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Synthesis of α-galactosyl ceramide and the related glycolipids for evaluation of their activities on mouse splenocytes

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Abstract—Phytosphingosine and its short-chain analog were efficiently synthesized with 19% overall yield in 10 steps, respectively, starting from an inexpensive D-lyxose. Galactosyl donors of sulfide and phosphite types bearing benzoyl protecting groups of 4- and 6-OH underwent glycosylation in excellent α-anomeric selectivity. A variety of α-galactosyl, fucosyl and glucosyl ceramides and serine-type lipids were prepared, and their activities involved in the proliferation of mouse splenocytes and the expression of cytokines were elucidated. Besides α-galactosyl ceramides, a galactosyl serine-type lipid also exhibited substantial effect on the expression of cytokines IFN-γ and IL-4.

1. Introduction

CD1 molecules are β2-microglobulin-associated proteins that are related to MHC I and II molecules.[1] Four CD1 isoforms, CD1a, CD1b, CD1c, and CD1d, have been found in humans.[2] Only human CD1d is homologous to mouse and rat CD1 molecules.[3] This protein is normally expressed by thymocytes and various cells with antigen-presenting functions, such as B cells and dendritic cells. The primary function of CD1 proteins is to present glycolipid antigens through lipid–protein interactions with receptors on T-cells, and thus activating the immune system.[4] The analysis of amino acid sequences reveals that CD1 molecules have highly hydrophobic antigen-binding grooves,[1] The X-ray diffraction analysis further indicates that the crystal structure of mouse CD1d has a hydrophobic antigen-presenting groove with two large pockets, which can probably accommodate the lipid tails of antigens.[5]

In 1993, six species of bioactive glycolipids having α-galactosylceramide structures were isolated from the marine sponge *Agelas mauritianus*.[6] Years later, KRN7000 (also called α-GalCer for common use) was chosen from the derivatives of these structures as a candidate for clinical applications.[7] The α-GalCer can be recognized by an entire population of mouse and human CD1d-restricted lymphocytes.[8] An unusual feature of α-GalCer is the α anomeric linkage of galactose to the lipid, unlike the ubiquitous β-glycosidic bond in nearly all known natural glycosphingolipids of normal mammalian cells. α-GalCer stimulates the fast release of large amounts of cytokines from most mouse NKT cells,[8] which are characterized by expression of an invariant Vα14 TCR.[9] Interaction of α-GalCer with CD1 receptors causes T cells to secrete primarily interferon γ (IFN-γ) and interleukin-4 (IL-4) resulting in TH1 and TH2 immune responses, respectively.[10] This activation raises the prospect of novel, lipid-based vaccines and adjuvants.[11] An analogue of α-GalCer, with a truncated sphingosine chain, was recently shown to induce the production predominantly of IL-4 by NKT cells.[12] Modification of the lipid chain in the α-GalCer structure likely causes immunoactivity switching to demonstrate a profound relationship between structure and activity.[13]

In continuation of our efforts on the development of glycolipids as vaccine adjuvants, we are particularly interested in the study of α-GalCer and its analogs. Although a few syntheses of α-GalCer have been described,[7,14] most of previous methods require extensive synthetic steps and the use of expensive starting material for

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synthesizing phytosphingosine,[15] the lipid moiety of α-GalCer. In this article, we report an efficient route for constructing phytosphingosine from commercially available D-lyxose, and several methods for synthesizing α-GalCer and its analogs in stereoselective manners.

2. Result and discussion

Figure 1 shows a retrosynthetic pathway of α-GalCer **1**. The azido group in synthon **3** would be reduced to amine, and the obtained galactosyl phytosphingosine can be coupled with appropriate fatty acids to give **1** and its analogues, e.g. the short-chain derivative **2**. Phytosphingosine **8** in the protected form is a key intermediate that can be obtained by Wittig olefination with the D-lyxose derivative **11**, followed by replacement of the C4 hydroxyl group by an azido group. Execution of this synthetic plan is shown in Scheme 1. The 2,3-dihydroxy groups of D-lyxose were selectively protected as an acetal using 2,2-dimethoxypropane,[16] and the primary hydroxyl group was subsequently protected as a trityl ether,[17] giving **11** in 71% yield. Wittig olefination of **11** using $Ph_3PC_{13}H_{27}Br$ or $Ph_3PC_4H_9Br$ in the presence of lithium hexamethyldisilazide (LHMDS)[18] yielded alkenes **12** (93% yield) and **13** (85% yield). The *E*/*Z* ratio of **12** was estimated to be 2:1 and 3:1 for **13** according to the ^1H NMR spectral analysis. Saturation of double bonds in **12** and **13** by catalytic hydrogenation afforded **14** and **15**, respectively, in 91 and 88% yields. The hydroxy group in **14** (or **15**) was activated as a triflate, which underwent an S_N2 reaction with tetramethylguanidinium azide (TMGA) to give azido compound **16** (or **17**) with inverted configuration.[19] As attempts of selective removal of the trityl group in **16** (or **17**) failed, simultaneous deprotection[15b] of the acetal and trityl groups were carried out by treating with acetic acid in MeOH at 60 °C to yield triol **18** (or **19**). The sphingosine derivatives **20** and **21** suitable to glycosylation were thus prepared from **18** and **19** by a sequence of tritylation, benzylation and de-tritylation. The whole synthetic process took 10 steps to convert D-lyxose into phytosphingosines (**20** and **21**) in 19% overall yield.

