyl,
R^2 is hydrogen or $-OR^5$, wherein R^5 is selected from hydrogen, a substituted or unsubstituted C_{1-6} alkyl, or a substituted or unsubstituted C_{2-6} acyl, preferably R^5 is hydrogen,
the bond ⌐¯¯¯ may be a double or a single bond when R^2 is hydrogen, or is a single bond when R^2 is $-OR^5$,
R^3 is hydrogen, a substituted or unsubstituted C_{1-6} alkyl, or a substituted or unsubstituted C_{1-6} acyl, preferably hydrogen,
R^4 is selected from hydrogen, a substituted or unsubstituted aryl, a heteroalkyl, a substituted or unsubstituted C_{2-32} acyl,

and wherein the sialic acid donor is 3´-sialyllactose.

2. The method according to claims 1, further comprising a step of adding an enzyme having β-galactosidase activity.

3. The method according to claims 1 or 2, further comprising a nanofiltration step.

4. The method according to any one of claims 1 to 3, further comprising a step of isolating the sialylated glycosphingolipid.

5. The method according to any one of claims 1 to 4 wherein R^4 of the glycosphingolipid of formula (1) is hydrogen, and wherein R^1 is a C_{13}-C_{17} alkyl, preferably a C_{13} alkyl.

6. The method according to any one of claims 1 to 5, wherein W of the glycosphingolipid of formula (1) is a glycosyl moiety selected from the group consisting of Galβ1-, Galβ1-4Glc1-, and Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc1-.

7. The method according to any one of claims 1 to 6, wherein the glycosphingolipid of formula (1) is selected from the group consisting of psychosine, lactosylsphingosine, and N-lyso-GM1a.

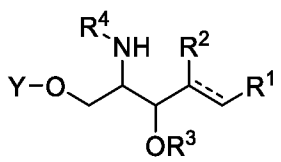
8. The method according to any one of claims 1 to 7, wherein the glycosphingolipid is lactosylsphingosine.

9. The method according to any one of claims 1 to 8 wherein the enzyme having trans-sialidase activity is a trans-sialidase originating from *Trypanosoma cruzi*, *Trypanosoma congolense*, or *Trypanosome brucei*, preferably from *Trypanosoma cruzi*, or a functional analogue thereof.

10. The method according to any one of claims 1 to 9, wherein enzyme having trans-sialidase activity is a mutant of the trans-sialidase originating from *Trypanosoma cruzi*.

11. The method according to any one of claims 2 to 10, wherein the enzyme having β-galactosidase activity is a β-galactosidase originating from Aspergillus oryzae, or a functional analogue thereof.

12. The method according to any one of claims 1 to 11, wherein the sialylated glycosphingolipid is a compound of formula (2):



(2),

wherein

Y is a glycosyl moiety carrying at least one sialic acid unit,

R^1, R^2, R^3, R^4, and the bond ⁼⁼⁼ are as defined as for the glycosphingolipid of formula (1).

13. The method according to claim 12 wherein R^4 of the sialylated glycosphingolipid of formula (2) is hydrogen, and wherein R^1 is a C_{13}-C_{17} alkyl, preferably a C_{13} alkyl.

14. The method according to claims 12 or 13, wherein Y of the sialylated glycosphingolipid of formula (2) is a glycosyl moiety selected from the group consisting of Neu5Acα2-3Galβ1-, Neu5Acα2-3Galβ1-4Glc1-, and Neu5Acα2-3Gal1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glc1-.

15. The method according to any one of claims 12 to 14, wherein the sialylated glycosphingolipid of formula (2) is selected from the group consisting of *N*-lyso-GM4, *N*-lyso-GM3 and *N*-lyso-GD1a.

16. The method according to any one of claims 12 to 15, wherein the sialylated glycosphingolipid of formula (2) is *N*-lyso-GM3.

