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COMMUNICATION

Glycosphingolipid synthesis employing a combination of recombinant glycosyltransferases and an endoglycoceramidase glycosynthase†‡

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Glycosynthase mutants of *Rhodococcus* sp. *endo*-glycoceramidase II efficiently synthesize complex glycosphingolipids. Glycosyl fluoride donors may be assembled *via* sequential glycosyltransferase-catalysed glycosylation of lactosyl fluoride. Alternatively, lactosyl fluoride may be coupled to sphingosine prior to subsequent glycosylation steps.

Many important roles for glycosphingolipids (GSLs, Fig. 1) in human health and disease are now widely acknowledged.[1] For instance, as characteristic components of the cell membrane GSLs contribute to lipid raft organization and participate in signalling and pathogen engagement.[2–4] An increasing appreciation of their roles in various physiological processes has heightened demand for quantities of homogeneous GSLs.

Isolation of GSLs from natural sources is common but subject to several notable limitations, including potential transmission of disease, heterogeneity, and, in some cases, scarcity. Chemical synthesis can provide exquisite control over structural features and allow access to modified GSLs.[5–7] However, the appeal of large-scale chemical synthesis is tempered by requisite protecting group manipulations and chromatography of stereoisomeric glycosylation products. Complex carbohydrate synthesis is increasingly conducted wholly or in part using enzymes, normally *via* stepwise addition of monosaccharides to the non-reducing terminus of the growing oligosaccharide.[7,8]

In recent years access to the glycosyltransferases (GTs) and gylcoside hydrolases (GHs) required to construct a wide range of linkages in complex oligosaccharides has substantially improved. Likewise, problems associated with GT sugar nucleotide donor cost and instability may be minimized through improved chemical syntheses and chemoenzymatic preparation by native and engineered enzymes.[9–11] Yields of GH-catalysed tranglycosylations are typically low since (i) reaction products are necessarily substrates subject to enzyme-catalysed hydrolysis, and (ii) the reaction must be driven forward by use of a high reactant concentration. Glycosynthases are mutant retaining GHs in which the catalytic nucleophile has been replaced by an alternative amino acid residue that is unable to generate a covalent glycosyl-enzyme intermediate.[12] When supplied with an activated glycosyl fluoride (of the opposite configuration to the linkage that is normally cleaved) and an appropriate acceptor alcohol, glycosynthases catalyse glycosidic bond formation. Since a functional catalytic nucleophile is required for hydrolysis, the enzyme cannot degrade its product appreciably, resulting in high yields for the transglycosylation-like reaction.

An obvious approach to enzymatic GSL synthesis would entail stepwise GT-catalysed addition of constituent monosaccharides to sphingosine. This process remains impractical since the first enzyme, glucosylceramide synthase, is not readily available and the poor aqueous solubility of the glucosyl-lipid would complicate scale-up. An alternative approach is to assemble the oligosaccharide fluoride in aqueous solution, and in turn couple this glycosyl fluoride with sphingosine using an appropriate glycosynthase.

Endoglycoceramidase II (EGCase) from *Rhodococcus* sp. strain M-777 is a retaining GH (CAZy GH5) that catalyses the cleavage of the glycosyl ceramide bond in many GSLs that contain a β-linked D-*erythro*-sphingosine lipid core.[13] We have demonstrated the glycosynthase activity of the EGCase nucleophile mutant E351S in the course of efficient syntheses of *lyso*-forms of G_{M1}, G_{M3}, and the P^k antigen, and have subsequently

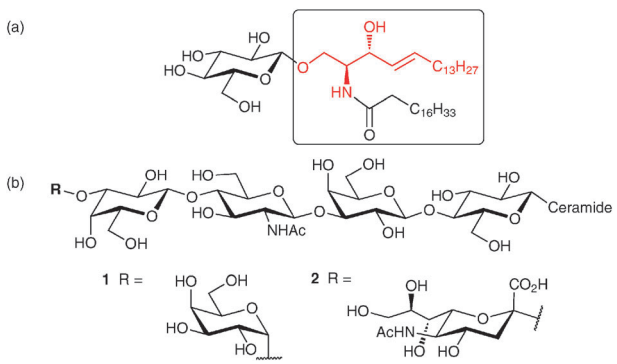

Fig. 1 Glycosphingolipids. (a) Glucosyl ceramide. Red: D-*erythro*-sphingosine. Boxed: ceramide. (b) Structures of glycosphingolipids ceramide pentahexoside (**1**) and sialosyl neolactotetraosylceramide (**2**).

