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Facile synthesis of biotin-labelled α-galactosylceramide as antigen for invariant natural killer T cells

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ABSTRACT

When iNKT cells are stimulated by the CD1d presented exogenous α-galactosylceramide, a myriad of cytokines are released that trigger a variety of immune responses. However, the invisibility and poor aqueous solubility of α-galactosylceramide during bioassays have rendered it difficult to study the aforementioned biological responses. Accordingly, a biotin-labelled α-galactosylceramide was prepared to facilitate detection of this antigen in the immune process. The problematic low solubility was also remedies via incorporation of a truncated acyl chain.

1. Introduction

In adaptive immunity, T helper (T_H) and T cytotoxic (T_C) cells are two well-defined subpopulations of T lymphocytes which recognize peptide antigens presented by class II major histocompatibility complexes (MHC) and class I MHC, respectively. In contrast, invariant natural killer T (iNKT) cells, a newly discovered subpopulation of T lymphocytes which express conserved, semi-invariant αβ (mouse Vα14-Jα18/Vβ8 or human Vα24-Jα18/Vβ11) T cell receptors (TCR) and natural killer (NK) receptors, can be stimulated by glycolipid antigens presented by the MHC class I-like protein CD1d.[1] It has been found that there are five classes of such β_2-microglobulin-associated CD1 proteins in humans. These five classes are further divided into the following two groups: group I CD1 (CD1a, CD1b and CD1c) and group II CD1 (CD1d and CD1e). In contrast, only a single class of CD1 proteins (mCD1d) is found in mice, and it is homologous to the human isoform CD1d. Once stimulated, the aforementioned iNKT cells then rapidly secrete copious amounts of cytokines within only a few hours, including the characteristic T_H1 cytokine interferon-γ (IFN-γ) and T_H2 cytokine interleukin-4 (IL-4). These cytokines play important roles in activating B cells, T_C cells, macrophages, and various other cells that participate in the immune response.

Such stimulation of cytokine release is initiated in part by the glycolipid antigen, α-Galactosylceramide (α-GalCer) (Fig. 1). α-GalCer was originally discovered in an extract from a marine sponge and was found to strongly activate iNKT cells in very low concentrations.[2] The salient structural feature that distinguishes exogenous α-GalCer from endogenous mamalian glycoceramides is the α-linkage between the saccharide and ceramide moieties. When this molecule presented to iNKT cells by antigen presenting cells (APC), large amounts of cytokines are secreted, causing a myriad downstream immune responses, including responses to pathogens, tumors, tissue graft, allergens, and others.

The structural features of this glycolipid antigen that are essential for iNKT cell activation have been rationalized through

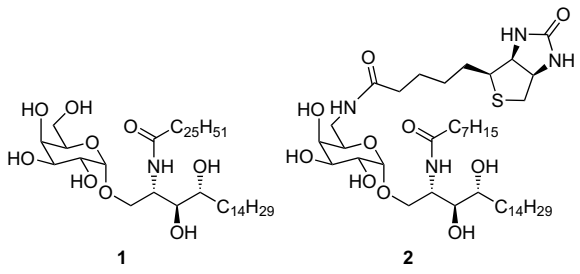

Figure 1. Structures of the optimized α-GalCer **1** (KRN7000) and designed biotin-labelled α-GalCer **2**.

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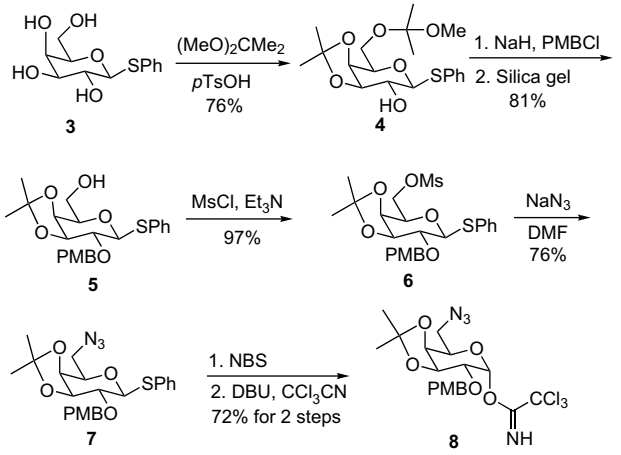
crystal structures of α-GalCer/CD1d complexes. It has been found, for example, that CD1d has a deep hydrophobic cleft that can accommodate the two lipid chains.[3] Specifically, the ceramide moiety of α-GalCer contributes to the binding with CD1d by anchoring α-GalCer in a distinct orientation for recognition by the iNKT cell TCR. Both alkyl chains are initially inserted perpendicularly to the β-sheet platform and then extend more laterally toward the ends of the A′ and F′ pockets. In regards to the sugar moiety, structure–activity-relationship studies showed that the C2 hydroxyl group is crucial for α-GalCer activity with the C3 hydroxyl group also demonstrating importance for binding with CD1d.[4] Furthermore, α-glucosylceramide (α-GlcCer), in which the C4 hydroxyl group is in an equatorial position, still showed activity in NKT cell stimulation, although to a lesser extent than α-GalCer.[5] When this position is capped by an additional sugar, however, the resulting dihexosylceramide becomes inactive.[6] In contrast, the C6 position is more flexible and can tolerate minimal modifications, such as the attachment of a small molecule, without losing its activity. This fact has been rationalized by the latest ternary crystal structure of the TCR/α-GalCer/CD1d complex, which unveiled that the C6 hydroxyl group is located on the outer of the cavity of the TCR and CD1d and thus does not form any H-bonds with them.[7]

2. Discussion

In spite of all this information, one of the lingering challenges for studying the role of α-GalCer in the immune process is its invisibility during assays. However, since the C6 position of the sugar moiety can tolerate some small molecules, and these small molecules protrude outside the complex, incorporation of a bio-visible functional group on this position should remedy this problem without affecting the activity of α-GalCer. Savage et al. suggested that attaching a biotin group would be a good tool to detect α-GalCer in flow cytometry studies.[8] Furthermore, α-GalCer is notorious for it low solubility in aqueous solutions during the assay due to the two long hydrophobic chains. It was found, however, that truncating the acyl chain to an 8-carbon unit could dramatically improve the solubility of α-GalCer without changing iNKT cell stimulation profile.[9] Herein, we accordingly report the facile synthesis and bioassay of biotin-labelled α-GalCer **2** with a truncated acyl chain (Fig. 1).

