Design, Synthesis, and Functional Activity of Labeled CD1d Glycolipid Agonists

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S Supporting Information

ABSTRACT: Invariant natural killer T cells (iNKT cells) are restricted by CD1d molecules and activated upon CD1d-mediated presentation of glycolipids to T cell receptors (TCRs) located on the surface of the cell. Because the cytokine response profile is governed by the structure of the glycolipid, we sought a method for labeling various glycolipids to study their in vivo behavior. The prototypical CD1d agonist, α-galactosyl ceramide (α-GalCer) **1**, instigates a powerful immune response and the generation of a wide range of

cytokines when it is presented to iNKT cell TCRs by CD1d molecules. Analysis of crystal structures of the TCR−α-GalCer−CD1d ternary complex identified the α-methylene unit in the fatty acid side chain, and more specifically the pro-S hydrogen at this position, as a site for incorporating a label. We postulated that modifying the glycolipid in this way would exert a minimal impact on the TCR−glycolipid−CD1d ternary complex, allowing the labeled molecule to function as a good mimic for the CD1d agonist under investigation. To test this hypothesis, the synthesis of a biotinylated version of the CD1d agonist threitol ceramide (ThrCer) was targeted. Both diastereoisomers, epimeric at the label tethering site, were prepared, and functional experiments confirmed the importance of substituting the pro-S, and not the pro-R, hydrogen with the label for optimal activity. Significantly, functional experiments revealed that biotinylated ThrCer (S)-**10** displayed behavior comparable to that of ThrCer **5** itself and also confirmed that the biotin residue is available for streptavidin and antibiotin antibody recognition. A second CD1d agonist, namely α-GalCer C20:2 **4**, was modified in a similar way, this time with a fluorescent label. The labeled α-GalCer C20:2 analogue (**11**) again displayed functional behavior comparable to that of its unlabeled substrate, supporting the notion that the α-methylene unit in the fatty acid amide chain should be a suitable site for attaching a label to a range of CD1d agonists. The flexibility of the synthetic strategy, and late-stage incorporation of the label, opens up the possibility of using this labeling approach to study the in vivo behavior of a wide range of CD1d agonists.
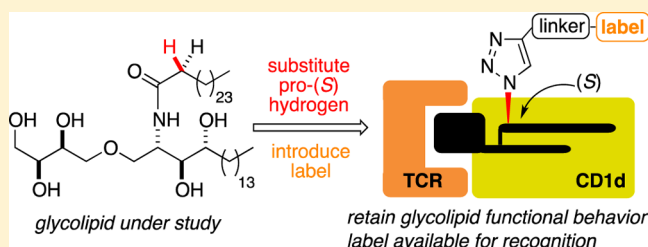
■ INTRODUCTION

Invariant natural killer T (iNKT) cells are a distinctive subset of T lymphocytes that express an invariant $\alpha\beta$ T cell receptor (TCR)[1−3] and play an important role in infectious diseases,[4,5] tumors,[6,7] and autoimmune diseases,[8] such as type I diabetes[9,10] and lupus.[11] In contrast to conventional CD4+ and CD8+ T lymphocytes, which recognize foreign peptides bound to the major histocompatibility complex (MHC) class I or MHC class II molecule, iNKT cells recognize a range of foreign and endogenous lipids bound to the nonpolymorphic CD1d molecule.[12,13] α-Galactosyl ceramide (α-GalCer) **1** is the prototypical iNKT cell agonist (Figure 1). Its presentation by CD1d molecules to TCRs located on iNKT cells results in a powerful immune response involving the secretion of both pro-inflammatory cytokines, including interferon-γ (IFNγ) (Th1-type), and regulatory cytokines, including interleukin-4 (IL-4) (Th2-type).[14] α-GalCer **1** has served as a valuable template for

preparing structural analogues,[15] such as compound OCH **2**, a more Th2 cytokine-biasing CD1d agonist,[16,17] and the C-glycosyl analogue of α-GalCer, **3**[18,19] (Figure 1), which causes a more Th1 cytokine-biasing immune response upon iNKT cell activation.[20−22] Threitol ceramide (ThrCer) **5** (Figure 1) also successfully activates iNKT cells and, significantly, overcomes the problematic iNKT cell activation-induced anergy, which is associated with iNKT cell stimulation by α-GalCer.[23−25] Moreover, we have recently shown that amide isosteres of this attractive nonglycosidic CD1d agonist can also be used to bias the cytokine response toward Th1-type cytokines.[26]