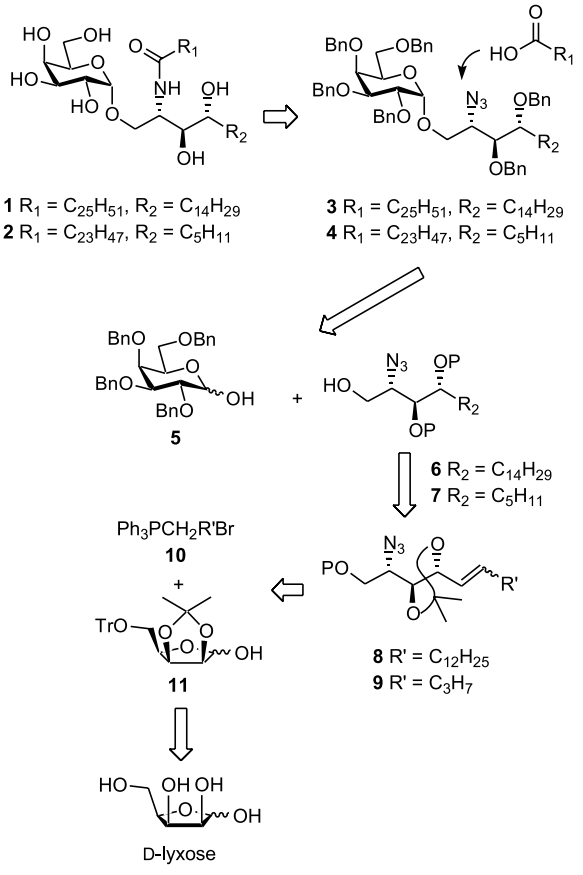
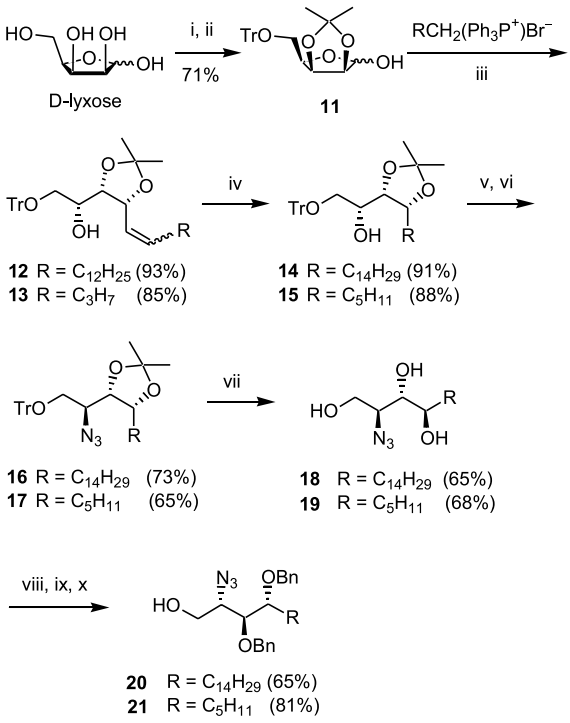
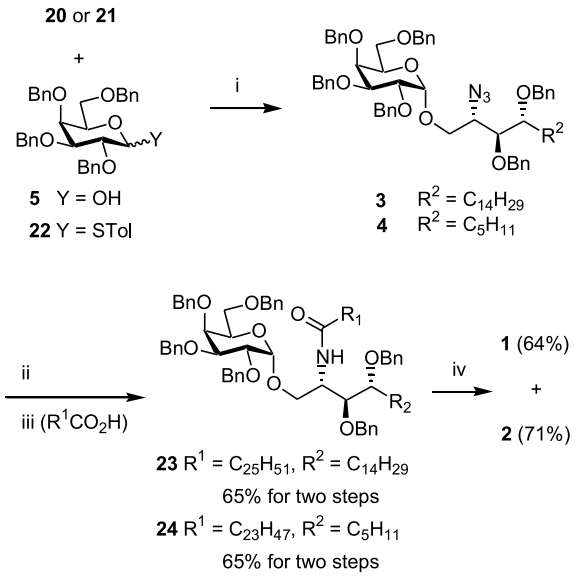


Figure 1. *Retro*-synthesis of α-GalCer (**1**) and a short-chain analogue **2**.



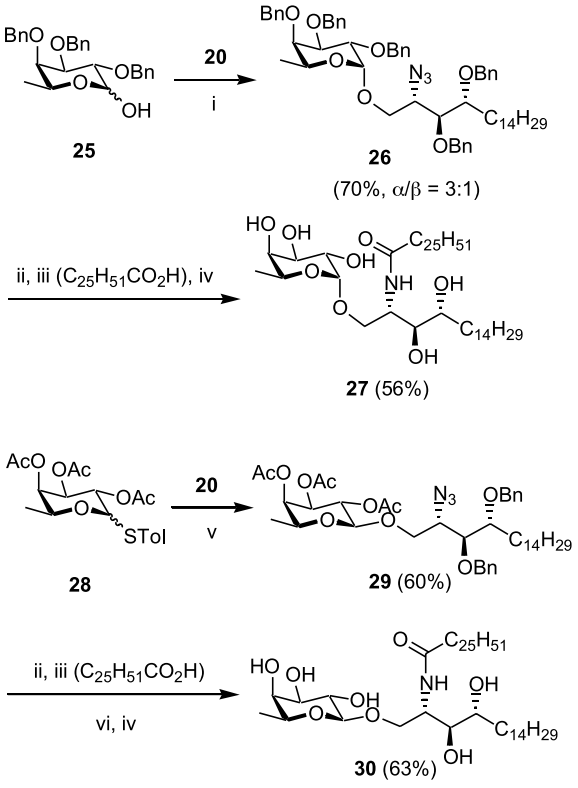
Scheme 1. Synthesis of phytosphingosind derivatives **20** and **21**. Reagents and conditions: (i) 2-methoxypropene, CSA. (ii) TrCl, pyridine, 80 °C, 6 h. (iii) LHMDS, THF. (iv) H_2, $Pd(OH)_2$, EtOAc. (v) Tf_2O, 2,6-lutidine, CH_2Cl_2. (vi) Tetramethylguanidinium azide. (vii) AcOH, MeOH, 60 °C. (viii) TrCl, pyridine. (ix) BnBr, NaH, DMF. (x) AcOH, H_2O.

