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A Diversity-Oriented Strategy for Chemoenzymatic Synthesis of Glycosphingolipids and Related Derivatives

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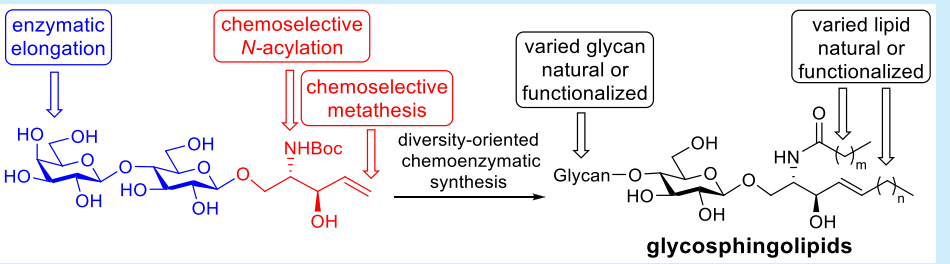

ABSTRACT: A diversity-oriented strategy combining enzymatic glycan assembly and on-site lipid remodeling via chemoselective cross-metathesis and *N*-acylation was developed for glycosphingolipid (GSL) synthesis starting from a common, simple glycoside. The strategy was verified with a series of natural GSLs and GSL derivatives and showed several advantages. Most notably, it enabled two-way diversification of the glycan and lipid, including introduction of designed molecular tags, to provide functionalized GSLs useful for biological studies and applications.

Glycosphingolipids (GSLs) are glycolipids consisting of a glycan and a ceramide moiety that is linked to the glycan reducing end via a β-glycosidic bond (Figure 1). GSLs are a

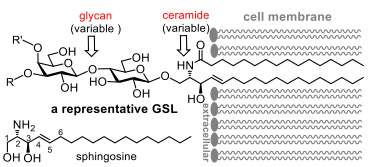

Figure 1. GSL attachment to the cell membrane.

major component of the cell membrane, having the lipid tail inserted into the lipid bilayer and the glycan exposed on the cell surface (Figure 1). Besides interacting with other biomolecules, GSLs can also self-assemble to create membrane microdomains.[1] These properties of GSLs have contributed to their functioning as key signaling molecules,[2] allowing them to play an essential role in many biological processes[3,4] and diseases such as cancer.[5]

Biological studies on GSLs require pure and structurally defined samples, including GSL derivatives containing different lipids, glycans, and tags. However, currently this is difficult to achieve. Isolation of GSLs from natural sources is not only tedious but gives mixtures, whereas total synthesis of GSLs, which is considered the only pragmatic means to access homogeneous GSLs and derivatives, remains challenging, despite its great progress.[6] This has hindered detailed studies and understanding of GSLs.

GSL total synthesis has several intrinsic issues associated with ceramide. For chemical synthesis, if ceramide is introduced early, its bulk can affect glycosylations. In contrast, if ceramide is introduced at a later stage, its glycosylation with large glycans can be difficult.[7−9] To address this dilemma, creative methods such as cassette synthesis[8] and use of macrocyclic acceptors[9,10] have been explored. For enzymatic synthesis,[11,12] if ceramide is introduced at the beginning, the substrate would be insoluble in water, hindering enzymatic reactions. However, if ceramide is introduced at the final stage, e.g., after the glycan is assembled, it would be a daunting task to couple the glycan and ceramide.[13−15] To address this issue, the Withers group[16−19] developed an elegant method based on on-site assembly of ceramide using engineered enzymes. Another issue about current methods is that they are target-oriented, i.e., they are designed for the synthesis of one GSL at a time. Thus, although they can give satisfactory results for specific GSLs, it is difficult to tailor them for the synthesis of a bunch of GSLs or GSL derivatives at once. Given the structural diversity of natural GSLs and derivatives, a facile and generally applicable method for rapid assembly of these biomolecules is

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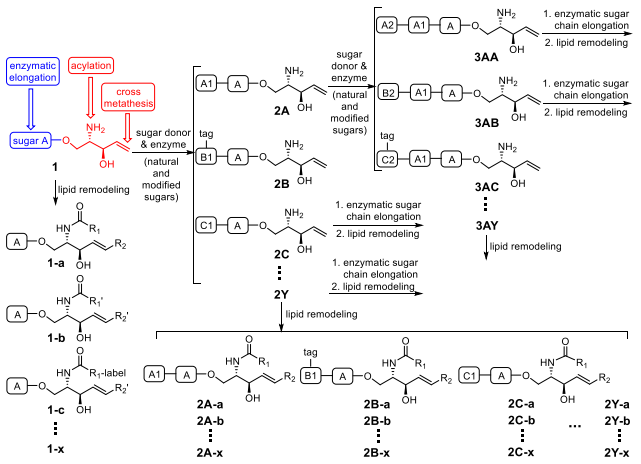
highly demanded. This work aimed to address the problem by developing a novel diversity-oriented synthetic method.

