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One-pot multienzyme (OPME) chemoenzymatic synthesis of brain ganglioside glycans with human ST3GAL II expressed in *E. coli*

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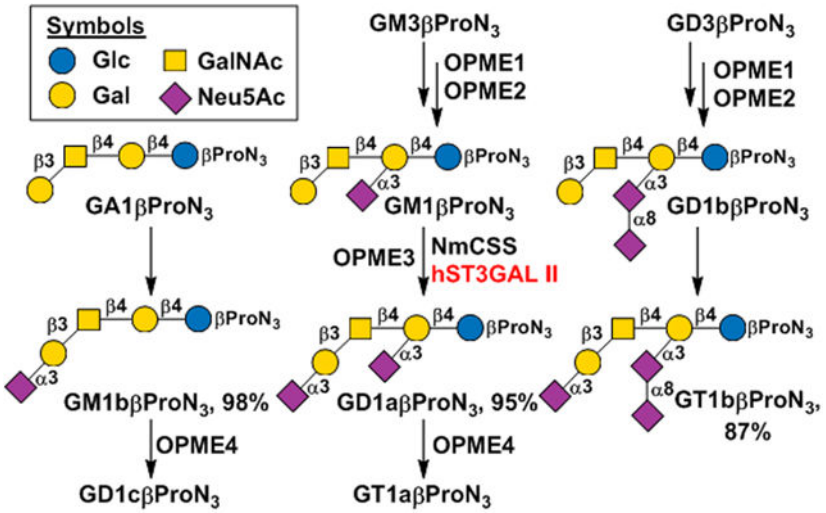
Abstract

A human sialyltransferase ST3GAL II (hST3GAL II) was successfully expressed in *Escherichia coli* as an active soluble fusion protein with an N-terminal maltose-binding protein (MBP) and a C-terminal hexa-histidine tag. It was used as an efficient catalyst in a one-pot multienzyme (OPME) sialylation system for high-yield production of the glycans of ganglioside GM1b and highly sialylated brain gangliosides GD1a and GT1b. Further sialylation of GM1b and GD1a glycans using a bacterial α2–8-sialyltransferase in another OPME sialylation reaction led to the formation of the glycans of GD1c and brain ganglioside GT1a, respectively. The lower reverse glycosylation activity of the recombinant hST3GAL II compared to its bacterial sialyltransferase counterpart simplifies the handling of enzymatic synthetic reactions and has an advantage for future use in automated chemoenzymatic synthetic processes.

Graphical Abstract



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A human sialyltransferase ST3GAL II (hST3GAL II) was expressed in *E. coli*, characterized, and used for highly efficient synthesis of highly sialylated ganglioside glycans in one-pot multienzyme (OPME) sialylation system with high yields.

Keywords

biocatalysis; carbohydrate; chemoenzymatic synthesis; ganglioside glycan; human sialyltransferase

Gangliosides are sialic acid-containing glycosphingolipids that are ubiquitously found in the plasma membrane of vertebrate cells and are the major sialic acid-containing glycoconjugates in animal nervous systems.[1] *N*-Acetylneuraminic acid (Neu5Ac) is the most abundant sialic acid form in nature[2] and the major sialic acid form in brain gangliosides.[3]

GM1a (**1**, also named as GM1) and more highly sialylated gangliosides GD1a (**2**), GD1b (**3**), and GT1b (**4**) (Figure 1) constitute the majority (>87%) of gangliosides in the brains of mammals.[4] Among these four, gangliosides GD1a (**2**) and GT1b (**4**) with two and three Neu5Ac residues, respectively, are the most abundant in the mammalian brain and are considered as the “major brain gangliosides”.[5] Alteration in the levels of different brain gangliosides has been linked to Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington disease (HD), and amyotrophic lateral sclerosis (ALS).[4c, 5b, 6] For example, decreased expression of GD1a (**2**) and GT1b (**4**) was shown in the substantia nigra of the male patients with PD.[7] Systemic deficiency of GM1a (**1**) was shown to correlate to sporadic PD. The brains of patients with ALS were found to have decreased levels of GD1b (**3**), GT1b (**4**), and GQ1b.[8] On the other hand, ganglioside GM1b (**5**) has been shown to be an influenza virus receptor[9] and the presence of its antibodies is associated with neurological diseases.[10]