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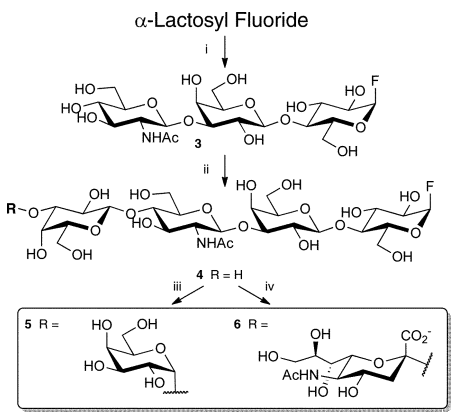
employed directed evolution to engineer tolerance of other lipid substrates.[14,15]

The glycosynthase approach to GSL synthesis holds broad appeal given the yields, flexibility with respect to oligosaccharide structure, and the demonstrated potential to alter lipid specificity in our earlier reports.[14,15] Furthermore, glycosyl-sphingosine products should readily undergo a variety of chemical reactions to generate diverse natural and unnatural GSLs. Ready access to glycosyl fluorides is key to this approach.

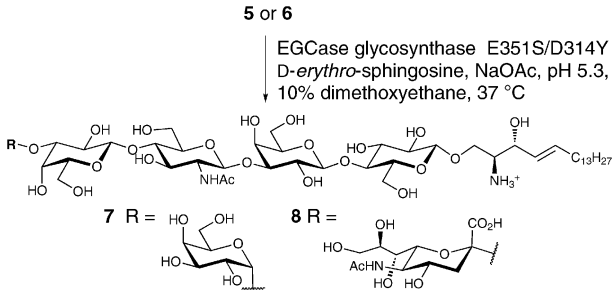
We have explored two distinct strategies for chemoenzymatic GSL synthesis, each of which involves synergistic application of GTs and EGCase glycosynthase. In the first, complex glycosyl fluorides are constructed *via* GT-catalysed sequential glycosylation of lactosyl-fluoride (Lac-F) and are then transferred *en bloc* to D-*erythro*-sphingosine by the glycosynthase. Alternatively, lactosyl sphingosine (Lac-sphing) can be assembled first from Lac-F using EGCase glycosynthase, and then be elaborated with the appropriate GTs.

Lacto-*N*-neotetraose (Galβ1,4-GlcNAcβ1,3-Galβ1,4-Glcβ-) is a structural subunit of *neolacto*- GSLs including those of potential therapeutic value such as ceramide pentahexoside **1** and LM_1 (sialosyl neolactotetraosylceramide) **2** (Fig. 1).[16,17] Neither are commercially available and they have received limited synthetic attention.[18,19] To determine whether EGCase glycosynthase could effectively synthesize *neolacto*- GSLs **1** and **2** we required glycosyl fluorides **5** and **6** (Scheme 1).

Pentasaccharides **5** and **6** were prepared from Lac-F in a series of glycosylation reactions catalysed by *H. pylori* β1,3-GlcNAcT HP-39, *H. pylori* β1,4-GalT HP-21, bovine α1,3 GalT, and SiaT Cst-I from *C. jejuni*.[20–23] Following addition of β1,3-linked N-acetylglucosamine (GlcNAc), the enzyme was removed by centrifugal filtration and excess UDP-GlcNAc was separated from **3** by ion exchange chromatography in order to prevent side reactions in the subsequent β-galactosyltransferase-catalysed step. In contrast, following addition of the second galactosyl residue to form tetrasaccharide **4**, subsequent galactosylation or sialylation to afford the pentasaccharides could be achieved without intermediate removal of enzyme or donor. Thin-layer chromatography suggested the complete glycosylation of each acceptor at each step. Following three successive enzymatic glycosylation steps to form either **5** or **6**, an unidentified more polar contaminant (≤20%) that could



Scheme 1 GT-catalysed synthesis of glycosyl fluorides. (i) *Helicobacter pylori* β1,3GlcNAcT HP-39 (ii) *H. pylori* β1,4GalT HP-21 (iii) Bovine α1,3GalT BOV-10 (iv) *Campylobacter jejuni* α2,3SiaT CST-06.



Scheme 2 EGCase glycosynthase reactions.

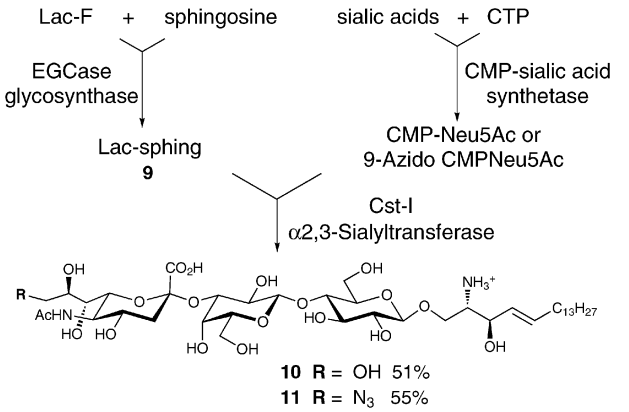
not be completely removed by gel permeation chromatography was in each case evident. Rather than risk further hydrolysis of the glycosyl fluoride, we opted for purification and characterization at a later stage. Fluorides **5** and **6** were stable for months at −20 °C.