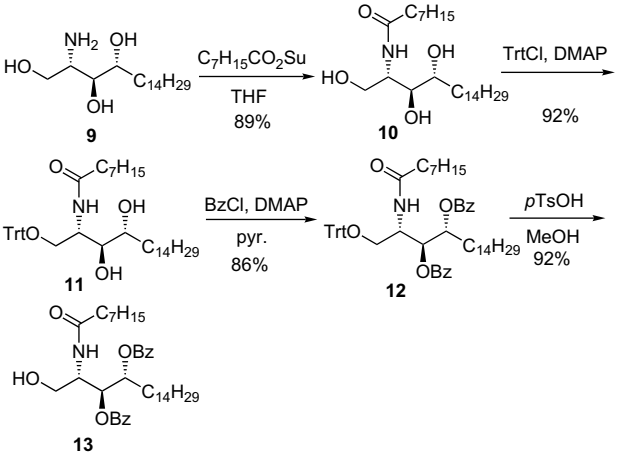
The formation of the α-isomer is the key in the synthesis of the biotin-labelled α-GalCer **2**. Both of the protecting groups on carbohydrate part and ceramide part play important effects on the outcomes of glycosidation. We already noticed that electron withdrawing protecting groups on the ceramide part favor the formation of α-linkage during the glycosidation, while the electron donating groups prefer the formation of β-isomer.[10] The protecting group on the C2 position of carbohydrate part that doesn't exert a neighboring group effect is also crucial i our synthetic design. The use of ether groups is the widely accepted method for this purpose as they are easily introduced and possess versatile deprotection protocols. For example, the benzyl ether group can be conveniently removed by palladium catalyzed hydrogenation or sodium mediated reduction in ammonia. However, due to the sulfur atom in biotin, these hydrogenation-dependent protecting groups are not compatible. Therefore, the *p*-methoxybenzyl (PMB) group was selected since it can be removed not only by hydrogenation, but also oxidation with DDQ or CAN, as well as by mild acidic conditions.

Accordingly, preparation of the *C*6-azido galactosyl donor commenced with treatment of the thiophenyl compound **3**[11] with a trace amount of *p*-toluenesulfonic acid in 2,2-dimethoxylpropane (Scheme 1). This afforded protection of the C3 and C4 hydroxyl groups as a five-membered isopropylidene ketal ring, while the C6 hydroxyl group was protected as the 1-methoxyl-1-methylethyl ether.[12] Subsequent treatment with NaH followed by PMBCl introduced the PMB-ether at C2, thus precluding any neighboring group effects. After the desired product was extracted from the reaction mixture, the crude preparation was stirred with silica gel in methanol to remove the ether on C6 and furnish compound **5**. In order to install the azido group on the C6 position, the previously deprotected hydroxyl group was first activated via formation of the mesylate. Heating in the presence of sodium azide then afforded the azido-galactose derivative **7**. However, when **7** was used as a donor for the glycosylation of ceramide **13** through activation with *N*-iodosuccinimide and triflic acid, both the α- and β-isomers were produced. In an effort to improve this anomeric selectivity, **7** was converted to the trichloroacetimide type donor **8**, a molecule which had worked well in the glycosylation of a ceramide in our previous research.[10] Specifically, the thiophenyl group was removed via treatment with *N*-bromosuccinimide,[13] after which trichloroacetonitrile and 1,8-diazabicylco-[5.4.0]-undec-7-ene (DBU) were introduced to give the donor **8**.[14]



Scheme 1. Synthesis of the 6-azido-galactosyl donor **8**.

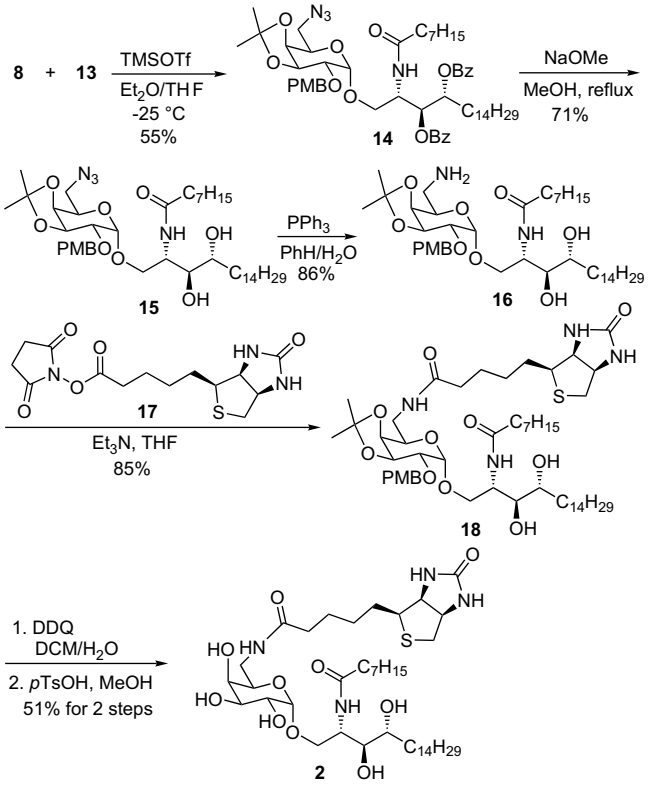
With the desired donor in hand, ceramide acceptor **13** was prepared from the commercially available phytosphingosine **9** using the procedures previously established for the 26-carbon acyl chain[10] (Scheme 2). This entailed introduction of the short acyl chain through condensation with the octoic acid *N*-hydroxysuccinimide ester to give the amide **10**. Subsequent selective protection of the primary hydroxyl group with trityl chloride in pyridine followed by protection of the two secondary hydroxyl groups with benzoyl groups afforded **12**. Such protection of the secondary hydroxyl groups was carried out so as to avoid formation of the β-isomer from an S_N1 reaction. Lastly, removal of trityl group



Scheme 2. Synthesis of the short acyl chain acceptor **13**.

was achieved via treatment with *p*-toluenesulfonic acid in methanol.

