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A General Chemoenzymatic Strategy for the Synthesis of Glycosphingolipids

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Abstract

A concise, prototypical, and stereoselective strategy for the synthesis of therapeutically and immunologically significant glycosphingolipids has been developed. This strategy provides a universal platform for glycosphingolipid synthesis by block coupling of enzymatically prepared free oligosaccharideglycans to lipids using glycosyl *N*-phenyltrifluoroacetimidates as efficient activated intermediates. As demonstrated here, two different types of glycosphingolipids were obtained in excellent yields using the method.

Graphical Abstract

Firstly, three oligosaccharideswere prepared by one-pot multi-enzyme system (OPME). Subsequently, the oligosaccharides were easily converted to *N*-phenyltrifluoroacetimidate donors using benzoyl as protecting group, followed by block coupling of enzymatically prepared sugar parts to lipid acceptors with TMSOTf as a promotor to produce corresponding GSLs.

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Supporting Information: Experimental procedure for enzymatic synthesis of oligosaccharides, synthesis of glycosphingolipid procedure, characterization data, and NMR spectra of all new obtained compounds.

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Keywords

chemoenzymatic; glycosphingolipids; enzymatic synthesis; glycosylation; *N*-phenyltrifluoroacetimidate

Glycosphingolipids (GSLs) are sugar-containing lipids that represent a major class of glycoconjugates biosynthesized by eukaryotes. The structural variation of the lipid parts and especially the diversity of the carbohydrate components lead to an enormously large depository of natural GSLs.[1] These biomacromolecules play numerous crucial functions related to human physiology and pathology,[2] such as protein sorting, signal transduction, membrane trafficking, viral and bacterial infection, as well as cell-cell communications.[3] Due to the increasing recognition of their vital roles and their potential biomedical applications, naturally occurring GSLs are attractive targets as cancer biomarkers and vaccines.[4] Despite numerous reports on preparing GSLs including chemical synthesis and isolation from natural sources,[5] the absence of an efficient method to access diverse GSLs in large scale and in homogenous form hampers the studies in developing GSL-based vaccines and therapeutics.

In nature, the assembly of most GSLs starts from the formation of glucosylceramide catalyzed by glucosylceramide synthase by transferring a monosaccharide, glucose (Glc), to a ceramide and this core is extended to reach complex glycans by using various glycosyltransferases (Scheme 1). Such an approach is difficult to reproduce in the flask owing to the low solubility of the ceramide.

Chemical synthetic approaches that have been developed are primarily based on building the complex glycan portion of the GSLs by chemical synthesis followed by coupling to the azido-sphingosine which can be further modified to ceramide by successive reduction and amidation. These synthetic methodologies usually encompass extensive selective protection and deprotection steps for the preparation of individual sugar donors/acceptors. Nevertheless, the main challenge resides in the regio- and stereoselective transferring glycosyl donors onto lipids which usually results in low yield production of GSLs. Withers and co-workers cleverly developed an chemoenzymatic strategy to overcome some of the drawbacks in chemical synthesis of GSLs. The strategy uses mutants of an endoglycoceramidase (which are called endoglyceramidase glycan synthases) and chemoenzymatically prepared α-glycosyl fluorides[2a, 6] for coupling the glycan and the sphingosine components. Nevertheless, the α-glycosyl fluorides have to be enzymatically synthesized from chemically synthesized α-lactosyl fluoride. Substrates are restricted to those that can be tolerated by the endoglycoceramidase synthases developed. Herein, we describe a general, highly expeditious, and scalable chemoenzymatic strategy for rapid accessing GSLs (Scheme 1). As chemical coupling is applied, it has less restriction to the structures of glycans and lipids that can be used compared to the endoglycoceramidase synthase-catalyzed reactions.

Recent advances in the identification and characterization of an increasing number of highly effective and stable glycosyltransferases and other carbohydrate biosynthetic enzymes in large amounts[7] have a profound effect on synthetic carbohydrate chemistry by allowing the

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access to a diverse array of oligosaccharides and glycoconjugates via enzymatic and chemoenzymatic synthesis. Our groups have been actively focused on identifying enzymes and developing highly effective one-pot multienzyme (OPME) glycosylation systems for the preparation of a large array of complex glycans including blood group antigens[8] and sialosides.[9] On the other hand, versatile routes for the synthesis of various lipid portions of GSLs in large scales have been well documented.[10] Therefore, a chemoenzymatic approach that combines the highly efficient enzymatic synthesis of glycans and a subsequent universal chemical block coupling strategy could dramatically shorten current routes for preparing GSLs and provide invaluable avenues for large-scale synthesis of these therapeutically valuable compounds. Here we report such a vital effort and success on synthesizing two different types of glycosphingolipids including blood group antigens and gangliosides.

