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Glycosynthase-Mediated Synthesis of Glycosphingolipids

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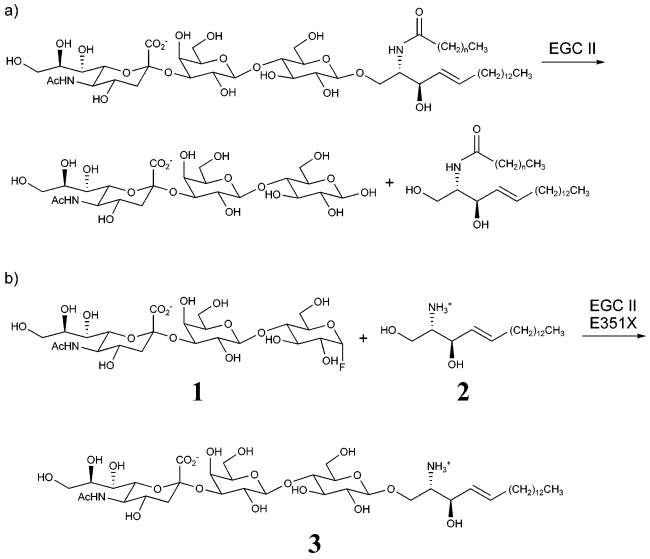
Glycosphingolipids, particularly the sialic acid-containing gangliosides, have numerous crucial functions within fundamental processes of human physiology. Predominantly localized to neuronal cell membranes (although present in all mammalian cell membranes), these compounds are extremely important in biochemical signaling and appear to play a critical role in virtually every stage of the cell life cycle, including growth, proliferation, differentiation, adhesion, senescence, and apoptosis.[1−6] Recent evidence suggests that clinical administration of particular gangliosides may alleviate symptoms of cancer[7] and diabetes[4] and may also promote nerve tissue regeneration,[8,9] which can counter the effects of Alzheimer's[10,11] and Parkinson's[12] diseases, stroke,[13] fetal alcohol syndrome,[14] and autoimmunity in transplant cases.[15] As such, there is great potential for health benefits stemming from the therapeutic utilization of these glycolipids.

A major limiting factor in applying these glycolipids to disease treatment is the great difficulty associated with producing large quantities of pure samples in an efficient and economical fashion. Commercial suppliers of gangliosides rely on that isolated from natural sources such as bovine brain and canine blood, which is impractical for large-scale preparation and also poses risks for possible contamination by prions or other infectious agents. Chemical syntheses of gangliosides have been reported;[16−19] however, significant challenges exist in these approaches with regard to control of stereo- and regiochemistry, the need for multiple protection/deprotection steps, poor product yields, difficult purification, and the unsuitability for industrial production.

Enzymatic syntheses offer significant improvements over purely chemical routes in the preparation of glycosylated compounds due to the ability to form specific glycoside linkages in the presence of many chemically similar hydroxyl groups. The most obvious route to the enzymatic synthesis of glycosphingolipids would be to recreate the natural biosynthetic pathway, which involves the sequential addition of glycosides to the core lipid acceptor.[20] This route, however, is problematic. First, the glucosyl transferase responsible for transferring the first carbohydrate residue is not currently available. Additionally, all subsequent transfer steps would have to be performed on the hydrophobic glycolipid, which could present problems in scale-up and product isolation.

We envisioned an alternative approach to the synthesis, involving preassembly of the glycosyl headgroup using known glycosyl transferases under normal aqueous conditions, followed by transfer of the oligosaccharide to the lipid acceptor. Although there are no known glycosyl transferases that catalyze this reaction, there is a well-studied endoglycoceramidase enzyme that catalyzes the reverse reaction, i.e. the hydrolysis of glycosphingolipids to their constituent lipid and sugar moieties (Scheme 1a).[21−24] This enzyme appeared to be a good candidate for conversion to a synthetic enzyme using glycosynthase methodology.

Scheme 1. Reactions Catalyzed by Wild-Type (a) and Glycosynthase (b) EGC II Enzymes



The glycosynthase approach utilizes retaining glycosidase variants in which the catalytic nucleophilic residue has been replaced by a residue unable to perform this function. When supplied with glycosyl fluorides with anomeric configuration opposite that of the normal cleaved linkage, these enzymes are often able to transfer the glycosyl moiety to an appropriate acceptor.[25] In this study, the parent glycosidase from which the glycosynthase was generated was endoglycoceramidase II (EGC II) from *Rhodococcus* strain M-777.[21−24] The *Escherichia coli* codon-optimized gene for this enzyme (minus 30 N-terminal residues constituting a secretion signal sequence) was synthesized (Blue Heron Biotechnology, Inc.) and expressed at high levels (180−200 mg protein per liter of culture) in an *Escherichia coli* host.