The benzyl groups were adopted as the protecting groups in both glycosyl acceptors (e.g. **20** and **21**) and donors (e.g. **5** and **22**) because catalytic hydrogenation can be applied for complete deprotection in the final step to obtain α-GalCer **1** and its truncated phytosphingosine analogue **2** (Scheme 2). Coupling of phtytosphingosine **20** with galactosyl donor **22** (*S*-glycoside, R=STol) by using *N*-iodosuccinimide (NIS) and trifluoromethanesulfonic acid (TfOH) as promoters[20] afforded **3** in 93% yield, albeit in no anomeric selectivity (α/β=1:1). When tetrabenzyl galactose **5** (R=OH) was used in dehydrative glycosylation[21] with **20**, 83% yield of **3** was obtained in a better α-selectivity (α/β=3:1). Compound **4** was similarly obtained in α/β=3:1 by coupling compound **21** with **5**. In principle, the azido and benzyloxyl group can be reduced concurrently. However, reductions by using Raney Ni, Birch reduction and hydrogenation using different catalysts [e.g. Pd/C and $Pd(OH)_2$] and various solvents (e.g. EtOAc, MeOH, EtOH and HOAc etc.) under a hydrogen pressure of 1 atm or 50 kg/cm^2 resulted in complicated mixture. Thus, azide **3**



Scheme 2. Synthesis of α-GalCer (**1**) and a short-chain analogue **2**. Reagents and conditions: (i) using **5**: Me_2S, 2-Cl-pyridine, Tf_2O, CH_2Cl_2, 4 Å MS; using **22**: NIS, TfOH, CH_2Cl_2, 4 Å MS. (ii) Ph_3P, pyr., H_2O, 60 °C, 6 h. (iii) R^1CO_2H, EDC, HOBt, 20 h. (iv) H_2, Pd/C, 40 kg/cm^2.

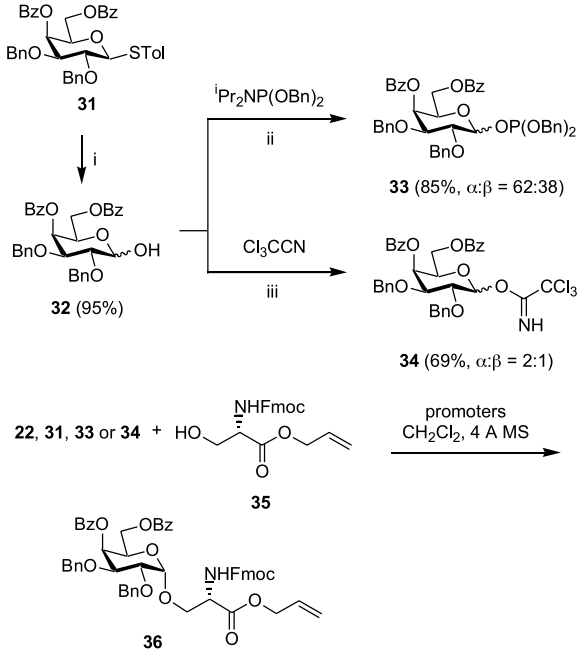
was reduced using Staudinger reaction[22] to give the amine intermediate, which coupled with hexacosanoic acid by using EDC and HOBT as the promoters to give compound **23** in 65% yield. The similar reduction of **4** and the subsequent coupling reaction with tetracosanoic acid yielded **24**. Finally, removal of the benzyl groups culminated in α-GalCer **1** and the short-chain analogue **2**.



Scheme 3. Synthesis of α- and β-fucosyl ceramides **27** and **30**. Reagents and conditions: (i) Me_2S, 2-Cl-pyridine, Tf_2O, CH_2Cl_2, 4 Å MS. (ii) Ph_3P, pyr., H_2O. (iii) PyBop, CH_2Cl_2. (iv) H_2, Pd/C, 40 kg/cm^2. (v) NIS, TfOH, 4 Å MS, CH_2Cl_2, −15 °C. (vi) NaOMe, MeOH.

Fucosyl ceramide does not occur in nature, so the synthesis and examination of the bioactivities of L-fucosyl ceramides (FucCers) **27** and **30** are of interest. Dehydrative glycosylation of phytosphingosine **20** with tribenzylfucose **25**[23] was performed to give an anomeric mixture of **26** (α/β=3:1), whereas the coupling reaction with *S*-tolyl triacetylfucoside **28** afforded only β-anomer **29** (Scheme 3). By the procedure similar to that for the synthesis of α-GalCer **1**, the α-FucCer **27** was synthesized from **26** in 56% overall yield by a sequence of azide reduction, amide formation and debenzylation. β-FucCer **30** was similarly prepared except for removal of the acetyl groups by sodium methoxide in methanol.

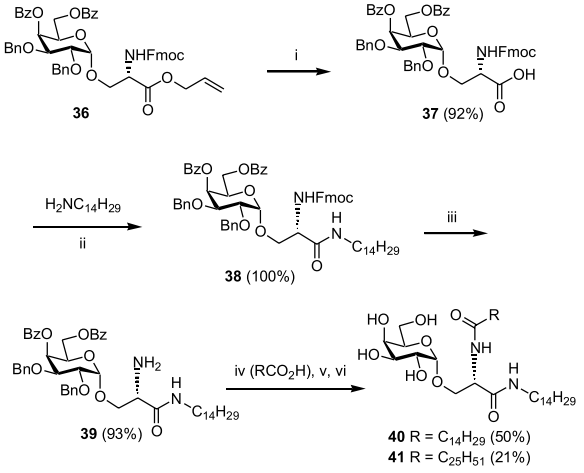
Serine-based lipid has been reported to exhibit the similar bioactivity as a ceramide mimic.[24] It would be interesting to know if serine-based lipid or its amide analogue could mimic the functions of phytosphingosine. In order to pursue the α-selective glycosylation with the L-serine derivative **35**, different types of galactosyl donors including sulfide **22**, sulfide **31**, phosphite **33** and imidate **34** were investigated (Scheme 4). Hydrolysis of sulfide **31** with NBS in the presence of water,[25] followed by treatments with *i*-Pr_2-$NP(OBn)_2$ or trichloroacetonitrile according to standard carbohydrate chemistry, gave dibenzyl phosphite **31**[26] and trichloroimidate **34**,[27] respectively. Glycosylation reactions of **35** generally afforded high yields (89–95%) of **36**, however, in varied anomeric selectivities depending on the use of different galactosyl donors.[26,28]