We envisioned preparing GSLs by enzymatic glycan assembly and chemical lipid remodeling as outlined in Scheme 1. The key intermediate is **1**, having a sphingosine headgroup

Scheme 1. Diversity-Oriented Chemoenzymatic Synthesis of GSLs

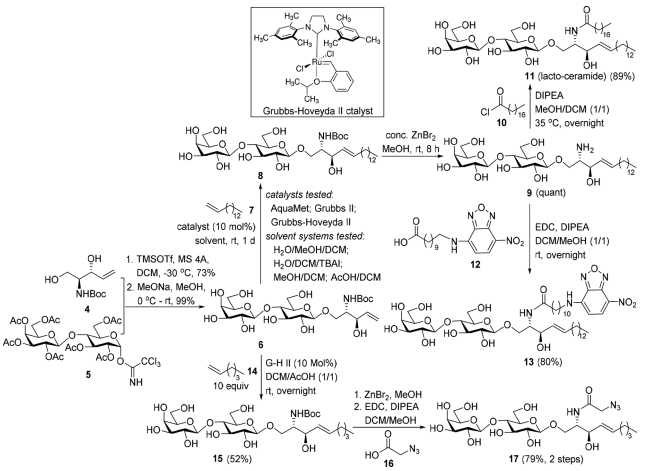


preinstalled on the GSL core, a mono/disaccharide. Glycoside **1** should be water-soluble for enzymatic glycosylations. Then, ceramide can be created on-site via chemoselective cross-metathesis[20] and *N*-acylation based on the sphingosine headgroup. In fact, lipid remodeling may be carried out after each enzymatic glycosylation using both natural and functionalized lipids, while either natural or modified sugars can be utilized for glycosylations as well, enabling two-way diversification and rapid access to a large variety of GSLs and derivatives. For instance, lipid remodeling of **1** using various olefins and fatty acids for cross-metathesis and *N*-acylation, respectively, would give GSLs **1-a**, **1-b**, and **1-x** carrying natural ceramide or its analogues, including those with molecular labels. Alternatively, the glycan in **1** can be elongated using natural/unnatural sugar donors and appropriate enzymes to obtain **2A**, **2B**, ..., and **2Y** containing natural or labeled sugar units. Thereafter, each of the products can be subjected to lipid remodeling by the same protocols to provide different series of natural and modified GSLs **2A-a** to **2A-x**, **2B-a** to **2B-x**, ..., and **2Y-a** to **2Y-x**. Similarly, the sugar chains in **2A**, **2B**, ..., and **2Y** can be enzymatically elongated further and the lipid in each glycosylation product can be remodeled to provide more complex GSLs and derivatives or analogues.

This synthetic strategy can have several advantages. First, **1** has only a simple lipid linked to a short mono- or disaccharide, and thus, it can be easily and efficiently prepared on a large scale by conventional methods. Second, the strategy can take advantage of enzymatic glycosylations, which are highly regio- and stereoselective and achieved with free sugar donors and acceptors.[21−23] Third, it should be easy to assemble diversified glycans by methods such as one-pot multienzyme (OPME) synthesis.[22] Fourth, since lipid remodeling occurs at the final synthetic stage, it should be easy to realize lipid diversification; moreover, since the reaction conditions for lipid remodeling are mild and compatible with various functionalities, it should be easy to include molecular labels in the lipid chain. Fifth, the strategy has almost completely gotten rid of protection−deprotection operations; furthermore, with optimized and standardizable protocols for enzymatic glycosylation and lipid remodeling, respectively, GSL synthesis by this strategy can be robust. Most notably, through diversification of the glycan and lipid in each step, it can provide an enormous array of GSLs and GSL derivatives rapidly.

To validate the strategy, we applied it to a series of GSLs. Since lactose is the core structure of most GSLs,[24] we started the synthetic studies with lactoside **6**, which was easily prepared on a gram scale in good yields after regio- and stereoselective glycosylation of **4** with lactosyl imidate **5** and then de-*O*-acetylation (Scheme 2). Its regio- and stereo-

Scheme 2. Synthesis of Lacto-ceramide and Its Analogues 13 and 17



chemistry was supported by the NMR data. It is noteworthy that the *tert*-butyloxycarbonyl (Boc) group in **6** was kept because it would help inhibit homodimerization of **6** during cross-metathesis.[25] In turn, **4** was prepared from L-serine by a reported method.[26]