In the final sequence, glycosylation of acceptor **13** with donor **8** was performed by using TMSOTf as a promoter in a mixed solvent composed of Et_2O and THF (Scheme 3). These conditions afforded only the α-isomer of the glycolipid in a moderate yield. Attempts to subsequently reduce the azido group to the amine via the Staudinger method failed due to the formation of the phosphinimine, a species which proved too stable to be decomposed under mild conditions.[15] It was intriguing, however, that when the two benzoyl groups in the phytosphingosine were first hydrolyzed, reduction of the azido group with triphenylphospine proceeded smoothly. The resultant amine **16** was treated with the biotin ester **17** to afford compound **18**. Since the PMB group can be removed under acidic condition, we then tried to deprotect both of the PMB and isopropylidene functionalities with *p*-toluenesulfonic acid or trifluoroacetic acid. However, TLC showed that only a trace amount of final product formed while many by-products were generated. The complexity of this reaction might arise from an issue with the PMB group because some of the by-products were still UV visible and existed even after prolonging the reaction time or increasing the amount of acid. To overcome this problem, these two protecting groups were accordingly removed separately. Specifically, the PMB was first deprotected by oxidation with DDQ,[16] after which the isopropylidene group was removed by treatment with *p*-toluenesulfonic acid to furnish the final product **2** in 51% yield over two steps.



Scheme 3. Preparation of the biotin-labelled α-GalCer **2**.

The Vα14*i* NKT hybridoma DN3A4-1.2 cells, which produce high amounts of IL-2 cytokines, were used to measure the stimulatory activities of biotin-labelled glycolipid antigen with a previously reported assay.[6] It's discovered that the acyl chain truncated compound **2** can be easily dissolved in aqueous without detergent, while the 26-carbon compound **1** (KRN7000) is very poorly soluble and normally different detergents are required to facilitate its dissolution. Specifically, the glycolipid antigens are up taken and presented by A20/CD1 cells which express CD1d on the surface. When the DN3A4-1.2 hybridoma cells are co-cultured with the A20/CD1 cells, cytokine IL-2 will be secreted. The results in Figure 2A indicate that although biotin-labelled α-GalCer **2** is less efficient than **1**, it still can stimulate iNKT hybridoma cells to release cytokine IL-2. They also further prove that the iNKT cells can tolerate some small molecules linked on the C6 position of the sugar moiety.

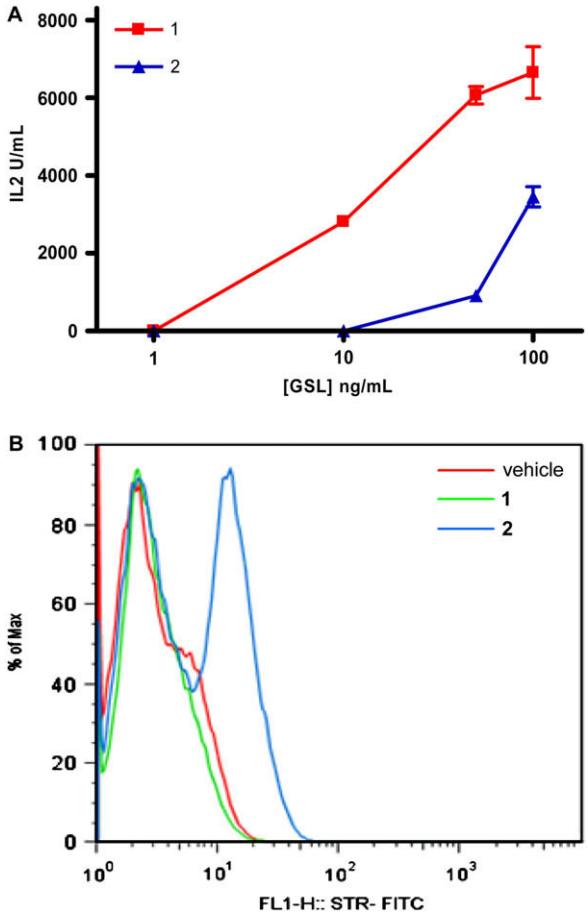


Figure 2. (A) Stimulating the mouse iNKT hybridoma cells with the α-galactosylceramides **1** and **2**. The release of IL-2 cytokine was measured by ELISA assay. (B) Detecting the glycosylceramide **1** and **2** loaded A20/CD1 cells by flow cytometry.

To prove the visibility of biotin-labelled α-GalCer **2**, the A20/CD1 cells treated with **2** were stained by fluorescein isothiocyanate (FITC) conjugated stravidin and analyzed by flow cytometry. As shown in Figure 2B, A20/CD1 cells with either vehicle or α-GalCer **1** could not be stained by stravidin–FITC. However, a significant FITC positive A20/CD1 cells population could be detected when they were co-cultured with biotin-labelled α-GalCer **2**. This assay indicates that compound **2** can be detected after being presented on the cell surface. Compound **2** can be further used to characterize which proteins, organelles, cells and organs are involved in glycolipids trafficking, presentation and recognition.

3. Conclusion

In summary, an efficient method of preparation of C6 biotin-labelled α-galactosylceramide was developed. The convergent synthetic pathway included the preparation of a 6-azido-galactosyl donor and the di-benzoyl protected ceramide acceptor. The yield of donor preparation is 33%, and acceptor is 65%, respectively. The following glycosidation and further installation of biotin group give

the final product in 15% yield. To improve the solubility of the glycosylceramide in bioassay, a short acyl chain was incorporated. The assay results indicated that the appending of a small probe on C6 did not affect the presentation of α-galactosylceramide by CD1d of APC and recognition by TCR of iNKT cells. This biotin-labelled α-GalCer **2** can be easily detected during assays such as flow cytometry.