Therapeutic applications of exogenous gangliosides, especially GM1a (**1**) and analogues, to spinal cord injury, PD, stroke, and AD, have been explored.[4c, 5b, 11] It is worth to note that the glycan component of GM1a (**1**) was shown to be able to promote tropomyosin receptor kinase A (TrkA)-dependent neurite growth.[12] A GM1a glycan was also shown to reduce the symptoms of sporadic PD in a mouse model.[13] Therefore, ganglioside glycans, in addition to gangliosides, may also have potential therapeutic applications.

Gangliosides in humans are synthesized by a series of glycosyltransferase-catalyzed reactions that sequentially add monosaccharides one at a time to simpler glycosphingolipids.[14] Due to their important biological functions, gangliosides and their corresponding glycans have been attractive synthetic targets for the development of potential therapeutics. Their synthesis is challenging due to their structural complexity and the presence of one or more sialic acids. Chemoenzymatic synthesis of highly sialylated ganglioside glycans reported recently[15] used a bacterial sialyltransferase *Pasteurella multocida* α2–3-sialyltransferase 1 (PmST1) that has relatively high reverse sialylation activity (previously identified as sialoside product cleavage/sialidase activity).[16] The reactions thus had to be monitored closely and the reactions had to be stopped promptly in order to minimize the

product cleavage.[15] The amount of the sialyltransferase used also needed to be controlled precisely. A sialyltransferase with lower reverse sialylation activity is desirable to simplify the synthetic procedures. This is especially important for the development of automated chemoenzymatic synthetic processes.[15b, 17] Herein we show that human ST3GAL II can be expressed in *Escherichia coli* in a soluble active form. The recombinant human ST3GAL II does not have significant product cleavage activity in the presence of cytidine 5'-monophosphate (CMP) and is highly efficient in a one-pot multienzyme (OPME) α2–3-sialylation system for catalyzing the synthesis of highly sialylated brain ganglioside glycans including the glycans of GD1a, GT1b, and GM1b from GM1a, GD1b, and GA1, respectively.

Human CMP-N-acetylneuraminate-β-galactosaminide-α-2,3-sialyltransferase 2 (hST3GAL II) (E.C.2.4.99.4)[18] is a type II membrane protein. It shares protein sequence similarity with other mammalian sialyltransferases in the carbohydrate active enzyme (CAZy) database (www.cazy.org)[19] glycosyltransferase GT29 family. Overexpression of hST3GAL II was shown to be related to skin aging.[20] A higher mRNA level of ST3Gal II was found to be related to advanced oral cancer.[21] Mouse[22] ST3Gal II and human ST3GAL II were reported to be the key enzymes involved in the synthesis of GD1a and GT1b.[23] Human ST3GAL II was also shown to be responsible for the synthesis of ganglioside stage-specific embryonic antigen 4 (SSEA4) which is overexpressed in various cancer cells.[24] We have been interested in expressing hST3GAL II in *E. coli* to explore its application in chemoenzymatic synthesis of oligosaccharides and glycoconjugates.

Among the twenty known human sialyltransferases, only three including hST3GAL I,[25] hST6GAL I,[25–26] hST6GALNAC I[27] have been reported to be expressed in *E. coli* as soluble active recombinant enzymes. Expressing additional human glycosyltransferases in amounts sufficient for preparative-scale synthesis of carbohydrates can expand the tool box of readily accessible synthetically useful carbohydrate biosynthetic enzymes which may have properties different from currently available enzymes that have been expressed in *E. coli*.