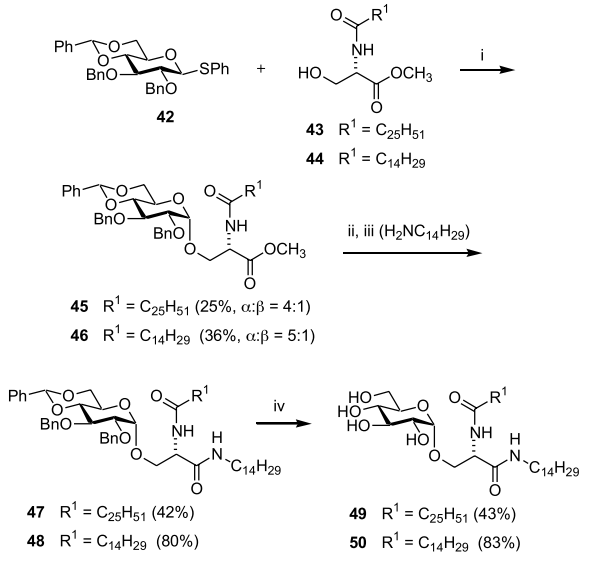
Scheme 4. Synthesis of galactosyl donors and coupling with serine derivative **35**. Reagents and conditions: (i) NBS, Me_2CO/H_2O=9:1, rt, 2 h. (ii) 1*H*-tetrazole, THF, rt, 2 h. (iii) DBU, THF, rt, 30 min.

Using tetrabenzyl *S*-galactoside **22**, the glycosylation was realized by the promotion of NIS and TfOH to give α and β-anomers (2:1) of **36**. The α/β selectivity was increased to 9:1 when *S*-galactoside **31** with benzoate protecting groups at 4- and 6-positions, differing from the benzyl groups in **22**, was applied in the glycosylation. Incorporation of benzoate

group at 4- or 6-position of galactoside or glucoside, in comparison to benzyl group, is known to enhance the α-selectivity of thiotolyl donor.[28] We were surprised and fortunate to find that coupling of an α/β mixture (6:1) of the phosphite donor **31** with the serine derivative **35** in the presence of TfOH gave only the α-glycosylation product **36** in 95% yield. On the other hand, glycosylation of imidate **34** (α anomer) in the presence of TMSOTf gave predominantly the β-anomor of **36** (α/β=1:10). It was presumed that glycosylation of phosphite donor proceeded with an S_N1-like mechanism, whereas that of imidate donor followed an S_N2-like pathway. During the glycosylation of phosphite **33**, the benzoyl groups at 4- and 6-positions might participate in stabilization of the oxonium intermediate. As the β-face was blocked, the serine derivative **35** could only have access to the oxonium intermediate from the α-face.



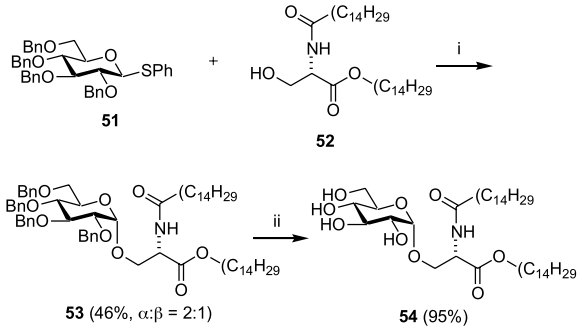
Scheme 5. Synthesis of galactosyl serine-type ceramide analogues **40** and **41**. Reagents and conditions: (i) $Pd(PPh_3)_4$, THF. (ii) HBTU, HOBt, CH_2Cl_2. (iii) 40% H_2NEt/THF. (iv) EDC, HOBt, DMF. (v) MeONa, MeOH. (vi) H_2, $Pd(OH)_2$, EtOH, $CHCl_3$, 50 kg/cm^2.



Scheme 6. Synthesis of α-glucosyl serine-type ceramides **49** and **50**. Reagents and conditions: (i) NIS, cat. TfOH, 4 Å MS, CH_2Cl_2, −15 to −10 °C. (ii) aq. NaOH (1 N), THF. (iii) EDC, HOBT, CH_2Cl_2. (iv) H_2, $Pd(OH)_2$/C, cat. AcOH, EtOAc/MeOH (1:1).

As shown in Scheme 5, the allyl group of **36** was removed by using $Pd(PPh_3)_4$ catalyst,[29] and the resulting acid was coupled with 1-tetradecylamine to yield amide **38**. Deprotection of the Fmoc group gave amine **39**. Amidation of **39** with carboxylic acids, followed by removal of benzoyl and benzyl groups, thus gave **40** and **41** with serine-type long-chain amide moieties as the structurally simpler mimics of α-GalCer.

In order to evaluate the role of 4-OH group of α-GalCer in immunoactivity, the α-glucosyl serine-type ceramides (e.g. **49** and **50**) were prepared. Glycosylation of *S*-glucoside **42** with the *N*-acyl derivatives of methyl L-serine (**43** and **44**) was carried out by the promotion of NIS and TfOH to give **45** and **46** predominating in the α-anomers (Scheme 6). Saponification and amidation of **45** and **46** with tetradecylamine, followed by romoval of the benzyl and benzylidene protecting groups, led to the α-glucosyl serine-type ceramides **49** and **50**. By a similar procedure, the α-glucosyl serine-type lipid **54** was also prepared (Scheme 7).



Scheme 7. Synthesis of α-glucosyl serine-type ester **54**. Reagents and conditions: (i) NIS, cat. TfOH, 4 Å MS, CH_2Cl_2, −15 to −10 °C. (ii) H_2, $Pd(OH)_2$/C, cat. AcOH, EtOAc/MeOH (1:1).