4. Experiments

4.1. General

All solvents were dried with solvent-purification system from Innovative Technology, Inc. All reagents were obtained from commercial sources and used without further purification. Analytical TLC was carried out on silica gel 60 F254 aluminum-backed plates (E. Merck). The 230–400 mesh size of the same absorbent was utilized for all chromatographic purifications. ^{1}H and ^{13}C NMR spectra were recorded at the indicated field strengths. The high-resolution mass spectra were collected at The Ohio State University Campus Chemical Instrumentation Center.

*4.1.1. Phenyl 1-thio-3,4-O-isopropylidene-6-O-(1-methoxyl-1-methylethyl)-β-D-galactopyranoside (**4**)*

To a solution of phenyl 1-thiogalactoside **3** (2.7 g, 10.0 mmol) in 2,2-dimethoxylpropane (25 mL) was added *p*-toluenesulfonic acid monohydrate (150 mg, 0.8 mmol). The resulting mixture was stirred for 4 h at room temperature. The reaction was quenched by addition of Et_3N (0.5 mL). After removal of the solvent, the residue was dissolved in ethyl acetate, and washed with water. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by silica gel chromatography (hexane/ethyl acetate 3:1) to give 2.8 g of **4** as colorless oil in 76% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.60 (m, 2H), 7.35 (m, 3H), 4.48 (d, J=10.2 Hz, 1H), 4.22 (dd, J=5.5, 2.1 Hz, 1H), 4.10 (t, J=6.7 Hz, 1H), 3.91 (ddd, J=7.1, 5.4, 2.1 Hz, 1H), 3.77 (dd, J=9.9, 7.0 Hz, 1H), 3.69 (dd, J=9.9, 5.2 Hz, 1H), 3.59 (ddd, J=9.8, 6.9, 2.4 Hz, 1H), 3.25 (s, 3H), 2.49 (br s, 1H), 1.44 (s, 3H), 1.40 (s, 3H), 1.39 (s, 3H), 1.35 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 132.4, 128.9, 127.9, 110.2, 100.2, 87.8, 79.1, 76.1, 73.8, 71.6, 60.5, 48.6, 28.1, 26.3, 24.4.

*4.1.2. Phenyl 1-thio-2-O-p-methoxybenzyl-3,4-O-isopropylidene-β-D-galactopyranoside (**5**)*

A solution of galactose derivative **4** (1.9 g, 5.2 mmol) in dry DMF (30 mL) was treated with 60% NaH (0.25 g, 6.2 mmol) for 30 min. PMBCl (0.84 mL, 6.2 mmol) was added by syringe and the reaction mixture was continued stirring for 5 h. The solvent was removed in vacuo. The residue was dissolved in ethyl acetate, washed with water. The organic layer was separated and dried over anhydrous Na_2SO_4. After concentrated, it was dissolved in methanol, stirred with silica gel (5 g) for 1 h. When TLC showed the reaction is completed, it was concentrated and purified by silica gel chromatography (hexane/ethyl acetate 4:1) to give 1.8 g of **5** as colorless oil in 81% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53 (m, 2H), 7.36 (d, J=8.5 Hz, 2H), 7.33–7.27 (m, 3H), 6.90 (d, J=8.5 Hz, 2H), 4.77 (d, J=11.0 Hz, 1H), 4.66 (d, J=9.5 Hz, 1H), 4.63 (d, J=11.0 Hz, 1H), 4.30 (t, J=6.0 Hz, 1H), 4.20 (dd, J=5.7, 1.7 Hz, 1H), 3.96 (dd, J=11.6, 7.3 Hz, 1H), 3.83 (s, 3H), 3.81 (m, 1H), 3.77 (dd, J=11.6, 3.9 Hz, 1H), 3.54 (dd, J=9.4, 6.3 Hz, 1H), 1.44 (s, 3H), 1.37 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.4, 133.5, 132.0, 131.5, 130.0, 129.9, 129.89, 129.1, 128.9, 127.5, 114.1, 113.8, 110.4, 86.0, 79.8, 78.0, 76.7, 73.9, 73.1, 62.6, 55.3, 27.8, 26.3; HRMS calcd for $C_{23}H_{28}O_6SNa$ ([M+Na]$^+$) 455.1504, found 455.1498.

*4.1.3. Phenyl 1-thio-2-O-p-methoxybenzyl-3,4-O-isopropylidene-6-O-methanesulfonyl-β-D-galactopyranoside (**6**)*

To a solution of the 6-hydroxyl-galactopyranoside **5** (1.13 g, 2.6 mmol) and Et_3N (0.72 mL, 5.2 mmol) in dry CH_2Cl_2 (15 mL) was added methanesulfonyl chloride (0.30 mL, 3.9 mmol) dropwise at 0 °C. After stirred for 30 min, the reaction was allowed to warm to room temperature and continued stirring for another 2 h. The reaction mixture was diluted with CH_2Cl_2 and washed with water. Dried over anhydrous Na_2SO_4 and purified by silica gel chromatography (hexane/ethyl acetate 3:1) to give 1.29 g of **6** as white solid in 97% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 (d, J=7.2 Hz, 2H), 7.35–7.25 (m, 5H), 6.89 (d, J=7.2 Hz, 2H), 4.75 (d, J=11.1 Hz, 1H), 4.73 (d, J=8.9 Hz, 1H), 4.62 (d, J=11.1 Hz, 1H), 4.49 (dd, J=11.4, 8.2 Hz, 1H), 4.39 (dd, J=11.4, 3.7 Hz, 1H), 4.33 (t, J=6.0 Hz, 1H), 4.19 (dd, J=5.9, 2.1 Hz, 1H), 4.05 (dt, J=6.9, 3.5 Hz, 1H), 3.82 (s, 3H), 3.56 (dd, J=8.9, 6.1 Hz, 1H), 2.92 (s, 3H), 1.45 (s, 3H), 1.36 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.4, 133.6, 131.5, 129.9, 129.7, 129.0, 127.5, 113.8, 110.6, 85.7, 79.1, 73.9, 73.0, 72.96, 69.2, 55.3, 37.5, 27.6, 26.2; HRMS calcd for $C_{24}H_{30}O_8S_2Na$ ([M+Na]$^+$) 533.1280, found 533.1293.