Based on topological model predictions of protein transmembrane domains, a codon optimized gene for a truncated hST3GAL II with the predicted N-terminal transmembrane domain being removed was cloned in pMAL-c4X vector to express a recombinant fusion protein with an N-terminal maltose-binding protein (MBP) and a C-terminal His_6-tag (MBP-Δ27hST3GAL II-His_6) (Figure S1). Expression of MBP-Δ27hST3GAL II-His_6 in *E. coli* BL21 (DE3) followed by Ni^{2+}-nitrilotriacetic acid (Ni^{2+}-NTA) affinity column purification provided a soluble active enzyme. About 9 mU of purified enzyme was routinely obtained from *E. coli* cells cultured in one liter of LB media. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Figure S3A) showed that the purified protein has a molecular weight close to the calculated value of 79 kDa although a weaker band with a smaller molecular weight was also observed.

Recombinant hST3GAL II expressed in various mammalian cell cultures has been reported. It was shown to catalyze the transfer of Neu5Ac from CMP-Neu5Ac to the terminal Gal residue in Galβ1–3GalNAc oligosaccharides or glycoconjugates to form an α2–3-sialyl

linkage.[28] We carried out acceptor substrate specificity studies for MBP-Δ27hST3GAL II-His_6 using a high-performance liquid chromatography (HPLC)-based quantitative assay. Several structurally defined synthetic disaccharides (**6–11**) and a monosaccharide (**12**) containing a hydrophobic benzyloxycarbonyl (Cbz)-protected propylamine (ProNHCbz)[29] or a methyl 2-anthranilic acid ester (2AA) tag[30] at the reducing end were used as potential acceptor substrates. As shown in Table 1, MBP-Δ27hST3GAL II-His_6 was the most reactive towards type IV glycan Galβ1–3GalNAcβProNHCbz (**6**) and type III or Core 1 glycan Galβ1–3GalNAcαProNHCbz (**7**). It was active, but with lower efficiencies, towards type I glycans Galβ1–3GlcNAcβProNHCbz (**8a**) and Galβ1–3GlcNAcβ2AA (**8b**) as well as the corresponding disaccharide analogue Galβ1–3GlcNAcαProNHCbz (**9**) with an α-linkage at the GlcNAc. No activity was observed for type II LacNAcβ2AA (**10**) and type VI LacβProNHCbz (**11**) glycans, or monosaccharide glycoside GalNAcαProNHCbz (**12**). The preference of MBP-Δ27hST3GAL II-His_6 towards Galβ1–3GalNAc-type acceptors was in consistent with previous reports regarding acceptor substrate specificity of purified and recombinant human ST3GAL II.[28] Using UV-detectable hydrophobic tag conjugated synthetic glycans, we demonstrated here that Galβ1–3GlcNAc-type glycans are also suitable although weaker acceptor substrates for the recombinant MBP-Δ27hST3GAL II-His_6. The preference of β1–3-linked galactoside acceptors by MBP-Δ27hST3GAL II-His_6 is complimentary to that of *Pasteurella multocida* sialyltransferase 3 (PmST3) which prefers β1–4-linked galactoside acceptors.[31] The information learned about acceptor substrate specificity of sialyltransferases will be very useful for choosing proper enzymes for selective sialylation of desired galactosyl branches in complex acceptor substrates containing different types of terminal galactosyl linkages.