We first studied remodeling of the lipid in **6** to find the optimal reactions/conditions for on-site ceramide construction in aqueous media. This was anticipated to be the most challenging step, as many reports have exhibited the feasibility of enzymatic assembly of glycans bearing a simple aglycon.[21−23,27,28] In contrast, no aqueous lipid modification via cross-metathesis[26] has been reported for GSLs. Thus, we examined the cross-metathesis conditions for **6** and 1-pentadecene (**7**) using AquaMet (Scheme 2). A mixture of water, methanol (MeOH), and dichloromethane (DCM) (1/3/3) was used as the solvent to ensure that both **6** and **7** were dissolved. However, the reaction gave the desired product **8** (J = 15 Hz, Z-only) in only a low yield (20%). Actually, **6** was not completely consumed even after prolonged time. Several factors, including dimerization of **7** and catalyst decomposition, might be responsible for the low conversion rate. To probe the issue, we repeated the reaction using excessive **7** (30 equiv) and 30 mol % AquaMet at a higher temperature, as it was reported that high temperature could enable internal alkenes formed from homodimerization to react.[25] Under the modified conditions, however, **7** dimerized more quickly, and the majority of **6** remained unreacted. As the different solubilities of **6** and **7** might have some impact on their reactivity, we examined different solvents composed of varied ratios of water, MeOH, and DCM, but all failed to give

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satisfactory yields even in the presence of the phase-transfer catalysts tetrabutylammonium iodide or TPGS-750-M.[29]

Another potential reason for the low conversion rate can be catalyst decomposition. Subsequently, we examined different catalysts. Grubbs II and Grubbs−Hoveyda II catalysts were found to provide improved yields. Especially, the latter showed good stability in protic solvents and gave the highest yields (ca. 50% with ∼70% conversion of **6**) in MeOH and DCM. On the basis of the properties of Grubbs catalysts,[30] we switched MeOH-based solvent systems to acetic acid (AcOH)-based ones (AcOH/DCM = 1/1 v/v), and the reaction gave further-improved yield (60%) with ca. 20% recovery of **6**.

After the conditions for metathesis were optimized, we studied the protocol to attach the *N*-acyl group. Considering that some glycosidic bonds in GSLs, such as that of sialic acid, are labile toward Bronsted acids, we tested Lewis acids for Boc group removal and found that the saturated methanol solution of zinc bromide gave almost quantitative yields without any impact on the glycan. Thereafter, crude **9** was applied to the next step without purification. Many methods are available for chemoselective *N*-acylation in aqueous media, but we decided to probe acyl chlorides first, as many fatty acid chlorides are commercially available. Moreover, acyl chlorides are relatively reactive, and thus, if they work, other acylation methods should work as well. The reaction of **9** with **10** in the presence of diisopropylethylamine (DIPEA) in MeOH and DCM went smoothly to afford the desired lacto-ceramide **11**, a natural GSL.

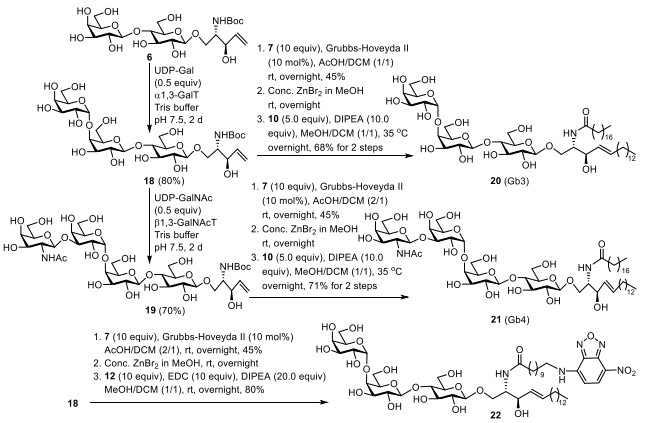
To demonstrate that the strategy can be used to prepare valuable labeled GSLs, we probed the incorporation of the common fluorophore nitrobenzoxadiazole (NBD) in lacto-ceramide (Scheme 2). The reaction of **9** with NBD-aliphatic acid **12** in the presence of 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDC) provided NBD-labeled lacto-ceramide **13** in a good yield (80%). It is noteworthy that the rotation of lipids in **13** may be impeded by NBD, as implicated by its broadened ^{1}H NMR signals, which may result in the unique membrane behaviors of NBD.[31]

The lipid of GSLs also accounts for a great portion of the structural diversity, which should be readily achievable by our synthetic strategy. As depicted in Scheme 2, cross-metathesis of **6** with 1-hexene (**14**) followed by removal of the *N*-Boc group in **15** and chemoselective *N*-acylation using azidoacetic acid and EDC produced lacto-ceramide analogue **17** containing an azido group and a short sphingosine. The azido group would allow further modification of the GSL analogue by click reaction.