The glycolipids **1**, **2**, **27**, **30**, **40**, **41**, **50** and **54** were submitted to evaluate their activities. First, colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT)[30] was conducted to evaluate the proliferation of glycolipid-stimulated mouse spleen cells in the presence of various concentrations of the glycolipids. α-GalCer **1** and the truncated analog **2** at a concentration of 100 ng/mL significantly promoted the cell proliferation activity comparing to the control samples (Fig. 2). The galactosyl serine-type lipid **40** at the same concentration also slightly enhanced the cell proliferation activity.

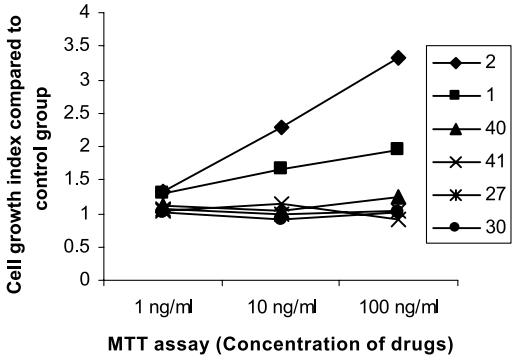


Figure 2. MTT assay for glycolipids **1**, **2**, **27**, **30**, **40** and **41**.

The expression levels of the cytokines, IFN-γ and IL-4, in mouse spleen cells were determined by an ELISA assay (enzyme-linked immunosorbent assay)[31] using 100 ng/mL glycolipids. Only compounds **1**, **2** and **40** were observed to stimulate IFN-γ and IL-4 expression after 24 h (Fig. 3). The related long-term cytokine stimulation by **1** and **2** is shown in Figure 4. Both compounds had similar effect on INF-γ production while compound **2** had a stronger effect on IL-4 production. Notably, these results are not the same as that obtained for compounds **1** and **2** on NKT cells.[12] The

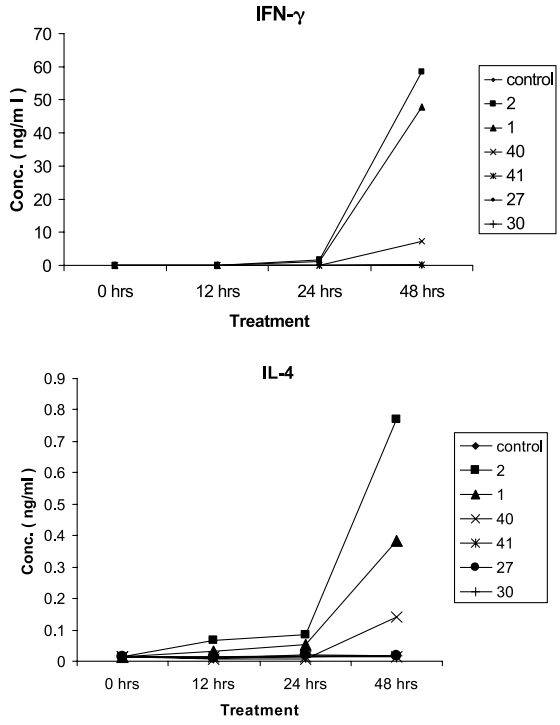


Figure 3. Cytokine assays for α-GalCer (**1**) and other glycolipids. For clarity, the lines of inactive compounds **50** and **54** are omitted. Untreated cells are taken as the control.

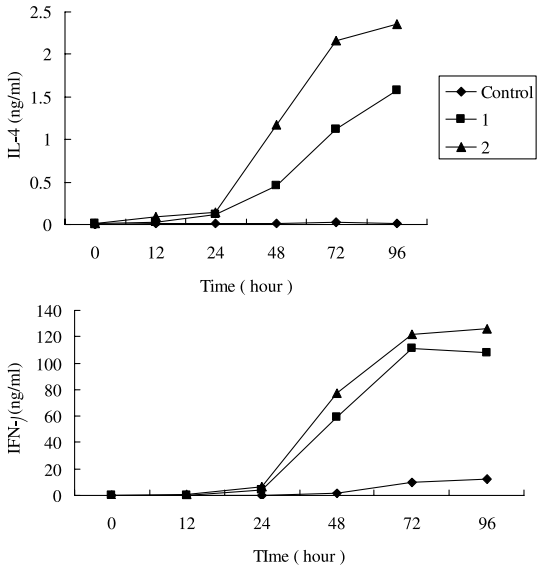


Figure 4. Long-term cytokine assays for α-GalCer (**1**) and a short-chain analogue **2** using untreated cells as the control.

simplified α-GalCer analog **40**, having a serine-type lipid to replace phytosphingosine, still retained some immunoactivity. Although compounds **1** and **2** are better antigens to stimulate the production of cytokines, compound **40** has the advantage of simple synthesis. The fucosyl ceramides (**27** and **30**) and glucosyl serine-type lipids (**50** and **54**) did not show any immuno-activity.

In conclusion, we have devised an expedient method for the synthesis of glycosphingolipids, starting from an inexpensive sugar, D-lyxose. We have also carried out the syntheses of galactosyl, fucosyl and glucosyl ceramides in α-anomeric selectivity. The bioassays indicated that α-galactosyl ceramides **1** and **2** exhibited substantial effects on the proliferation of mouse splenocytes as well as the expression of cytokines IFN-γ and IL-4. The galactosyl serine-type ceramide **40** also showed similar bioactivities, though to less degrees. Works on the synthesis of a glycolipid library and extensive evaluation of the immuno-modulating activities of these immuno-stimulators are in progress.

3. Experimental

Compounds **1**,[32] **3**,[33] **5**,[34] **11**,[35] **18**,[15a] **19**,[15a] **20**,[36] **22**,[20] **23**,[35] **25**,[37] **28**,[38] **35**,[39] **42**[40] and **51**[41] have previously been reported, and our prepared samples showed consistent ^{1}H and ^{13}C NMR spectral data to the structural assignments.