*4.1.4. Phenyl 1-thio-2-O-p-methoxybenzyl-3,4-O-isopropylidene-6-deoxy-6-azido-β-D-galactopyranoside (**7**)*

To a solution of the above compound **6** (1.28 g, 2.5 mmol) in DMF (10 mL) was added NaN_3 (0.85 g, 13.0 mmol). The resulting suspension was stirred at 80 °C for 36 h. The solvent was removed in vacuo, and the residue was dissolved in ethyl acetate, washed with water twice. The organic layer was separated and dried over anhydrous Na_2SO_4, purified by silica gel chromatography (hexane/ethyl acetate 5:1) to give 0.87 of **7** as white solid in 76% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 (m, 2H), 7.36 (d, J=8.6 Hz, 2H), 7.32 (m, 3H), 6.91 (d, J=8.6 Hz, 2H), 4.77 (d, J=11.0 Hz, 1H), 4.69 (d, J=9.1 Hz, 1H), 4.66 (d, J=11.0 Hz, 1H), 4.31 (t, J=6.0 Hz, 1H), 4.16 (dd, J=5.9, 2.1 Hz, 1H), 3.85 (m, 1H), 3.83 (s, 3H), 3.65 (dd, J=12.8, 7.9 Hz, 1H), 3.56 (dd, J=9.0, 6.1 Hz, 1H), 3.42 (dd, J=12.9, 5.1 Hz, 1H), 1.46 (s, 3H), 1.38 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.4, 133.5, 132.3, 129.9, 129.86, 128.9, 127.6, 113.8, 110.4, 86.5, 79.2, 77.6, 75.1, 73.6, 73.1, 55.3, 51.3, 27.6, 26.3; HRMS calcd for $C_{23}H_{27}N_3O_5S$ Na ([M+Na]$^+$) 480.1569, found 480.1582.

*4.1.5. 2-O-p-Methoxybenzyl-3,4-O-isopropylidene-6-deoxy-6-azido-α-D-galactopyranosyl-(1,1)-trichloroacetimide (**8**)*

A solution of the thiogalactose **7** (0.36 g, 0.79 mmol) in a mixture of acetone and water (9:1, 10 mL) at 0 °C was treated with NBS (0.32 g, 1.80 mmol) for 1 h. The reaction was quenched by addition solid $NaHCO_3$. The organic solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate and washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4 and purified by silica gel chromatography (hexane/ethyl acetate 2:1) to give 0.26 g of product as white solid in 91% yield.

To a solution of the above compound (0.26 g, 0.71 mmol) in dry CH_2Cl_2 (8 mL) were added CCl_3CN (0.71 mL, 7.1 mmol) and DBU (54 μL, 0.36 mmol) successively. After stirring for 2 h at room temperature, the solvent was evaporated and the residue was purified by silica gel chromatography (hexane/ethyl acetate 6:1) to give 0.28 g of **8** was white solid in 80% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.67 (s, 1H), 7.29 (d, J=8.4 Hz, 2H), 6.89 (d, J=8.4 Hz, 2H), 6.39 (d, J=3.5 Hz, 1H), 4.75 (d, J=11.8 Hz, 1H), 4.65 (d, J=11.8 Hz, 1H), 4.47 (t, J=6.4 Hz, 1H), 4.39 (m, 1H), 4.27 (dd, J=6.0, 2.4 Hz, 1H), 3.82 (s, 3H), 3.80 (m, 1H), 3.57 (dd, J=12.7, 7.6 Hz, 1H), 3.42 (dd, J=12.8, 5.6 Hz, 1H), 1.44 (s, 3H), 1.37 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 161.0, 159.4, 129.9, 129.5, 129.4, 114.0, 113.8, 110.0, 94.3, 91.2, 74.7, 74.0, 73.0, 72.5, 69.8, 55.3, 50.8, 27.5, 26.0; HRMS calcd for $C_{19}H_{23}Cl_3N_4O_6Na$ ([M+Na]$^+$) 531.0581, found 531.0591.

*4.1.6. (2S,3S,4R)-1-O-Triphenylmethyl-2-octanoylamino-octadecan-1,3,4-triol (**11**)*

To a solution of amide **10** (1.84 g, 4.15 mmol) in pyridine (40 mL) were added TrtCl (5.78 g, 20.7 mmol) and DMAP (100 mg,

0.82 mmol) and stirred at 50 °C for overnight. The solution was concentrated under reduced pressure. The residue was dissolved by EtOAc and washed by water. The organic layer was dried over anhydrous Na_2SO_4. The concentrated residue was purified by silica gel chromatography (hexane/ethyl acetate 4:1) to give 2.62 g of **11** in 92% yield as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42–7.44 (m, 6H), 7.31–7.35 (m, 6H), 7.25–7.28 (m, 3H), 6.06 (d, J=8.4 Hz, 1H), 4.27 (m, 1H), 3.59 (m, 1H), 3.52 (m, 2H), 3.42–3.35 (m, 2H), 3.15 (d, J=8.4 Hz, 1H), 2.28 (d, J=7.6 Hz, 1H), 2.17 (t, J=7.6 Hz, 2H), 1.71–1.60 (m, 4H), 1.45 (m, 2H), 1.32–1.25 (m, 28H), 0.90 (t, J=6.4 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.2, 143.2, 128.5, 128.1, 127.4, 87.7, 75.6, 73.2, 65.9, 63.0, 50.4, 36.9, 33.3, 31.9, 31.7, 29.7, 29.6, 29.4, 29.3, 29.0, 25.8, 25.7, 22.7, 22.6, 14.1, 14.06; HRMS calcd for $C_{45}H_{67}NO_4Na$ ([M+Na]$^+$) 708.4968, found 708.4962.