17. The method according to any one of claims 1 to 16, wherein the method further comprising a step of reacting the sialylated glycosphingolipid, produced according to any of claims 1 to 16, with a mixture comprising sialic acid, cytidine monophosphate (CMP), a nucleoside triphosphate, and at least five enzymes, wherein the at least five enzymes comprise at least one enzyme having sialyl-transferase activity, at least one enzyme having a *N*-acylneuraminate citydyltransferase activity, at least one enzyme having inorganic diphosphatase activity, and at least two enzymes having kinase activity.

18. The method according to claim 17, wherein the nucleoside triphosphate is adenosine 5'-triphosphate (ATP), and wherein the at least five enzymes comprise the following enzyme:

- an enzyme having sialyl-transferase activity,
- an enzyme having cytidine monophosphate kinase activity,
- an enzyme having nucleoside diphosphate kinase activity,
- an enzyme having *N*-acylneuraminate cytidyltransferase activity, and
- an enzyme having inorganic diphosphatase activity.

19. The method according to claims 17 or 18, wherein the step of reacting the sialylated glycosphingolipid, produced according to any of claims 1 to 16, with a mixture

comprising sialic acid, cytidine monophosphate (CMP), a nucleoside triphosphate, and at least five enzymes, is performed in the presence of a cyclodextrin, and wherein the cyclodextrin is preferably selected from α-cyclodextrin, β-cyclodextrin, γ-cyclodextrin, or derivatives thereof, more preferably the cyclodextrin is β-cyclodextrin.

20. The method according to any one of claims 17 to 19, wherein the enzyme having nucleoside-diphosphate kinase activity is an enzyme originating from Mycobacterium tuberculosis complex, or a functional analogue thereof, and the enzyme having cytidine monophosphate kinase activity is an enzyme originating from *Mycobacterium tuberculosis*, or a functional analogue thereof.

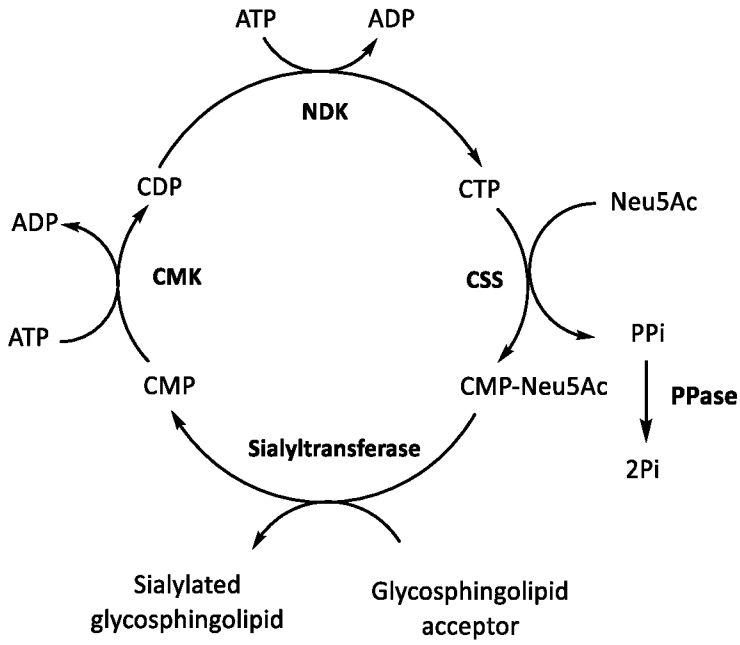

ATP
ADP
NDK
CDP
CTP
Neu5Ac
ADP
CMK
CSS
ATP
PPi
CMP
CMP-Neu5Ac
PPase
Sialyltransferase
2Pi
Sialylated glycosphingolipid
Glycosphingolipid acceptor