We also explored the donor substrate specificity of MBP-Δ27hST3GAL II-His_6 using in situ-generated CMP-sialic acids and derivatives. A two-step process was established to do this. In the first step, CMP-sialic acid or it derivative was prepared from sialic acid, its derivative, or its precursor using a recombinant *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS)[32] with (when a sialic acid precursor was used as the starting material) or without (when sialic acid or its derivative was used as the starting material) *Pasteurella multocida* sialic acid aldolase (PmAldolase)[33] and sodium pyruvate in the presence of cytidine 5'-triphosphate (CTP). In the second step, MBP-Δ27hST3GAL II-His_6 sialylation reactions were carried out for 20 minutes or 16 hours using Galβ1–3GalNAcβProNHCbz (**6**)[29] as the acceptor substrate and the reaction mixtures from the first step as the sources of CMP-sialic acid donors. As shown in Table 2, MBP-Δ27hST3GAL II-His_6 was able to tolerate a variety of CMP-sialic acids and derivatives as donor substrates. CMP-Neu5Ac was generated highly effectively from either Neu5Ac or ManNAc, and was used efficiently by MBP-Δ27hST3GAL II-His_6 which was also efficient in using *in situ*-generated CMP-Kdn (generated from Kdn or mannose) and CMP-Neu5Ac9N_3 (generated from ManNAc6N_3[16a]). In comparison, CMP-Neu5N_3 (generated from Man2N_3[32]) and CMP-Neu5Ac7N_3 (generated from ManNAc4N_3[35]) were suitable but less efficient donor substrates for MBP-Δ27hST3GAL II-His_6.

pH profile studies (Figure S4) using Galβ1–3GalNAcβProNHCbz (**6**)[29] as the acceptor substrate showed that MBP-Δ27hST3GAL II-His_6 was active in a broad pH range of 4.5–

10.0. It was the most active in a pH range of 4.5–5.5 with an optimal activity at pH 5.0 in MES buffer. About 50% of its maximal activity was observed at pH 6.0 and in the pH range of 8.0–9.5. And 32–43% of its maximal activity was observed in the pH range of 6.5–7.5. The sialyltransferase activity fell to minimal when the pH was at or below 4.0 or when it was at or above 10.5. Reaction temperature profile studies (Figure S5A) showed that MBP-Δ27hST3GAL II-His_6 was the most active at 45 °C and 42 °C. More than 50% of the optimal activity was observed in the range of 35–45 °C. Minimal activity was observed when the temperature was at or below 25 °C or at or above 55 °C. Thermostability studies (Figure S5B) showed that the enzyme lost more than 60% activity by incubation at 35 °C for 1 hour and incubation at 37 °C for 1 hour retained only 14% of its activity. Incubation at 40 °C or a higher temperature for 1 hour completely abolished the enzyme activity. Altogether, these data showed that 30 °C is a well suited reaction temperature for MBP-Δ27hST3GAL II-His_6 for preparative-scale synthesis of sialosides.

Homologs of hST3GAL II have been identified in various higher vertebrates. Differences in acceptor specificity and tissue-specific expression have been observed for hST3GAL II[28a, 28b] versus mouse ST3Gal II.[36] Reversible sialylation activity has been shown previously for both rat and mouse ST3Gal II.[37] Although the reversible glycosylation activities of glycosyltransferases can be used for accessing some targets, they are undesirable for enzymatic synthesis utilizing the main glycosyltransferase functions as if the reaction progresses were not monitored and controlled closely they may lead to low yields due to the cleavage of the target products.[15a, 16c, 38] To investigate whether MBP-Δ27hST3GAL II-His_6 has a potential reverse sialylation activity, Neu5Acα2–3Galβ1–3GalNAcβProNHCbz (**13**) was synthesized from Galβ1–3GalNAcβProNHCbz (**6**) using a one-pot two-enzyme sialylation system (see ESI and **OPME3** in Scheme 1 below) containing NmCSS[32] and MBP-Δ27hST3GAL II-His_6. An excellent 96% yield was achieved for a preparative-scale synthetic reaction at 30 °C for 16 h followed by product purification using a simple C18-cartridge-based process. The potential reverse sialylation activity which can be presented as the desialylation of the sialylated product such as Neu5Acα2–3Galβ1–3GalNAcβProNHCbz (**13**) in the presence of CMP to form asialo-acceptor substrate of the enzyme (e.g. Galβ1–3GalNAcβProNHCbz **6**) was then assayed. To our delight, no significant conversion of compound **13** to compound **6** by MBP-Δ27hST3GAL II-His_6 was observed when it incubated at different concentrations (190, 19, and 1.9 μU sialyltransferase activities) with sialoside **13** (the product of the sialyltransferase activity) and CMP for 1 h (≤1.4±0.1%) and 20 h (≤3.9±0.1%) (Table 3). This indicated that the sialylated product formed from the reaction catalyzed by MBP-Δ27hST3GAL II-His_6 was not hydrolyzed significantly either with an elongated incubation time or with an excess amount of the enzyme, thus minimizing the necessity for close reaction monitoring which is essential for using sialyltransferases with reverse sialylation or product cleavage activity such as PmST1.[15–16]