4.1.7. *(2S,3S,4R)-1-O-Triphenylmethyl-3,4-O-dibenzoyl-2-octanoylamino-octadecan-1,3,4-triol (**12**)*

To a solution of **11** (2.8 g, 4.08 mmol) in pyridine (40 mL) were added BzCl (2.8 mL, 24.5 mmol) and DMAP (49 mg, 0.4 mmol) and the reaction mixture was stirred for 8 h. The solution was concentrated under reduced pressure. The residue was partitioned between ethyl acetate and water. The organic layer was separated and washed with cooled 1 N HCl, saturated aqueous $NaHCO_3$ and brine. After dried over anhydrous Na_2SO_4, it was purified by silica gel chromatography (hexane/ethyl acetate 15:1) to give 3.14 g of **12** in 86% yield as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, J=8.0 Hz, 2H), 7.90 (d, J=7.6 Hz, 2H), 7.62–7.55 (m, 2H), 7.42 (dd, J=14.8, 7.2 Hz, 4H), 7.33–7.31 (m, 6H), 7.19–7.17 (m, 9H), 6.02 (d, J=9.6 Hz, 1H), 5.81 (dd, J=8.8, 2.4 Hz, 1H), 5.37 (m, 1H), 4.60 (m, 1H), 3.35 (dd, J=12.2, 4.5 Hz, 1H), 3.31 (dd, J=12.1, 3.6 Hz, 1H), 2.23–2.16 (m, 2H), 1.91–1.86 (m, 2H), 1.68–1.62 (m, 2H), 1.40–1.23 (m, 32H), 0.91–0.88 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.8, 166.4, 165.1, 143.3, 133.1, 132.9, 130.2, 129.9, 129.8, 129.76, 128.6, 128.4, 129.36, 127.8, 127.0, 86.8, 74.2, 72.8, 61.7, 48.6, 36.9, 31.9, 31.7, 29.7, 29.66, 29.6, 29.57, 29.5, 29.4, 29.1, 28.5, 25.7, 25.69, 22.7, 22.66, 14.1, 14.09; HRMS calcd for $C_{59}H_{75}NO_6Na$ ([M+Na]$^+$) 916.5492, found 916.5497.

4.1.8. *(2S,3S,4R)-3,4-O-Dibenzoyl-2-octanoylamino-octadecan-1,3,4-triol (**13**)*

To a solution of **12** (1.8 g, 2.0 mmol) in CH_2Cl_2 and MeOH (2:1, 20 mL) were added *p*-toluenesulfonic acid monohydrate (400 mg, 2.1 mmol) and stirred for 3 h. The solution was quenched by addition Et_3N (0.2 mL). The solution was concentrated in vacuo. The concentrated residue was purified by silica gel chromatography (hexane/ethyl acetate 5:1) to give 1.2 g of **13** in 92% yield as colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.04 (d, J=1.0 Hz, 2H), 7.95 (d, J=1.0 Hz, 2H), 7.63 (t, J=7.5 Hz, 1H), 7.49–7.54 (m, 3H), 7.38 (d, J=7.5 Hz, 2H), 6.50 (d, J=9.5 Hz, 1H), 5.46 (dd, J=9.5, 2.5 Hz, 1H), 5.39 (dt, J=9.3, 3.1 Hz, 1H), 4.41 (tt, J=9.4, 2.6 Hz, 1H), 3.66 (dd, J=12.0, 2.0 Hz, 2H), 2.95 (br, 1H), 2.29 (t, J=7.5 Hz, 2H), 2.03 (m, 2H), 1.70 (m, 2H), 1.24–1.46 (m, 32H), 0.88 (J=7.0 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.3, 167.0, 166.4, 133.8, 133.1, 130.0, 129.9, 129.7, 129.2, 128.7, 128.4, 74.0, 73.7, 50.0, 36.9, 31.9, 31.7, 29.7, 29.68, 29.65, 29.6, 29.57, 29.4, 29.36, 29.3, 29.1, 28.5, 25.8, 22.7, 22.6, 14.1, 14.07; HRMS calcd for $C_{40}H_{61}NO_6Na$ ([M+Na]$^+$) 674.4397, found 674.4401.

4.1.9. *2-O-p-Methoxybenzyl-3,4-O-isopropylidene-6-deoxy-6-azido-α-D-galactopyranosyl-(1,1)-(2S,3S,4R)-2-octanoylamino-3,4-O-dibenzoyl-octadecan-1,3,4-triol (**14**)*

A suspension of the azido-galactosyl donor **8** (0.28 g, 0.55 mmol), ceramide acceptor **13** (0.26 g, 0.40 mmol) and 4 Å molecular sieve (0.5 g) in a mixture of ethyl ether and tetrahydrofuran (5:1, 4 mL) was stirred at room temperature for 30 min. After the mixture was cooled to −25 °C, TMSOTf (23 μL, 0.11 mmol) was added by syringe and continued stirring for 2 h. The reaction was quenched by addition Et_3N (0.2 mL) and filtered through Celite pad. The filtrate was concentrated and the residue was purified by silica gel chromatography (hexane/ethyl acetate 7:1) to give 218 mg of **14** as white solid in around 55% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.06 (m, 2H), 7.97 (m, 2H), 7.64 (m, 1H), 7.57 (m, 1H), 7.51 (m, 2H), 7.40 (m, 2H), 7.22 (d, J=8.6 Hz, 2H), 6.77 (d, J=8.6 Hz, 2H), 6.73 (d, J=9.5 Hz, 1H), 5.72 (dd, J=9.3, 2.8 Hz, 1H), 5.37 (m, 1H), 4.72 (d, J=3.5 Hz, 1H), 4.66 (d, J=11.8 Hz, 1H), 4.63 (m, 1H), 4.59 (d, J=11.8 Hz, 1H), 4.28 (t, J=6.1 Hz, 1H), 4.17 (m, 1H), 4.11 (dd, J=5.8, 2.4 Hz, 1H), 3.83 (dd, J=10.8, 3.1 Hz, 1H), 3.77 (s, 3H), 3.70 (dd, J=10.9, 3.6 Hz, 1H), 3.53 (dd, J=13.3, 8.3 Hz, 1H), 3.52 (dd, J=7.0, 3.4 Hz, 1H), 3.38 (dd, J=12.84.8 Hz, 1H), 2.24 (t, J=7.8 Hz, 2H), 1.93 (m, 2H), 1.65 (m, 2H), 1.43–1.26 (m, 32H), 1.36 (s, 3H), 1.31 (s, 3H), 0.90 (t, J=7.2 Hz, 3H), 0.89 (t, J=7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.1, 166.3, 165.4, 159.3, 133.4, 133.0, 130.1, 130.0, 129.8, 129.75, 129.7, 129.5, 128.6, 128.4, 113.7, 109.6, 98.4, 75.2, 75.1, 74.0, 73.4, 72.4, 72.3, 68.2, 67.8, 55.2, 51.2, 48.4, 36.8, 31.9, 31.8, 29.7, 29.69, 29.67, 29.64, 29.6, 29.57, 29.4, 29.3, 29.1, 28.2, 27.7, 26.1, 25.8, 25.7, 22.7, 22.68, 14.14, 14.1; HRMS calcd for $C_{57}H_{82}N_4O_{11}Na$ ([M+Na]$^+$) 1021.5878, found 1021.5881.