EGCase glycosynthases E351S and E351S D^{-1}314Y catalysed smooth conversion of fluorides **5** and **6** to the corresponding β;-linked glycosides **7** and **8** (Scheme 2). Reactions were conducted using 0.8–1.1 eq. of sphingosine, with isolated yields of 45–86% despite difficulties associated with handling these detergent-like molecules. The hydrophobic aglycon permitted C18 SepPak purification.

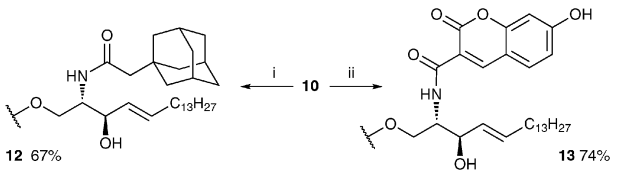
Linear assembly of natural or unnatural GSLs by stepwise GT catalysed addition of sugars to a lactosyl-sphingolipid is in some cases preferable to the convergent approach detailed above. Examples include instances where a complex mixture of inseparable glycosyl fluorides is obtained from GT-catalysed reactions, or where the glycosyl donor is of particular value. Glycosynthase catalysed synthesis of Lac-sphing proceeds in an overall yield of ~60%, taking into account the chemical synthesis of Lac-F, and we have shown that Lac-sphing is an acceptor for several relevant GTs. For instance as outlined in Scheme 3, *lyso*-G_{M3} **10** and related 9-azido analogue **11** could be prepared from Lac-F, sphingosine, cytidine-triphosphate, and the appropriate sialic acid in a three-step, three-enzyme process requiring only one SepPak purification. Crude Lac-sphing and the CMP-sialic acid were prepared with EGCase glycosynthase E351S and *Neisseria meningitidis* CMP-sialic acid synthetase respectively, and the crude products reacted in the presence of sialyltransferase Cst-I to afford the trisaccharide GSLs in 50–55% yield.[14,24,25]

Sphingosines, rather than ceramides, are the preferred acceptor alcohols in EGCase glycosynthase reactions owing to the solubility of the hydrochloride salt in aqueous buffer (~25 mM after sonication at 37 °C). While this necessitates an acylation step if glycosyl ceramides are ultimately desired, the approach offers a number of advantages including ease of handling, access to ceramides containing structurally diverse acyl chains, and an ability to generate unnatural GSLs.

Ceramides in naturally occurring GSLs display diversity among their *N*-acyl substituents, which dictate biological activity to some extent.[26] The acyl substituent has also been identified as a convenient position for conjugation of non-lipid substituents, including fluorescent tags.[27] Exemplifying this potential, acylation of one of the *lyso*-GSLs (**10**) synthesized with EGCase glycosynthase is shown in Scheme 4. Reaction with the activated ester of adamantane acetic acid or



Scheme 3 Three enzyme synthesis of *lyso*-G_{M3} and 9-N_3-*lyso*-G_{M3}.



Scheme 4 Acylation of *lyso*-G_{M3} **10**. (i) *N*-Succinimidyl adamantan-1-yl acetic acid, DMF, Et_3N. (ii) *N*-Succinimidyl 7-hydroxycoumarin-3-carboxylate, DMF, Et_3N.

7-hydroxy-coumarin-3-carboxylic acid afforded the corresponding amides **12** and **13** in reasonable yields.

The combined application of GTs and EGCase glycosynthase provides access to a broad range of GSLs. We have demonstrated that not only are simple GSLs excellent substrates for elaboration to more complex structures by glycosyltransferases, but that complex glycosyl fluorides assembled chemoenzymatically may themselves serve as acceptors in GT-catalysed reactions before undergoing transfer to sphingosine. *Lyso*-GSLs obtained using EGCase glycosynthase are water soluble at concentrations relevant to enzymatic synthesis, are readily purified on SepPak cartridges, and are amenable to acylation to form ceramides or analogues thereof. This approach allows for modification of the structures of both oligosaccharide and sphingolipid, reminiscent of the control available in chemical synthesis, yet it retains the advantages of efficiency inherent in enzymatic processes.

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