With a general understanding of the properties of MBP-Δ27hST3GAL II-His_6, its application in chemoenzymatic synthesis of ganglioside glycans was explored. To synthesize glycans of GM1b, GD1a, and GT1b containing a propylazide aglycone, GM3βProN_3 (**14**) and GD3βProN_3 (**15**)[34] were used as the starting materials. A one-pot four-enzyme

N-acetylgalactosamine (GalNAc)-activation and transfer system (**OPME1**, Scheme 1) [39] containing *Bifidobacterium longum* strain ATCC55813 N-acetylhexosamine-1-kinase (BLNahK),[40] *Pasteurella multocida* N-acetylglucosamine uridylyltransferase (PmGlmU), [41] *Pasteurella multocida* inorganic pyrophosphatase (PmPpA),[42] and *Campylobacter jejuni* β1–4-N-acetylgalactosaminyltransferase (CjCgtA)[39] was used to add a β1–4-linked GalNAc to GM3βProN$_3$ (**14**) and GD3βProN$_3$ (**15**) to form GM2βProN$_3$ (**16**) and GD2βProN$_3$ (**17**), respectively, in excellent 98% and 99% yields. A one-pot four-enzyme galactose-activation and transfer system (**OPME2**, Scheme 1) containing *Streptococcus pneumoniae* galactokinase (SpGalK),[43] *Bifidobacterium longum* UDP-sugar pyrophosphorylase (BLUSP),[44] PmPpA, and *Campylobacter jejuni* β1–3-galactosyltransferase (CjCgtB)[45] was then used to synthesize GM1βProN$_3$ (**18**) and GD1bβProN$_3$ (**19**), respectively, with excellent 99% and 94% yields. Similar to the previous reports, we observed that α2–3-sialylated lactosides but not their asialylated forms were suitable acceptor substrates for CjCgtA.[46] Therefore, GA1βProN$_3$ (**20**) was not able to be obtained directly from LacβProN$_3$ using sequential **OPME1** and **OPME2** reaction processes described above. It can, however, be readily obtained from GM1βProN$_3$ (**18**) using a suitable sialidase. We cloned an N-terminal 22 amino acid truncated sialidase from *Bacteroides fragilis* as a N-terminal His$_6$-tagged fusion protein (His$_6$-Δ22BfGH33C) (Figure S2)[47] (see ESI for cloning, expression, and purification, its SDS-PAGE analysis results are shown in Figure S3B). We found that it was highly efficient in synthesizing GA1βProN$_3$ (**20**) with an excellent 94% yield from GM1βProN$_3$ (**18**). Similarly, GA2βProN$_3$ (**21**) was readily obtained from GM2βProN$_3$ (**16**) by a His$_6$-Δ22BfGH33C-catalyzed reaction. With GA1βProN$_3$ (**20**), GM1aβProN$_3$ (**18**), and GD1bβProN$_3$ (**19**) in hands, MBP-Δ27hST3GAL II-His$_6$ was used together with NmCSS[32] in a one-pot two-enzyme sialylation system (**OPME3**, Scheme 1) for the synthesis of GM1bβProN$_3$ (**22**), GD1aβProN$_3$ (**23**), and GT1bβProN$_3$ (**24**), respectively, with 98%, 95%, 87% yields.