4.1.10. *2-O-p-Methoxybenzyl-3,4-O-isopropylidene-6-deoxy-6-azido-α-D-galactopyranosyl-(1,1)-(2S,3SR,4R)-2-octanoylamino-octadecan-1,3,4-triol (**15**)*

A solution of the protected glycoceramide **14** (200 mg, 0.2 mmol) and freshly prepared NaOMe (11 mg, 0.2 mmol) in dry MeOH (5 mL) was heated to refluxing for 24 h. After cooled to room temperature, it was neutralized by Dowex ion-exchange resin and filtered. The filtrate was concentrated and the residue was purified by silica gel chromatography (hexane/ethyl acetate 3:1) to give 112 mg of **15** as white solid in 71% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.28 (d, J=8.6 Hz, 2H), 6.89 (d, J=8.6 Hz, 2H), 6.33 (d, J=8.5 Hz, 1H), 4.86 (d, J=3.8 Hz, 1H), 4.79 (d, J=11.6 Hz, 1H), 4.61 (d, J=11.6 Hz, 1H), 4.32 (dd, J=7.2, 5.8 Hz, 1H), 4.27 (m, 1H), 4.16 (dd, J=5.7, 2.5 Hz, 1H), 4.07 (m, 1H), 4.00 (dd, J=10.5, 3.9 Hz, 1H), 3.83 (m, 1H), 3.81 (s, 3H), 3.57–3.53 (m, 3H), 3.49 (dd, J=6.2, 3.7 Hz, 1H), 3.40 (dd, J=12.9, 5.5 Hz, 1H), 2.20 (t, J=7.0 Hz, 2H), 1.63 (m, 2H), 1.49–1.26 (m, 34H), 1.43 (s, 3H), 1.35 (s, 3H), 0.89 (t, J=6.7 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.1, 159.6, 129.8, 129.3, 113.9, 109.7, 98.3, 76.1, 76.06, 75.4, 73.3, 73.2, 72.4, 69.9, 67.3, 36.8, 33.4, 31.9, 31.7, 29.7, 29.66, 29.3, 29.1, 27.9, 26.2, 25.9, 25.8, 22.7, 22.6, 14.1, 14.05; HRMS calcd for $C_{43}H_{74}N_4O_9Na$ ([M+Na]$^+$) 813.5354, found 813.5352.

4.1.11. *2-O-p-Methoxybenzyl-3,4-O-isopropylidene-6-deoxy-6-amino-α-D-galactopyranosyl-(1,1)-(2S,3S,4R)-2-octanoylamino-octadecan-1,3,4-triol (**16**)*

A solution of the azido-glycoceramide **15** (160 mg, 0.20 mmol) and PPh_3 (79 mg, 0.30 mmol) in benzene and trace amount of water was heated to 50 °C for 6 h. The solvent was evaporated and the residue was purified by silica gel chromatography (ethyl acetate to dichloromethane/methanol 5:1) to give 132 mg of amine **16** in 86% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.26 (d, J=8.6 Hz, 2H), 6.87 (d, J=8.6 Hz, 2H), 6.66 (d, J=8.3 Hz, 1H), 4.83 (d, J=3.6 Hz, 1H), 4.74 (d, J=11.8 Hz, 1H), 4.62 (d, J=11.8 Hz, 1H), 4.28 (t, J=6.5 Hz, 1H), 4.13 (dd, J=5.5, 2.1 Hz, 1H), 3.98 (m, 1H), 3.79 (s, 3H), 3.75 (dd, J=10.5, 4.1 Hz, 1H), 3.51–3.48 (m, 3H), 3.35 (br, 2H), 3.03 (dd, J=13.1, 8.6 Hz, 1H), 2.90 (dd, J=13.1, 3.6 Hz, 1H), 2.18 (t, J=7.4 Hz, 2H), 1.64 (m, 3H), 1.49 (m, 1H), 1.40 (s, 3H), 1.32 (s, 3H), 1.31–1.21 (m, 32H), 0.88 (t, J=6.8 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.4, 159.5, 129.7, 129.6, 113.8, 109.5, 98.1, 76.2, 75.5, 74.1, 72.7, 72.2, 68.5, 68.2, 55.2, 50.0, 42.3, 36.7, 33.7, 31.9, 31.7, 29.8, 29.78, 29.7, 29.66, 29.4, 29.3, 29.1, 28.0, 26.3, 26.0, 25.8, 22.7, 22.6, 14.1, 14.1; $C_{43}H_{76}N_2O_9Na$ ([M+Na]$^+$) 787.5449, found 787.5444.