On the basis of sequence alignments, EGC II has been assigned to glycosyl hydrolase family 5, which predominantly consists of cellulases (β-1,4-endoglucanases). Previous studies in this laboratory have permitted the identification of the catalytic nucleophile residue in this family.[26] Sequence similarities in the region of the nucleophile allowed us to tentatively assign this function to Glu351 in EGC II. Substitution of this residue with a series of residues expected to be catalytically incompetent in a hydrolytic context (Ser, Ala, Gly, and Asp) led to the loss of EGC II activity, supporting the postulated role for this residue in the mechanism.

When 3′-sialyllactosyl fluoride (**1**) and D-*erythro*-sphingosine (**2**) were combined at 5 mM each in 25 mM sodium acetate, pH 5.0 containing 0.2% (v/v) Triton X-100 and incubated in the presence

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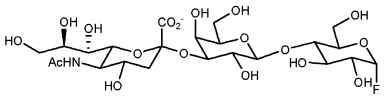
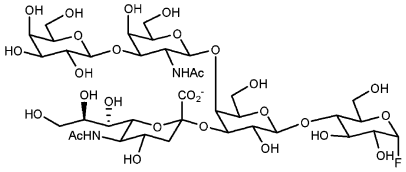
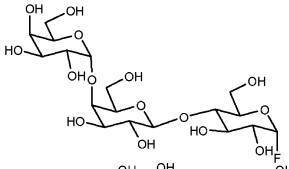
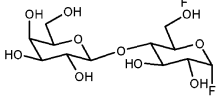
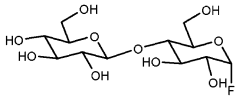
Table 1. Reaction Yields for EGC II E351S Glycosynthase Reactions with Various Glycosyl Fluoride Donor Sugars[a]

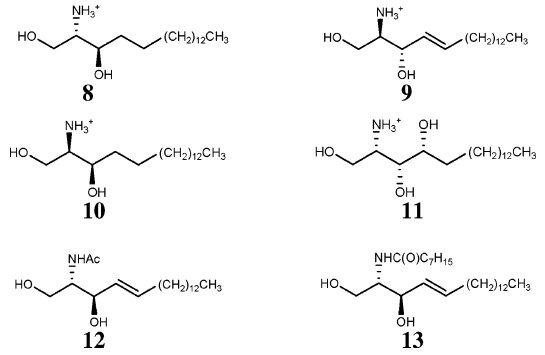
Compound	Structure	Yield (%)[b]
1		95
4		95
5		96
6		97
7		94

[a] Reaction conditions provided in Supporting Information. [b] Determined by thin-layer chromatography with quantification by spot densitometry (refer to Supporting Information for additional information).

of the candidate glycosynthase enzymes for 12 h at 25 °C, formation of the glycosynthase product *lyso*-G_{M3} ganglioside (**3**) was catalyzed by the Ser, Ala, and Gly mutant enzymes, but not the Asp mutant (Scheme 1b). By using excess **1** or **2**, it was possible to drive the reaction to near completion, with yields in excess of 90% obtained with the E351S enzyme. The reaction has been performed with up to 300 mg of **1** in the presence of excess **2**. In this larger-scale reaction, all of the glycosyl fluoride substrate was consumed, but the isolated yield was reduced to 73% due to losses during the purification, which required two C_{18} chromatographic steps. Further optimization of the purification should significantly improve the product yield.

As is the case for the hydrolytic activity of the wild-type enzyme,[22] the glycosynthase activity was enhanced by inclusion of detergent in the reaction. Among the detergents tested, Triton X-100 clearly gave the greatest rate acceleration. The use of 1,2-dimethoxyethane or glycerol at 10−15% in place of the detergent led to a similar rate enhancement, but this effect was not observed for any other solvents tested.

Reaction products were obtained in high yields using other glycosyl fluoride donor sugars (Table 1). In addition to *lyso*-G_{M3}, this approach was also effective in preparing the *lyso* analogue of G_{M1} ganglioside and the P^k antigen (precursor to the globoside series of glycosphingolipids), as well as lactosyl and cellobiosyl sphingosines by coupling donors **4**, **5**, **6**, and **7,** respectively, with D-*erythro*-sphingosine. Moreover, the enzyme was able to utilize several additional sphingosine analogues (**8**−**13**) as acceptors, illustrating the flexibility of this glycosynthase in the preparation of a range of natural and unnatural glycosphingolipids. Although *N*-palmitoyl-D-*erythro*-sphingosine (C16 ceramide) was not able to act as an acceptor substrate due to its very low aqueous solubility, intact G_{M3} ganglioside was easily prepared in near quantitative yield by acylation of the *lyso* analogue using *N*-palmitoyl succinimide as an acyl donor.



The ability of this enzyme to form the synthetically challenging glycosidic bond between the oligosaccharide and lipid portions of gangliosides and related glycolipids offers a new route to the efficient assembly of glycosphingolipids, which should provide avenues for the large-scale synthesis of these therapeutically valuable compounds.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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