Further sialylation of GM1bβProN$_3$ (**22**) and GD1aβProN$_3$ (**23**) by adding an α2–8-linked Neu5Ac at the non-reducing end using NmCSS and *Campylobacter jejuni* α2–3/8-sialyltransferase (CjCstII)[48] in another OPME sialylation reaction (**OPME4**, Scheme 1) led to the formation of GD1cβProN$_3$ (**25**), GT1aβProN$_3$ (**26**) in 78% and 68% yields, respectively. The relatively lower yields in these reactions were due to the formation of over-sialylated byproducts by CjCstII's capability in adding an additional α2–8-linked Neu5Ac to the desired products **25** and **26**, respectively. To purify the desired products, the pH of the reaction mixture was adjusted to 1.0–2.0 before it was loaded to a C18 cartridge to separate the product from nucleotides in the mixture. A second C18 cartridge purification at pH 8.0 was used to separate GD1cβProN$_3$ (**25**) from the minor byproduct with an additional α2–8-linked Neu5Ac.The same strategy was applied for the purification of GT1aβProN$_3$ (**26**).

In conclusion, a human sialyltransferase hST3GAL II was successfully expressed in *E. coli* as a soluble and active fusion protein with an N-terminal MBP and a C-terminal His$_6$-tag. The recombinant MBP-Δ27hST3GAL II-His$_6$ was highly efficient in chemoenzymatic synthesis of GM1b, GD1a and GT1b ganglioside glycans. A recombinant sialidase from *Bacteroides fragilis*, His$_6$-Δ22BfGH33c, was shown to be highly effective in the formation