3.1. Representative procedure for glycosylation. Using aldose (method A)

To a solution of galactosyl donor **5** (300 mg, 0.57 mmol), dimethylsulfide (54 μL, 0.74 mmol), 4 Å molecular sieve (100 mg) and 2-chloropyridine (150 μL, 1.58 mmol) in anhydrous CH_2Cl_2 (2 mL) under Ar at −45 °C was added trifluoromethanesulfonic anhydride (94 μL, 0.56 mmol). The reaction mixture was stirred for 20 min at 0 °C and 20 min at room temperature. Phytosphingosin derivative **21** (150 mg, 0.37 mmol) in CH_2CL_2 (2 mL) was slowly added via cannula under positive nitrogen pressure. The reaction mixture was stirred at room temperature for 20 h, and then filtered. The crude filtrate was partitioned between EtOAc and water. The organic layer was separated, washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed on a silica gel column (EA/Hex from 9:1 to 4:1) to give the product **4** as yellow oil (285 mg, 82%).

3.2. Using glycosyl sulfide (method B)

To a mixture of glycosyl sulfide **28** (200 mg, 0.5 mmol), phytosphingosin derivative **20** (220.2 mg, 0.42 mmol) and 4 Å molecular sieves (400 mg) in CH_2Cl_2 (2.5 mL) was added NIS (453.6 mg, 2.0 mmol) at 0 °C under Ar. The reaction mixture was stirred for 30 min, and TfOH (1.9 μL, 0.02 mmol) was added. After 30 min, the reaction mixture was concentrated under reduced pressure. The residue was diluted with EtOAc, and washed with aqueous $Na_2S_2O_3$, saturated $NaHCO_3$ and brine. The organic phase was dried over $MgSO_4$, and concentrated under reduced pressure. The

residue was chromatographed on a silica gel column (EtOAc/hexane, 1:2) to give product **29** (200.0 mg, 60%).

3.3. Using glycosyl phosphite (method C)

A mixture of serine derivative **35** (0.55 g, 1.5 mmol), galactosyl phosphite **33** ($\alpha/\beta=6/1$, 1.33 g, 1.6 mmol) and 4 Å molecular sieves in dried CH_2H_2 (20 mL) was stirred for 10 min at room temperature under Ar. Trifluoromethanesulfonic acid (26 μL, 0.3 mmol) was added, and the mixture was stirred at room temperature for another 1 h. In this period of reaction, compound **35** was completely consumed as shown by TLC analysis. The reaction mixture was filtered through a short pad of Celite. The filtrate was diluted with CH_2Cl_2, and washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, and concentrated under reduced pressure. The residue was chromatographed on a silica gel column (EtOAc/Hex: from 8:1 to 3:1) to obtain the product **36** (α-anomer) as yellow oil (1.30 g, 95%).

3.4. Representative procedure for Wittig reaction

To a mixture of triphenyl-tridecyl-phosphonium bromide (4.86 g, 9.2 mmol) in anhydrous THF (20 mL) was added lithium hexamethyldisilazide (LHMDS, 9.2 mL of 1 M solution in THF) at 0 °C under Ar, and stirred at 0 °C for 60 min. A solution of **11**[34] (2.0 g, 4.6 mmol) in anhydrous THF (10 mL) was treated with LHMDS (4.6 mL, 4.6 mmol) at 0 °C for 60 min under Ar to give the **11**-anion solution. The mixture of the above prepared phosphonium solution and **11**-anion solution was stirred at 0 °C to room temperature for 9 h, and then quenched with MeOH. After removal of volatiles under reduced pressure, the residue was partitioned between EtOAc and water. The organic layer was separated, washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed on a silica gel column (EtOAc/hexane, 9:1 to 3:1) to give the product **12** as colorless oil (2.58 g, 93%). The *E*/*Z* ratio is about 2:1 as determined by the ^1H NMR analysis.

3.5. Representative procedure for catalytic hydrogenation

To a solution of compound **24** (40 mg, 0.032 mmol) in a cosolvent system of $CHCl_3$ and EtOH (1:4, 1 mL) was added Pd/C (10 mg). The reaction was shaken under high pressure of hydrogen (50 kg/cm^2) for 6 h. The reaction mixture was filtered over a short pad of Celite, and the filtercake was washed with CH_2Cl_2/MeOH cosolvent (1:1). The filtrate was concentrated under reduced pressure, and the resulting residue was purified by LH20 (MeOH/$CHCl_3=1:1$) and then silica gel chromatography (MeOH/$CHCl_3=1:15$) to give the product **2** as white solid (16 mg, 71%).

3.6. Representative procedure for substitution of hydroxyl group by azide

To a mixture of **14** (91 mg, 0.15 mmol), 2,6-lutidine (21 μL, 0.18 mmol) and 4 Å molecular sieves (30 mg) in anhydrous CH_2Cl_2 (0.5 mL) under Ar at −40 °C was added trifluoromethanesulfonic anhydride (30 μL, 0.18 mmol). After stirring at −40 °C for 30 min, tetramethylguanidinium azide (71 mg, 0.45 mmol) was added in one portion, and the reaction mixture was slowly warmed to room temperature and stirred for 18 h. The mixture was filtered over a short pad of Celite, and the filtrate was partitioned between EtOAc and water. The organic layer was separated, washed with brine, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed on a silica gel column to give product **16** as colorless oil (76 mg, 73%).

3.7. Representative procedure for azide reduction and amide formation

To a solution of compound **4** (100 mg, 0.11 mmol) in pyridine (4 mL) and water (0.4 mL) cosolvent system was added triphenylphosphine (57 mg, 0.22 mmol). The reaction mixture was heated to 40 °C for 12 h, concentrated, and the residue was partitioned between EtOAc and water. The organic layer was separated, washed with brine, dried over anhydrous $MgSO_4$, and concentrated. The crude amine product was used for the next step without further purification.