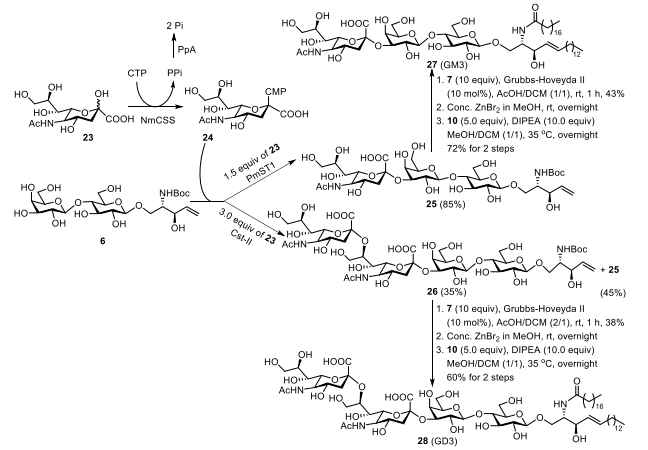
Next, we examined enzymatic glycan elongation. We chose Gb3 and Gb4, two neutral GSLs and cancer markers,[32,33] as our synthetic targets (Scheme 3). The reaction of **6** with UDP-Gal catalyzed by an α-1,4-galactosyltransferase[34] gave Gb3 trisaccharide **18** in a good yield. Then a GalNAc residue was attached to the new galactose 3-*O* position using UDP-GalNAc and a β-1,3-*N*-acetylgalactosaminyltransferase[35] to get Gb4 tetrasaccharide **19** in a 70% yield. Clearly, the lipid headgroup in **6** did not affect the enzymatic glycosylation. Finally, the lipid headgroup in **18** and **19** was remodeled by established protocols to obtain the target GSLs Gb3 (**20**) and Gb4 (**21**). Similarly, *N*-acylation of the cross-metathesis product of **18** with **12** provided fluorophore-labeled Gb3 analogue **22** in good overall yield (36%).

To show the application scopes of this strategy, we applied it to acidic GSLs GM3 and GD3 (Scheme 4), which are also

Scheme 3. Synthesis of Gb3, Gb4, and Gb3 Analogue 22



Scheme 4. Synthesis of GM3 and GD3



recognized cancer markers.[36,37] We selected them because they contain the most acid-labile sialyl linkages. Thus, if the strategy works for them, it should work for all GSLs. The addition of sialic acid residues to **6** was carried out by the OPME method[22] using CMP-sialic acid formed in situ as the sialyl donor. In the synthesis of GM3 trisaccharide **25**, sialylation was realized with PmST1, a sialyltransferase that catalyzes the formation of sialyl α-(2→3)-Gal linkages.[38] Again, the enzymatic reaction was smooth, and **25** was obtained in an impressive 85% yield. The stereochemistry of the new α-sialyl residue was confirmed by the chemical shifts of its H-3$_{ax}$ signal at 1.73 ppm and H-3$_{eq}$ signal at 2.86 ppm in the ^{1}H NMR spectrum. In the synthesis of GD3 tetrasaccharide **26**, Cst-II, a bifunctional α-2,3/α-2,8-sialyltransferase from *Campylobacter jejuni*, was utilized for double sialylations of **6**. Thus, excessive sialic acid and cytidine triphosphate (CTP) (3 equiv each) were used to generate CMP-sialic acid. The desired GD3 tetrasaccharide **26** (slight impurities were caused by decomposition of the sensitive sialyl-2,8-linkage in the NMR tube) was isolated together with trisaccharide **25** in 35% and 45% yield, respectively.[27] Lipid remodeling of **25** and **26** as described above furnished GM3 (**27**) and GD3 (**28**), respectively. However, for cross-metathesis performed in acetic acid, the reaction time was cut to an hour to avoid potential hydrolysis of the sialic acid residue(s). Although the reaction yield was slightly compromised, this precaution was deemed worthwhile because unreacted starting materials were recovered and product purification was kept simple.

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In brief, a new strategy combining enzymatic glycan assembly with chemoselective lipid remodeling was developed for GSL synthesis, which may have several advantages. Importantly, it can overcome the intrinsic problems associated with chemical or enzymatic GSL synthesis alone. It can also help minimize sugar protection/deprotection steps to significantly shorten and accelerate the synthesis. Thus, a series of natural GSLs and their derivatives were prepared from **6** in only three to five steps. Furthermore, in contrast to target-oriented syntheses, this strategy also facilitates the rapid synthesis of various natural and modified GSLs via two-way diversification of the glycan and lipid. This strategy, as well as its protocols, has shown wide applicability. Thus, we anticipated it to be readily adopted for other GSLs and various functionalized GSL derivatives, both of which are very powerful biological tools.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c02847.

Experimental procedures and 1D and 2D NMR spectra of all new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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