of GA1βProN$_3$ (**20**) and GA2βProN$_3$ (**21**) from GM1βProN$_3$ (**18**) and GM2βProN$_3$ (**16**), respectively. Ganglioside glycans GM1b, GD1a, GT1b, GD1c, and GT1a were chemoenzymatically synthesized with high yields using sequential OPME systems with *in situ* generation of sugar nucleotides from simple monosaccharides.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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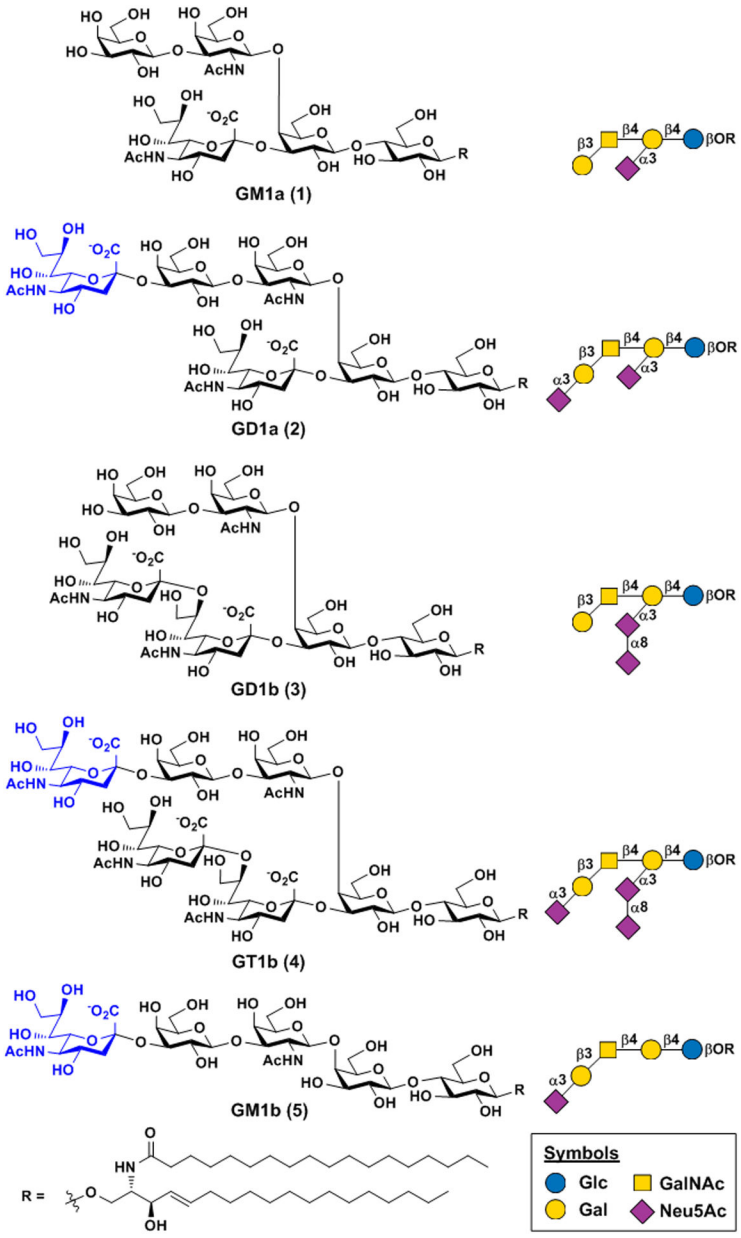
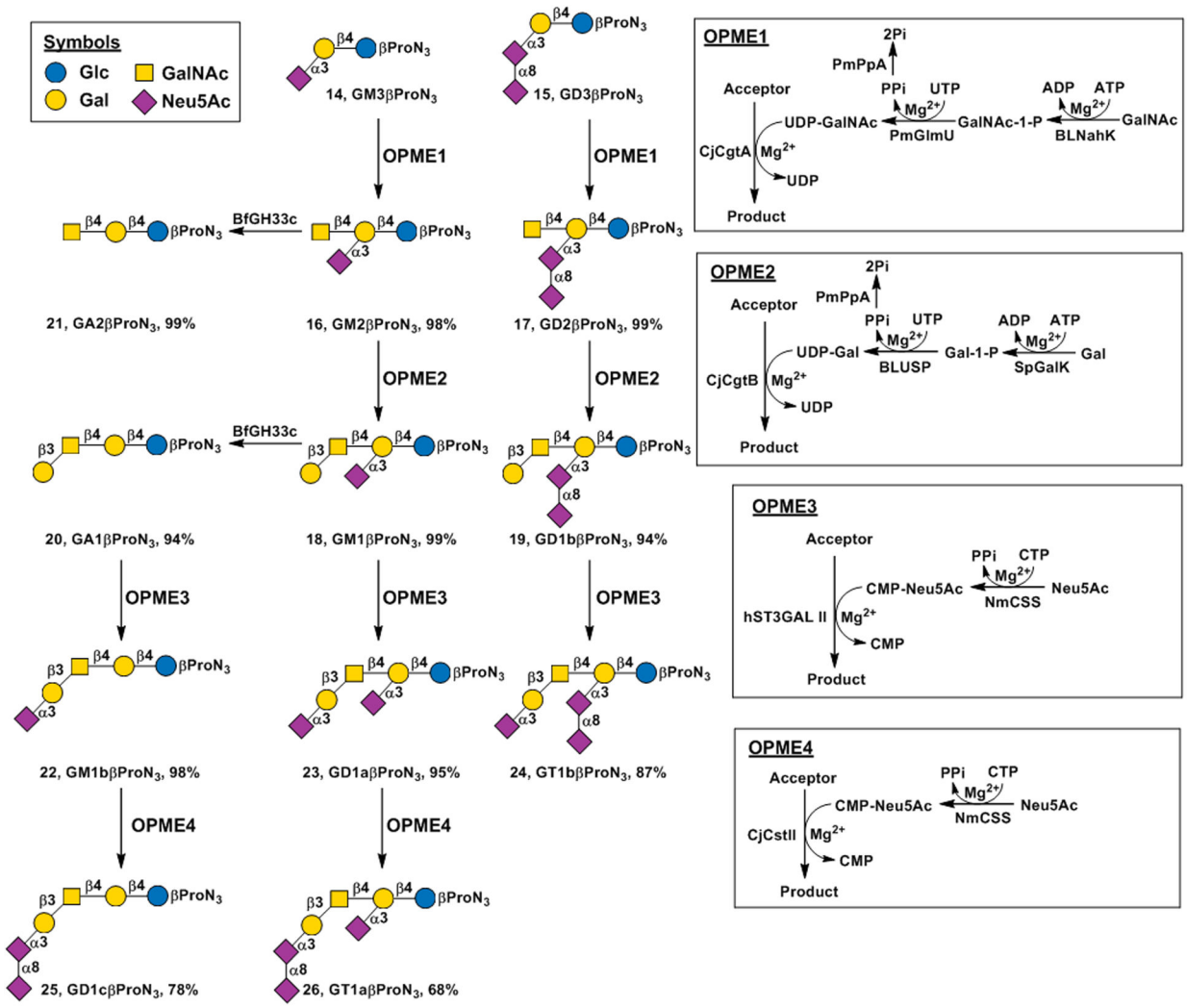