FIG. 1

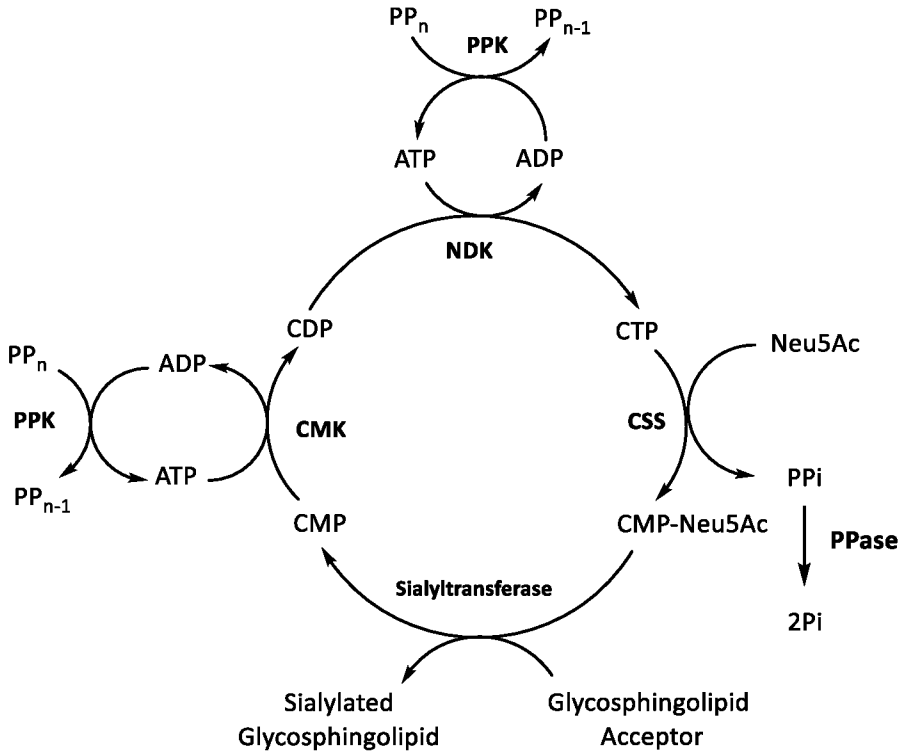

PPn
PPn-1
PPK
ATP
ADP
NDK
CDP
CTP
Neu5Ac
PPn
ADP
PPK
CMK
CSS
PPi
PPn-1
ATP
CMP
CMP-Neu5Ac
PPase
Sialyltransferase
2Pi
Sialylated
Glycosphingolipid
Glycosphingolipid
Acceptor

FIG. 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2024/057597

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P19/18 C07H15/10 A61K31/7028 C12N9/12
ADD.

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)
C12P C07H C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03/016469 A2 (NEOSE TECHNOLOGIES INC [US]; DEFREES SHAWN [US]; MCGUIRE EDWARD J [US]) 27 February 2003 (2003-02-27)	1,3-9, 12-16
Y	page 82 - page 92; figures 1-11; examples 1-8	2,10,11, 17-20
Y	WO 94/25614 A1 (SCRIPPS RESEARCH INST [US]) 10 November 1994 (1994-11-10) example (pages 22-24), page 23 (lines 20-32), page 17 (lines 6-15)	2,10,11, 17-20
A	WO 99/28491 A1 (CYTEL CORP [US]; DEFREES SHAWN [US]) 10 June 1999 (1999-06-10) cited in the application the whole document	1-20

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[x] Further documents are listed in the continuation of Box C.

[x] 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
17 December 2024	17/01/2025

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Zamarija, Ivica

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2024/057597

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 922 577 A (DEFREES SHAWN [US] ET AL) 13 July 1999 (1999-07-13) figure 10	1-20
	- - - - -	
A	US 5 278 299 A (WONG CHI-HUEY [US] ET AL) 11 January 1994 (1994-01-11) column 11	1-20
	- - - - -	
A	US 5 728 554 A (BAYER ROBERT J [US] ET AL) 17 March 1998 (1998-03-17) figure 2	1-20
	- - - - -	
L	IMBROGNO A. ET AL: "Molecular weight cut off (MWCO) determination in ultra- and nanofiltration: Review of methods and implications on organic matter removal", SEPARATION AND PURIFICATION TECHNOLOGY, vol. 354, 3 July 2024 (2024-07-03), page 128612, XP093227879, NL ISSN: 1383-5866, DOI: 10.1016/j.seppur.2024.128612 Figure 1A (and references cited therein)	
	- - - - -	

3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2024/057597

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2024/057597

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 9632491 A1	17-10-1996