To obtain human blood group H and A antigens in preparative scales, an efficient sequential one-pot multienzyme (OPME) strategy was developed to enzymatically extend lactose with an α1–2-linked fucose at the terminal galactose (Gal) residue in lactose by OPME1 followed by further addition of the Gal with an α1–3-linked *N*-acetylgalactosamine (GalNAc) by OPME2 (Scheme 2). In OPME1 system, a recombinant bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (FKP)[11] was used to generate guanosine 5′-diphosphate fucose (GDP-fucose) starting from fucose, adenosine 5′-trisphosphate (ATP), and guanidine 5′-triphosphate (GTP) to provide the donor substrate for *Helicobacter mustelae* α1–2-fucosyltranferase (Hmα1,2FT)[12] for the produce blood group H-antigen trisaccharide **1** (1.07 g, 2.19 mmol, 73%). *Pasteurella multocida* inorganic pyrophosphatase (PmPpA)[13] was used to drive the reaction forward by digesting the by-product pyrophosphate. containing and *H. mustelae* α1–3-GalNAc transferase (BgtA)[8b] was developed for the synthesis of In this system, Subsequently, in OPME2 system, *H. mustelae* α1–3-GalNAc transferase (BgtA) catalyzed the formation of Blood group A-antigen tetrasaccharide **2** (633 mg, 0.92 mmol, 92%) using UDP-GalNAc as the donor substrate which was generated from GalNAc, ATP and uridine 5′-triphosphate (UTP) by recombinant *Bifidobacterium infantis* *N*-acetylhexosamine-1-kinase (NahK)[14] and *Homo sapiens* UDP-GalNAc pyrophosphorylase (AGX1).[15]

As an example of ganglioside oligosaccharides, sialic acid-containing GM3 trisaccharide **3** (1.08 g, 1.71 mmol, 85%) was synthesized in gram scale from lactose using a one-pot two-enzyme sialylation system[7b, 16] containing *Neisseria meningitides* CMP-Sia synthetase (NmCSS)[17] and *Pasteurella multocida* sialyltransferase 1 M144D mutant (PmST1_M144D)[18] (Scheme 3).

With a considerable amount of oligosaccharides in hand, the next key step was to convert them to efficient glycosyl donors in a limited number of synthetic steps which can yield β-linked glycosphingosine analogs by forming a 1,2-trans-glycosidic bond. As previously reported, potentially attractive glycosyl donors could be glycosyl trichloroacetimidate,[19] glycal,[20] thioglycoside,[21] glycosyl halides[22] and *N*-phenyltrifluoroacetimidates[23] which can be prepared within few synthetic steps from the corresponding oligosaccharides. Based on our previous successes in synthesizing a series of GSLs,[24] it was obvious that Schmidt's glycosyl trichloroacetimidate with neighbouring group participation and 2-azido-sphingosine shown in Table 1 could be ideal building blocks for glycosylation.

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Initially, the blood group H-antigen trisaccharide **1** was used as a model to develop a robust and high-yielding synthetic route for the production of GSLs, for which both a high degree of stereoselectivity in the glycosylation reactions and minimal protecting group manipulations are of importance. Peracetylated glycosyl trichloroacetimidate was our first choice for a glycosyl donor for this case due to its relative preparative simplicity and easy removal. As shown in Scheme 4, trisaccharide **1** was treated with acetic anhydride in the presence of catalytic 4-dimethylaminopyridine (DMAP) to afford peracetylated trisaccharide. Selective deprotection of the anomeric acetyl group was achieved by reaction with benzylamine in tetrahydrofuran (THF). The resulting intermediate was then treated with trichloroacetonitrile in the presence of 1,8-diazabicycloundec-7-ene (DBU) to provide the desired trichloroacetimidate donor **4a** (75% yield over three steps).