Figure 1.

Structures of major brain gangliosides GM1a (**1**), GD1a (**2**), GD1b (**3**), and GT1b (**4**) as well as ganglioside GM1b (**5**) with a representative ceramide (d18:1–18:0) structure (R).



Scheme 1.

Sequential one-pot multienzyme (OPME) synthesis of highly sialylated ganglioside glycans.

Table 1.

Acceptor substrate specificity of MBP-Δ27hST3GAL II-His_6.[a]

Acceptor	30 min (%)	16 h (%)
Galβ1–3GalNAcβProNHCbz (**6**)	44.0 ±0.1	100
Galβ1–3GalNAcαProNHCbz (**7**)	35.1 ±0.7	100
Galβ1–3GlcNAcβProNHCbz (**8a**)	9.1 ± 0.3	83.6 ±0.5
Galβ1–3GlcNAcβPro2AA (**8b**)	8.3 ±3.0	84.9 ±1.9
Galβ1–3GlcNAcαProNHCbz (**9**)	13.6 ± 2.2	96.4 ± 0.6
LacβProNHCbz (**10**)	0	0
LacNAcβPro2AA (**11**)	0	0
GalNAcαProNHCbz (**12**)	0	0

[a]Quantitative HPLC methods were used to determine the yields.

Table 2.

Donor substrate specificity of MBP-Δ27hST3GAL II-His_6.

Donor precursor	CMP-sialic acid yield (%)	Sialoside yield	
		20 min (%)	16 h (%)
Neu5Ac	100 ±1.2	62.6 ±1.2	100 ±0
Kdn	91.2 ±1.6	41.8 ±2.5	87.6 ±2.3
ManNAc	89.9 ±0.1	53.1 ±2.4	98.3 ±2.4
Mannose	86.3 ±2.1	40.8 ±3.8	86.0 ±0.8
$Man2N_3$	78.3 ±4.5	28.9 ±1.1	46.3 ±5.5
ManNAc	96.9 ±3.1	62.3 ±0.4	100 ±0.0
$ManNAc4N_3$	85.3 ±2.8	27.5 ±2.4	64.0 ±1.9
$ManNAc6N_3$	90.3 ±1.2	48.1 ±1.4	95.3 1.2

Table 3.

Percentage (%) of sialoside Neu5Acα2–3Galβ1–3GalNAcαProNHCbz (**13**) (the product of the sialyltransferase activity) that is desialylated by different amounts of MBP-Δ27hST3GAL II-His_6 in the presence of CMP for 1 h or 20 h.

Reaction Time	190 μU[a]	19 μU[a]	1.9 μU[a]
1 h	1.4±0.1	0.2±0.3	ND[b]
20 h	3.9±0.1	2.3±0.1	0.6±0.1

[a]The amounts of enzyme used are shown in units for its sialyltransferase activity: 1 unit of the sialyltransferase activity is defined as the amount of the enzyme that catalyzes the formation of 1 μmol of sialylated product Neu5Acα2–3Galβ1–3GalNAcαProNHCbz from Galβ1–3GalNAcαProNHCbz at 37 °C per minute at pH 7.0.

[b]ND, not detected.