All of the aforementioned glycolipids activate iNKT cells in a similar fashion, namely through CD1d-mediated presentation

1 (α-GalCer) X = O, n = 13, R = $(CH_2)_{24}CH_3$,
2 (OCH) X = O, n = 4, R = $(CH_2)_{22}CH_3$,
3 (α-*C*-GalCer) X = CH_2, n = 13, R = $(CH_2)_{24}CH_3$,
4 (α-GalCer C20:2) X = O, n = 13,
R = (*Z*,*Z*)-$(CH_2)_9(CH=CHCH_2)_2(CH_2)_3CH_3$
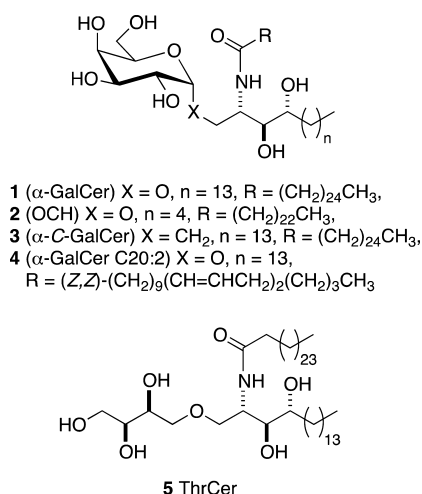
5 ThrCer

Figure 1. Glycolipid agonists of CD1d.

to TCRs located on these cells; however, the origins of the different immune responses observed in each case remain poorly understood.[27] Thus, while CD1d and TCR binding affinity likely play a role,[28,29] other factors, such as the cellular location of glycolipid loading on the CD1d molecule, may also be important in determining the ensuing immune response.[30] For example, the Th2 cytokine-biasing α-GalCer analogues, **2** and **4**, are loaded rapidly onto CD1d molecules located at the surface of antigen-presenting cells.[22,31] In contrast, the disaccharide Gal(α1→2)α-GalCer requires cellular internalization and lysosomal processing before loading can take place.[32] α-GalCer **1** also exhibits a requirement for endosomal loading before surface presentation can take place, principally within lipid rafts.[22,33] To investigate the cellular behavior of different CD1d agonists, we sought a method for labeling glycolipids, which would allow us to study the mechanisms that control their uptake by professional antigen-presenting cells (APC). Herein, we show that the methylene unit, and more specifically the *pro-S* hydrogen, α to the amide functionality provides a new site for appending a label to this class of molecules. We describe a general and flexible synthesis strategy that allows structural variation in the glycolipid as well as late-stage introduction of the label.

■ EXPERIMENTAL PROCEDURES

General Experimental Procedures. Melting points were determined using open capillaries and are uncorrected. Infrared spectra were recorded either neat or as thin films between NaCl disks. The intensity of each band is described as s (strong), m (medium), or w (weak), with the prefix v (very) and suffix br (broad) where appropriate. ^{1}H NMR spectra were recorded at 500, 400, or 300 MHz. ^{13}C NMR spectra were recorded at 125, 100, or 75 MHz. Chemical shifts are reported as δ values (parts per million) referenced to the following solvent signals: $CHCl_3$, δ_H 7.26; $CDCl_3$, δ_C 77.0; CH_3OH, δ_H 3.31; CD_3OD, δ_C 49.9. For spectra recorded in a 1:2 $CD_3OD/CDCl_3$ mixed solvent system, chemical shifts are referenced to the residual methanol peak. The term "stack" is used to describe a region in which resonances arising from nonequivalent nuclei are coincident and multiplet, m, to describe a resonance arising from a single nucleus (or equivalent nuclei) but where coupling constants cannot be readily assigned. Mass spectra were recorded utilizing electrospray ionization (and a MeOH mobile phase) and are

reported as *m/z* (%). HRMS spectra were recorded using a lock mass incorporated into the mobile phase.