Subsequently, high yielding reaction conditions for the block coupling of glycosyl donor to the azido-sphingosine employing different Lewis acid promoters were investigated. Under the glycosylation reaction conditions (Table 1) using glycosyl donor **4a** and acceptor **5a**[25] in the presence of boron trifluoride diethyl etherate as a promoter resulted in exclusive formation of glycoconjugate **6a** with β-configuration with an undesirable low yield (30%) (Table 1, entry 1). The low yield of the reaction was mainly due to the problem of acyl migration. In the context of increasing the yield of the reaction, TMSOTf was examined as an alternative promoter in the synthesis of **6a**. In this case, glycoside **6a** was obtained with a slight increase in yield (35%) (Table 1, entry 2) as the acyl migration persisted. To overcome the problem of acyl migration, we switched acetyl protecting group to benzoyl group. Reaction of perbenzoylated glycosyl trichloroacetimidate donor **4b** (prepared from **1** in 70% yield over three steps) and **5a** (acceptor) with TMSOTf as a promoter produced glycoside **6b** in low yield (40%) (Table 1 entry 3). The low yield was due to the formation of the corresponding amide by-product under the reaction conditions.[26] To minimize the unwanted side reactions, glycosylation was performed by an inverse addition strategy.[25] Interestingly, a noticeable increase in the yield from 40% to 62% for synthesizing **6b** was achieved (Table 1, entry 4). To further improve the coupling yield, *N*-phenyltrifluoroacetimidate **4c** was tested as a donor due to its accessibility, stability, and reactivity.[26b, 27] Here, *N*-phenyltrifluoroacetimidate **4c** was prepared from **1** in an excellent yield (78% for three steps), involving the following procedures: (1) perbenzoylation, (2) selective deprotection of the anomeric benzoyl group, and (3) introduction of the *N*-phenyltrifluoroacetimidate leaving group. Engaging *N*-phenyltrifluoroacetimidate **4c** as the glycosyl donor and **5a** as the acceptor in the presence of TMSOTf as a catalyst with inverse addition provided glycoside **6b**[14a] with a remarkable increase in yield (88%) (Table 1, entry 5). The superior reactivity of *N*-phenyltrifluoroacetimidates over the trichloroacetimidates was verified to be crucial to the increase of yield. On the other hand, exchanging *O*-benzoyl to *O*-PMB in azido-sphingosine **5b** produced **6c** with an insignificant change in the yield (90%) (Table 1, entry 6). Therefore, the use of *N*-phenyltrifluroacetimidate and PMB-protected azido-sphingosine along with catalytic TMSOTf with inverse addition glycosylation was found to be the ideal condition for high yield synthesis of GSL precursors. However, considering the number of synthetic steps accompanied by overall yield, 2-azido-3-*O*-benzoyl sphingosine **5a** was chosen as the glycosyl acceptor for the synthesis of desired GSLs.

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Scheme 5 outlines the final chemical steps required for the synthesis of blood group H GSL **8** from **6b**. The azide group was reduced to an amine using Staudinger reaction conditions, and the crude amine was N-acylated with hexacosanoic acid in the presence of DIPEA, EDC·HCl, and HOBt to provide amide **7** in an excellent yield. Finally, global deprotection of benzoyl protective groups using NaOMe/MeOH readily yielded the target GSL **8** (26mg, 0.02 mmol) in a quantitative yield[24] from **7**.

To confirm the generality of the block coupling methodin the synthesis of GSLs from the enzymatically synthesized complex sugars, two additional GSLs belonging to different types were synthesized. These include blood group antigen A derived GSL **11** and sialic acid-containing GM3 ganglioside **15** (Scheme 6). The enzymatically synthesized blood group A antigen tetrasaccharide **2** was converted to glycosyl *N*-phenyltrifluoroacetimidate donor **9** and block coupled to the azido-sphingosine with a yield of 67% over four steps using the similar chemical approach employed for the synthesis of **6b**. This glycoconjugate was converted to GSL **11** (25 mg, 0.02 mmol) by reduction, N-acylation followed by global deprotection with a yield of 87% over three steps (Scheme 6). For the synthesis of sialic acid-containing ganglioside GM3 **15** from **3**, the carboxyl group in the sialic acid was protected as benzyl ester **12** as our effort in protecting it as a methyl ester failed under the acidic conditions owing to the sensitive sialyl linkage. Sialyl glycan derivative **12** was then converted to glycosyl *N*-phenyltrifluoroacetimidate donor **13** and block coupled to azido-sphingosine to form **14** (60% over five steps). Reduction, *N*-acylation, and global deprotection leading to the formation of desired GM3 ganglioside **15** (26 mg, 0.02 mmol) were carried out by following the similar procedure in obtaining GSLs **8** and **11** (72% over three steps).