*4.1.12. 2-O-p-Methoxybenzyl-3,4-O-isopropylidene-6-deoxy-6-biotinamino-α-D-galactopyranosyl-(1,1)-(2S,3S,4R)-2-octanoylamino-octadecan-1,3,4-triol (**18**)*

The above amine **16** (40 mg, 0.052 mmol) was dissolved in THF (1.5 mL), and Et_3N (16 μL, 0.12 mmol) and biotin-OSu **17** (26 mg, 0.072 mmol) was added. The resulting mixture was stirred at room temperature overnight. The solvent was removed in vacuo, and the residue was purified by silica gel chromatography (dichloromethane/methanol 25:1) to afford 44 mg of **18** as white foam in 85% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.27 (d, J=7.6 Hz, 2H), 6.88 (d, J=7.6 Hz, 2H), 6.78 (br, 1H), 6.69 (br, 1H), 4.82 (d, J=2.6 Hz, 1H), 4.74 (d, J=11.8 Hz, 1H), 4.61 (d, J=11.8 Hz, 1H), 4.49 (br, 1H), 4.34–4.26 (m, 3H), 4.16 (d, J=4.3 Hz, 1H), 4.12 (d, J=7.8 Hz, 1H), 3.88 (m, 1H), 3.81 (s, 3H), 3.76 (m, 1H), 3.70 (m, 1H), 3.53–3.49 (m, 3H), 3.31 (t, J=10.8 Hz, 1H), 3.14 (m, 1H), 2.89 (d, J=10.9 Hz, 1H), 2.75 (d, J=12.4 Hz, 1H), 2.24 (m, 2H), 2.20 (t, J=7.2 Hz, 2H), 1.76–1.58 (m, 7H), 1.53–1.44 (m, 3H), 1.41 (s, 3H), 1,34 (s, 3H), 1.32–1.23 (m, 34H), 0.89 (t, J=6.7 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8, 173.5, 164.0, 159.5, 129.7, 129.6, 113.9, 109.6, 98.1, 76.3, 76.1, 75.4, 73.9, 72.8, 72.2, 68.3, 66.4, 62.1, 60.3, 55.7, 55.3, 49.7, 40.5, 36.7, 35.7, 33.7, 31.9, 31.8, 29.8, 29.79, 29.76, 29.7, 29.68, 29.4, 29.3, 29.1, 28.1, 28.0, 26.3, 26.0, 25.8, 25.6, 22.7, 22.66, 14.1, 14.12; HRMS calcd for $C_{53}H_{90}N_4O_{11}SNa$ ($[M+Na]^+$) 1013.6225, found 1013.6251.

*4.1.13. 6-Deoxy-6-biotinamino-α-D-galactopyranosyl-(1,1)-(2S,3S,4R)-2-octanoylamino-octadecan-1,3,4-triol (**2**)*

A solution of protected biotin-galactosylceramide **18** (26 mg, 0.026 mmol) in a mixture of CH_2Cl_2 and H_2O (30:1, 1.5 mL) was treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (8.9 mg, 0.039 mmol) for 4 h. After neutralized with Et_3N, it was purified by column. Then it was dissolved in MeOH, and *p*-toluenesulfonic acid (2.5 mg, 0.013 mmol) was added. The resulting mixture was stirred at room temperature for 6 h. After neutralized by addition of Et_3N and concentrated, the residue was purified by silica gel chromatography (chloroform/methanol/water 6:1:0.1) to give 11 mg of **2** as white solid in 51% yield. ^{1}H NMR (500 MHz, pyridine-d_6) δ 8.81 (t, J=5.2 Hz, 1H), 8.56 (d, J=8.6 Hz, 1H), 7.53 (br, 1H), 7.44 (br, 1H), 7.01 (br, 1H), 6.38 (br, 2H), 6.13 (br, 1H), 5.49 (d, J=3.4 Hz, 1H), 5.22 (br, 1H), 4.59 (dd, J=10.8, 5.0 Hz, 1H), 4.57 (dd, J=12.9, 3.5 Hz, 1H), 4.51 (t, J=6.6 Hz, 1H), 4.47 (t, J=6.2 Hz, 1H), 4.36 (t, J=4.5 Hz, 1H), 4.32–4.24 (m, 5H), 4.18 (m, 1H), 3.88 (m, 1H), 3.20 (dd, J=11.2, 7.6 Hz, 1H), 2.93 (dd, J=12.5, 4.9 Hz, 1H), 2.83 (d, J=12.4 Hz, 1H), 2.48–2.43 (m, 4H), 2.26 (m, 1H), 1.90 (m, 2H), 1.83–1.74 (m, 5H), 1.67–1.57 (m, 3H), 1.42–1.09 (m, 32H), 0.84 (t, J=6.4 Hz, 3H), 0.78 (t, J=6.5 Hz, 3H); ^{13}C NMR (125 MHz, pyridine-d_6) δ 173.6, 173.1, 164.2, 101.1, 76.5, 72.3, 71.0, 70.9, 70.4, 69.8, 68.3, 62.2, 60.4, 56.0, 51.0, 40.9, 40.8, 36.6, 36.0, 34.2, 31.9, 31.7, 30.2, 30.0, 29.8, 29.77, 29.7, 29.4, 29.39, 29.2, 28.8, 28.78, 26.3, 26.2, 26.0, 22.7, 22.6, 14.1, 14.0; HRMS calcd for $C_{42}H_{78}N_4O_{10}SNa$ ($[M+Na]^+$) 853.5336, found 853.5318.

4.2. Cytokine release assay

1 and **2** were dissolved in DMSO at a concentration of 1.0 mg/mL and then diluted with medium to the indicated concentration. They were pulsed to 100,000 cytoplasmic tail-deleted CD1d-transfected A20 cells for overnight. After washing with culture medium, the A20/CD1 cells were mixed with 50,000 Vα14*i* NKT hybridoma DN3A4-1.2 cells and co-cultured for 24 h. The released IL2 in the supernatant was measured by the ELISA assay. Data is representative of 3 independent experiments.

4.3. Flow cytometry assay

The A20/CD1 cells were treated overnight with compounds **1**, **2** and a DMSO control at the concentration of 1 μg/mL. After washing away the extra glycolipids in the medium, the treated cells were stained by Stravidin–FITC conjugate following the blocking procedure. The analysis was carried out on BD FACS Calibur and the data was analyzed by FLOWJO V.7.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2009.06.007.

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