To a solution of the crude amine product and tetracosanoic acid (53 mg, 0.14 mmol) in dried DMF (1 mL) was added triethylamine (30 μL, 0.22 mmol), EDC (33 mg, 0.17 mmol) and HOBt (23 mg, 0.17 mmol) at 0 °C under Ar. The reaction mixture was stirred at 0 °C to room temperature for 12 h, and then concentrated. The residue was partitioned between EtOAc and water. The organic layer was separated, washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was chromatographed on a silica gel column (EA/Hex = 1:19 to 1:9) to give the desired product **24** as white foam (88 mg, 65% in two steps).

3.8. Preparation of spleen cells

Male BALB/c mice about 8-weeks-old were sacrificed and the spleen cells were isolated. Briefly, spleen cells were aseptically prepared by mechanical disruption and red blood cells were removed by ACK lysis buffer (NH_4Cl 150 mM, $KHCO_3$ 1 mM, EDTA 0.1 mM) treatment. Viable cells were washed with PBS saline, then counted and resuspended in RPMI-1640 supplemented with 50 μM 2-mercaptoethanol, 10 mM HEPES, 2 mM L-glutamine and 10% FCS.

MTT assay. Spleen cell solution (100 μL of 2.5×10^6 cell/mL containing 1, 10 and 100 ng/mL of test compound or vehicle DMSO only) were dispensed into 96-well plate and cultured at 37 °C incubator containing 5% CO_2 for two days. After cultured, 20 μL of MTT stock solution (5 mg/mL dissolved in PBS and filtered) was added to the cultured cells and then incubated the cells for a further 4 h. For dissolving the dark blue formazan product (converted from MTT in active mitochondria), 120 μL of 0.04 N HCl in isopropyl alcohol was added to each wells. The colorimetric values were measured by microtiter plate reader with filter settings of 570 and 630 nm as reference. The cell growth index was calculated by the following formula: cell growth

index = (value$_{(test\ compounds)}$ − value$_{(blank)}$)/(value$_{(control)}$ − value$_{(blank)}$))×100. The value$_{(control)}$ was the detected value of the cells cultured with medium containing vehicle, while the value$_{(blank)}$ was that of medium only.

3.9. Cytokine assay

Spleen cells were suspended in RPMI-1640 medium containing 10% FCS in cell density of 5×10^6 cells/mL. The cell solution was added test compound to a final concentration of 100 ng/mL or none as control and was dispensed 200 μL/well into 96-well plate. The culture plates were incubated at 37 °C incubator containing 5% CO_2 for 0, 12, 24 and 48 h. At each time point, the culture supernatant was drawn for IFN-γ and IL-4 detection using standard sandwich ELISA. All procedures were conducted according to the standard protocol of the assay kits from Quantikine® M. (catalog number was MIF00 for INF-γ and M4000 for IL-4).

3.9.1. (2*S*,3*S*,4*R*)-1-*O*-(α-D-Galactopyranosyl)-2-(*N*-tetracosanoylamino)-1,3,4-nonanetriol (2). ^{1}H NMR (400 MHz, pyridine-d_5) δ 0.82 (t, J=7.2 Hz, 3H), 0.89 (t, J=6.8 Hz, 3H), 1.10–1.50 (br, 46H), 1.58–1.70 (m, 1H), 1.78–1.94 (m, 3H), 2.46 (t, J=7.4 Hz, 2H), 4.28–4.34 (m, 2H), 4.36–4.47 (m, 3H), 4.52 (t, J=5.6 Hz, 1H), 4.56 (d, J=3.2 Hz, 1H), 4.63–4.72 (m, 2H), 5.27 (m, 1H), 5.58 (d, J=3.6 Hz, 1H), 8.47 (d, J=8.4 Hz, 1H). ^{13}C NMR (100 MHz, pyridine-d_5) δ 14.7, 23.4, 23.5, 26.6, 26.9, 30.1, 30.2, 30.3, 30.5, 32.6, 32.9, 34.8, 37.3, 51.9, 63.1, 69.1, 70.2, 71.5, 72.1, 72.9, 73.5, 77.2, 102.0, 173.7. HRMS (MALDI-TOF, M+H$^+$) calcd for $C_{39}H_{78}NO_9$: 704.5677. Found: 704.5663.

3.9.2. (2*S*,3*S*,4*R*)-1-*O*-(α-L-Fucopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol (27). ^{1}H NMR ($CDCl_3$/MeOH=1/1, 400 MHz): δ 0.86 (t, 6H, J=6.8 Hz), 1.21 (d, 3H, J=6.4 Hz), 1.25 (m, 72H), 2.19 (t, 2H, J=7.6 Hz), 3.41 (dd, 1H, J=3.6, 10.0 Hz), 3.46–3.53 (m, 1H), 3.59–3.62 (m, 1H), 3.65–3.76 (m, 2H), 3.72 (dd, 1H, J=3.6, 6.8 Hz), 3.95–3.97 (m, 2H), 4.17–4.19 (m, 1H), 4.74 (d, 1H, J=3.6 Hz). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 13.0, 15.1, 21.9, 25.3, 28.7, 28.7, 28.9, 28.9, 29.0, 31.2, 63.5, 65.7, 66.7, 68.2, 69.6, 69.9, 71.5, 71.6, 98.6, 173.8. HRMS (MALDI-TOF, M+Na$^+$) calcd for $C_{50}H_{99}NO_8Na$: 864.7268, found 864.7252.

3.9.3. (2*S*,3*S*,4*R*)-1-*O*-(β-L-Fucopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol (30). ^{1}H NMR ($CDCl_3$/MeOH=1/1, 400 MHz) δ 0.87 (t, 6H, J=6.8 Hz), 1.25–1.28 (m, 75H), 2.19 (t, 2H, J=7.6 Hz), 3.45–3.49 (m, 3H), 3.56–3.63 (m, 2H), 3.67 (dd, 1H, J=5.2, 7.2 Hz), 3.87–3.88 (m, 2H), 4.07–4.11 (m, 1H), 4.23 (d, 1H, J=7.2 Hz). ^{13}C NMR ($CDCl_3$, 500 MHz) δ 12.9, 15.1, 21.8, 25.1, 25.2, 28.5, 28.6, 28.6, 28.7, 28.8, 28.9, 28.9, 29.0, 31.1, 35.7, 49.8, 68.4, 70.2, 70.4, 71.0, 71.6, 73.1, 73.3, 102.9, 174.1. HRMS (MALDI-TOF, M+H$^+$) calcd for $C_{50}H_{100}NO_8$: 842.7449, found 842.7440.