In summary, we have developed a general chemoenzymatic approach for convenient and efficient synthesis of GSLs with excellent yields, in which enzymatic synthesis of oligosaccharides and chemical block coupling were combined to overcome drawbacks of total chemical synthesis and enzymatic synthesis. This practical procedure was applied successfully to the synthesis of GSLs containing human blood group antigens, and sialic acid-containing GM3. Synthesis efforts of other GSLs that are of therapeutic potential are ongoing. This work provides a practical strategy using oligosaccharides which become increasingly readily available from enzymatic synthesis to generate GSLs library efficiently. The method described here can find broad application in developing glycosphingolipid-based cancer vaccine and drug development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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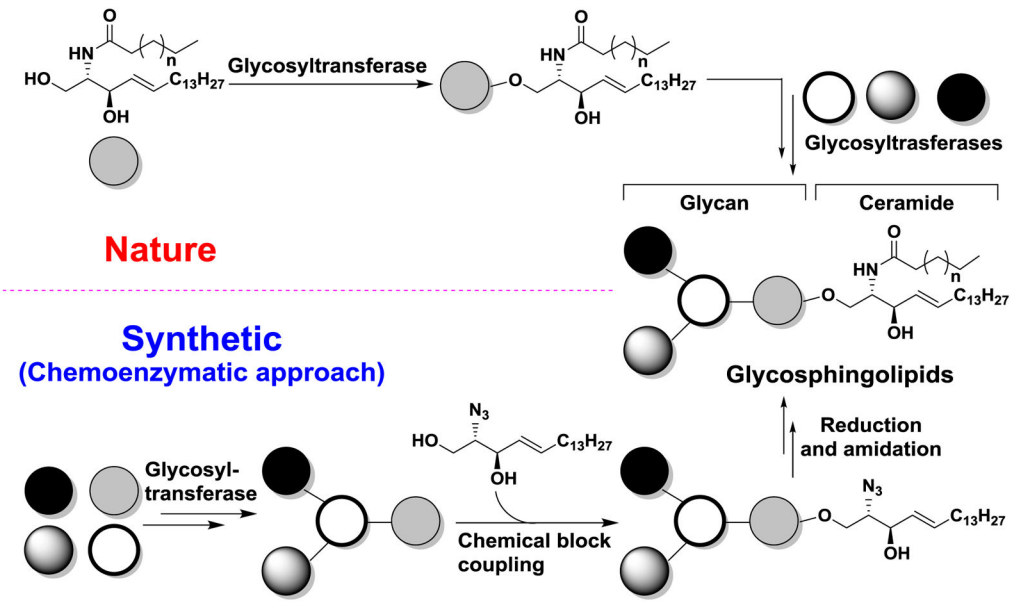

Scheme 1.

Schematic illustration of the synthesis of glycosphingolipids (GSLs) by the Nature and the chemoenzymatic synthetic route developed here.

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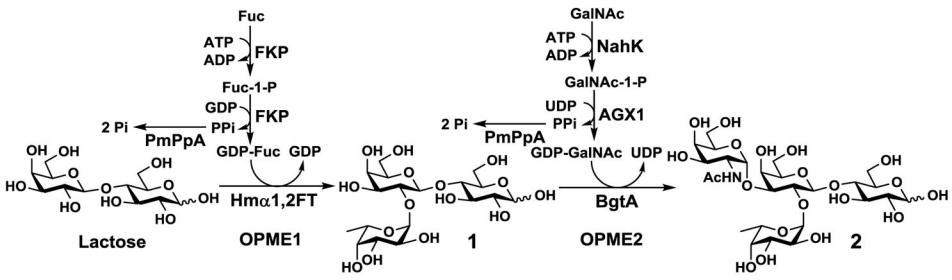

Scheme 2.

Synthesis of blood group H antigen trisaccharide **1** and blood group A antigen tetrasaccharide **2** using a sequential one-pot multienzyme (OMPE) strategy. Enzyme abbreviations: Hmα1,2FT,*Helicobacter mustelae* α1–2-fucosyltranferase; FKP, bifunctional fucokinase and GDP-fucose pyrophosphorylase; PmPpA, *Pasteurella multocida* inorganic pyrophosphatase; BgtA, *Helicobacter mustelae* α1–3-GalNAc transferase; NahK, *Biofidobacterium infantis N*-acetylhexosamine-1-kinase.

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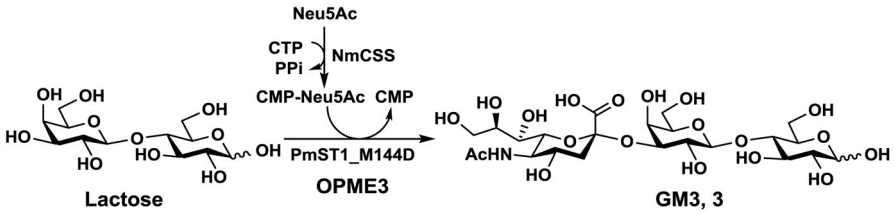

Scheme 3.

Synthesis of GM3 trisaccharide **3** using a one-pot two enzyme sialylation (OPME3) strategy. Enzyme abbreviations: NmCSS, *Neisseria meningitides* CMP-sialic acid synthetase; PmST1_M144D, *P. multocida* sialyltransferase 1 M144D mutant.

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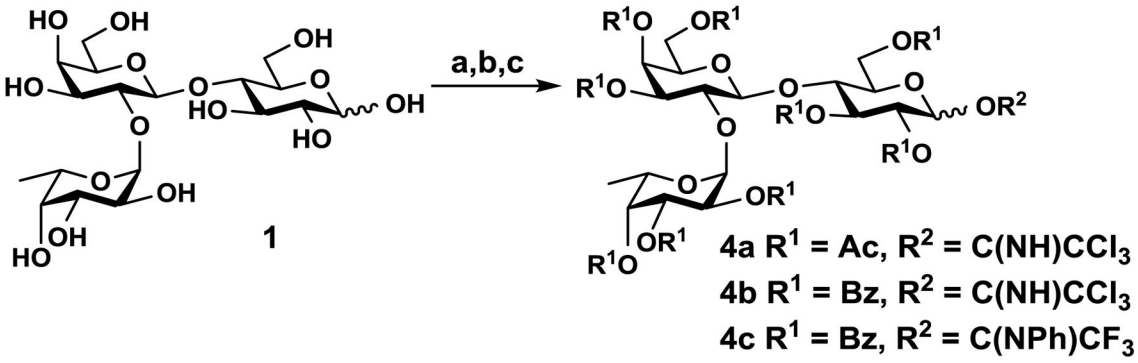

Scheme 4.

Synthesis of different glycosyl donors (**4a**–**4c**). Reagents and conditions: (a) Ac_2O, Py, DMAP for **4a**; BzCl, Py, DMAP for **4b** and **4c**; (b) $BnNH_2$, THF for **4a**; or hydrazine acetate, DMF for **4b** and **4c**; (c) $CNCCl_3$, DBU, DCM for **4a** (75% over three steps)and **4b** (70% over three steps); $ClC(NPh)CF_3$, DBU, DCM for **4c** (78% over three steps).

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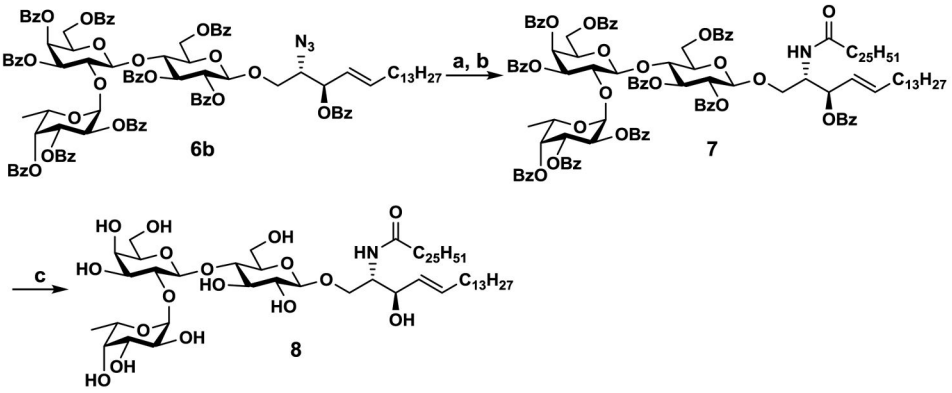
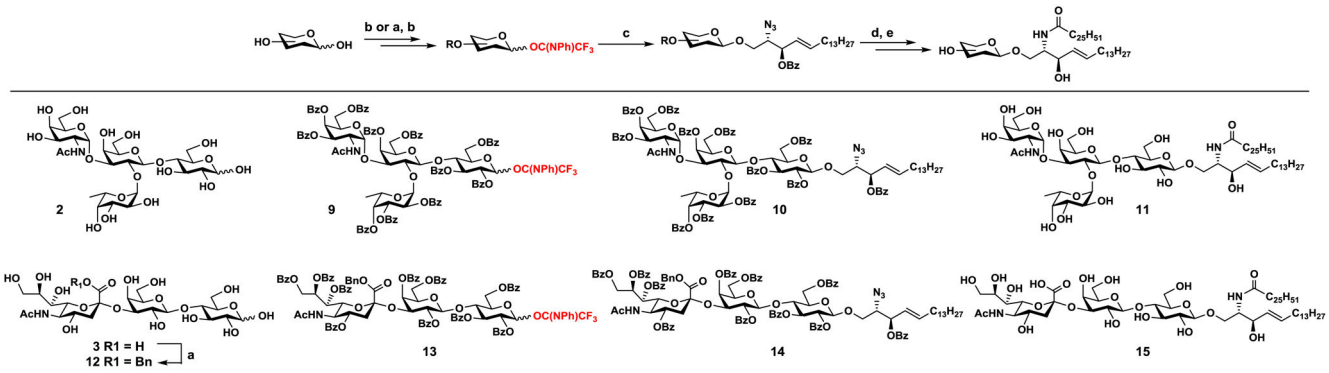