3.9.4. Pentadecanoic acid [1-tetradecylcarbamoyl-2-(α-D-galactopyranosyl)-ethylamide (40). ^{1}H NMR (400 MHz, pyridine-d_5) δ 0.87 (t, J=6.6 Hz, 6H), 1.14–1.48 (br, 44H), 1.56–1.68 (m, 2H), 1.72–1.85 (m, 2H), 2.47 (t, J=7.4 Hz, 2H), 3.47 (dd, J=6.8, 12.8 Hz, 2H), 4.26 (dd, J=7.4, 10.6 Hz, 1H), 4.35–4.52 (m, 4H), 4.55 (t, J=5.8 Hz, 1H), 4.60 (d, J=3.2 Hz, 1H), 4.68 (dd, J=3.8, 9.8 Hz, 1H), 5.35 (dt, J=5.2, 7.4 Hz, 1H), 5.56 (d, J=3.6 Hz, 1H), 8.85 (t, J=5.4 Hz, 1H). ^{13}C NMR (100 MHz, pyridine-d_5) δ 14.7, 23.4, 26.6, 27.80, 30.1, 30.1, 30.1, 30.3, 30.4, 32.6, 37.0, 40.4, 53.8, 63.2, 70.1, 70.7, 71.5, 72.01, 73.7, 102.0, 171.3, 173.8. HRMS (MALDI-TOF, M+H$^+$) calcd for $C_{38}H_{75}N_2O_8$: 687.5523. Found: 687.5527.

3.9.5. *O*-(α-D-Glucopyranosyl)-*N*-pentadecanoyl-L-serine tetradecyl amide (50). TLC (MeOH/$CHCl_3$=1:10) R_f 0.15; ^{1}H NMR (CD_3OD, 400 MHz) δ 4.86 (1H, H-1, mixed with water peak), 4.56 (1H, dd, J=6.2, 5.9 Hz, H-2), 3.88 (1H, dd, J=10.5, 6.2 Hz, H-1′), 3.85 (1H, dd, J=11.8, 2.4 Hz, H-6), 3.78 (1H, dd, J=10.5, 5.9 Hz, H-1′), 3.70 (1H, dd, J=11.8, 5.6 Hz, H-6), 3.63 (1H, dd, J=9.7, 9.2 Hz, H-3), 3.58 (1H, ddd, J=9.9, 5.6, 2.4 Hz, H-5), 3.45 (1H, dd, J=9.7, 3.8 Hz, H-2), 3.32 (1H, dd, J=9.9, 9.2 Hz, H-4), 3.26–3.21 (2H, m, H-3′), 2.30 (2H, t, J=7.5 Hz, H-4′), 1.67–1.64 (2H, m), 1.57–1.54 (2H, m), 1.50–1.10 (44H, br), 0.94 (6H, t, $CH_3\times2$); ^{13}C NMR (CD_3OD, 100 MHz) δ 176.4, 172.2, 101.0, 75.2, 74.2, 73.6, 71.8, 69.1, 62.8, 54.9, 40.8, 37.0, 33.2, 31.0, 30.9, 30.9, 30.8, 30.7, 30.6, 30.6, 30.5, 28.2, 27.0, 23.9, 14.6; FAB-MS *m*/*z* 687.5 (M$^+$+1); HRMS calcd. for $C_{38}H_{75}N_2O_8$ (M$^+$+H) 687.5523, Found: 687.5531.

3.9.6. *O*-(α-D-Glucopyranosyl)-*N*-pentadecanoyl-L-serine tetradecyl ester (54). TLC (MeOH/$CHCl_3$=1/7) R_f 0.33; ^{1}H NMR (CD_3OD, 400 MHz) δ 4.83 (1H, d, J=3.8 Hz, H-1), 4.69 (1H, dd, J=5.6, 4.4 Hz, H-2′), 4.18 (2H, t, J=6.6 Hz, H-3′), 3.99 (1H, dd, J=10.8, 4.4 Hz, H-1′), 3.95 (1H, dd, J=10.8, 5.6 Hz, H-1′), 3.84 (1H, dd, J=11.8, 2.3 Hz, H-6), 3.71 (1H, dd, J=11.8, 5.5 Hz, H-6), 3.64 (1H, t, J=9.3 Hz, H-3), 3.59 (1 H, ddd, J=9.8, 5.3, 2.3 Hz, H-5), 3.43 (1H, dd, J=9.7, 3.8 Hz, H-2), 3.33 (1H, t, J=9.4 Hz, H-4), 2.31 (2H, t, J=7.2 Hz, H-5′), 1.73–1.66 (4H, m, H-4′, 6′), 1.50–1.20 (44H, br), 0.94 (6H, t, $CH_3\times2$); ^{13}C NMR (C_5D_5N, 100 MHz) δ 174.0, 171.7, 102.3, 75.6, 75.1, 74.2, 72.5, 70.2, 65.9, 63.2, 54.4, 36.7, 32.5, 30.4, 30.3, 30.3, 30.2, 30.2, 30.1, 30.0, 29.9, 29.3, 26.6, 26.5, 23.4, 14.7; FAB-MS *m*/*z* 688.1 (M$^+$ +1); HRMS calcd. for $C_{38}H_{74}NO_9$ (M$^+$ +1) 688.5364, Found: 688.5389; HRMS calcd. for $C_{38}H_{73}NNaO_9$ (M$^+$+Na) 710.5183, Found: 710.5168.

Supporting information available. Synthetic procedure, characterization and NMR spectra of new compounds **4**, **12–17**, **19**, **21**, **24**, **26**, **29**, **36–39**, **41**, **45–48**, and **53**.

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