Scheme 5.

Final steps in the synthesis of GSL **8.** Reagents and conditions: (a) PPh_3, PhMe, H_2O, 50 °C; (b) $C_{25}H_{51}COOH$, EDCI, HOBt, DIPEA, 75% (two steps); (c) NaOMe, MeOH, quantitative.



Scheme 6.

A general approach for synthezing glycosphingolipids. Reagents and conditions: (a) BnBr, DMF; (b) i: BzCl, Py; ii: $AcOH{\cdot}NH_2NH_2$, DMF; ii: $ClC(NPh)CF_3$, DBU, DCM, 78% for **9** (three steps), 70% for **13** (four steps); (c) **5a**, TMSOTf, DCM, −20 °C, 86% for **10**, 85% for **14**; (d) i: PPh_3, PhMe, H_2O, 50 °C; ii: $C_{25}H_{51}COOH$, EDCI, HOBt, DIPEA, 73% for **10** (two steps), 72% for **14** (two steps); (e) (i): NaOMe, MeOH, quantitative for **11**; (ii): NaOMe, MeOH, then H_2O, quantitive for **15**.

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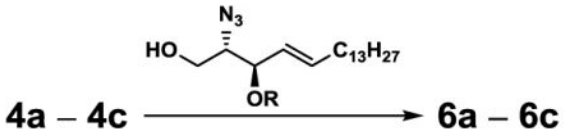
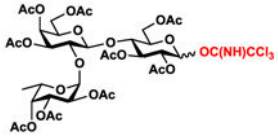
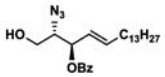
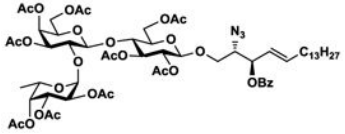
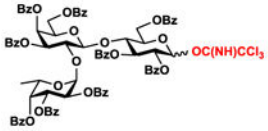
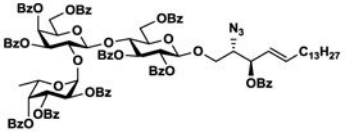
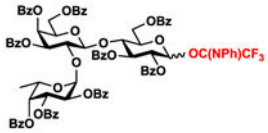
Table 1

Optimization of block coupling conditions using different glycosyl donors, azido-sphingosine acceptors, and Lewis acids. *Inverse addition.



5a R = OBz; 5b R = OPMB

Entry	Donors	Acceptors	Catalysts	Products	Yield (%)
1	 **4a**	 **5a**	$BF_3{\cdot}OEt_2$	 **6a**	30
2	**4a**	**5a**	TMSOTf	**6a**	35
3	 **4b**	**5a**	TMSOTf	 **6b**	40
4	**4b**	**5a**	TMSOTf	**6b**	62*
5	 **4c**	**5a**	TMSOTf	**6b**	88*

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Entry	Donors	Acceptors	Catalysts	Products	Yield (%)
6	4c	5b	TMSOTf	6c	90*