All reagents were obtained from commercial sources and used without further purification unless stated otherwise. Anhydrous solvents were stored over 4 Å molecular sieves and under an Ar atmosphere. All solutions are aqueous and saturated unless stated otherwise.

All reactions were monitored by TLC using precoated aluminum-backed ICN silica plates (60A F_{254}) and visualized by UV detection (at 254 nm) and staining with 5% phosphomolybdic acid in EtOH (MPA spray). Column chromatography was performed on silica gel (particle size of 40−63 μm mesh).

(2″S)-Biotinylated ThrCer [(S)-10]. A $CuSO_4$ solution (12 μL of a 0.5 M solution, 6 μmol) and a sodium ascorbate solution (26 μL of a 1.0 M solution, 26 μmol) were added to a solution of azide (S)-**17** (25 mg, 0.029 mmol) and alkyne **18** (13 mg, 0.029 mmol) in a *t*-BuOH/H_2O mixture (1 mL, 1:1) at room temperature. The reaction mixture was heated for 10 h at 50 °C, diluted with $CHCl_3$ (10 mL), and washed with brine (3 mL). The phases were separated, and the aqueous layer was extracted with $CHCl_3$ (2 × 5 mL). The combined organic layers were dried over Na_2SO_4, and the volatiles were removed under reduced pressure. Purification of the residue by flash column chromatography (10% MeOH in $CHCl_3$) afforded triazole (S)-**10** as a white paste (28 mg, 77%): R_f = 0.20 (10% CH_3OH in $CHCl_3$); the poor solubility of this amphiphilic compound at room temperature prevented us from obtaining reliable optical rotation data; ν_{max} (film) 3332br (O−H, N−H), 1672s (C=O) cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD, 2:1) δ 0.84 (t, *J* = 6.9 Hz, 6H, 2 × CH_2CH_3), 1.17−1.36 (stack, 70H), 1.38−1.46 (stack, 2H), 1.52−1.75 [stack, 4H, C(12‴)H_2 (middle of stack), C(14‴)H_aH_b (LHS of stack), C(14‴)H_aH_b (RHS of stack)], 2.01−2.09 [stack, 1H, C(3″)H_aH_b], 2.10−2.22 [stack, 3H, C(3″)H_aH_b, C(11‴)H_2], 2.71 [d, *J* = 12.8 Hz, 1H, C(18‴)H_aH_b], 2.89 [dd, *J* = 12.8, 5.0 Hz, 1H, C(18‴)H_aH_b], 3.13−3.19 [m, 1H, C(15‴)*H*], 3.33−3.38 [stack, 2H, C(9‴)H_2], 3.47−3.71 [stack, 18H, including C(1)H_aH_b, C(4‴)H_2], 3.73 [dd, *J* = 10.0, 5.0 Hz, 1H, C(1)H_aH_b], 3.75−3.79 (m, 1H), 4.17−4.22 [m, 1H, C(2)*H*], 4.30 [dd, *J* = 7.8, 4.3 Hz, 1H, C(16‴)*H*], 4.48 [dd, *J* = 7.8, 4.8 Hz, 1H, C(17‴)*H*], 4.65 [s, 2H, C(3″)H_2], 5.27 [dd, *J* = 8.5, 6.5 Hz, 1H, C(2″)*H*], 7.99 [s, 1H, C(1‴)*H*], exchangeable hydrogens not observed; ^{13}C NMR (125 MHz, $CDCl_3$/CD_3OD, 2:1) δ 14.3 [CH_3, C(18), C(26″) (resonance overlap)], 23.2 (CH_2), 26.1 [CH_2, C(12‴)], 26.2 (CH_2), 26.4 (CH_2), 28.8 [CH_2, C(14‴)], [29.1, 29.5, 29.87, 29.90, 30.1, 30.19, 30.22, 32.5 (CH_2, alkyl chain, some resonance overlap)], 36.2 [CH_2, C(11‴)], 39.8 [CH_2, C(9‴)], 40.8 [CH_2, C(18‴)], 51.3 [CH, C(2)], 56.3 [CH, C(15‴)], 60.9 [CH, C(17‴)], 62.6 [CH, C(16‴)], 63.9 [CH_2, C(4′)], 64.7 [CH, C(2″)], 64.8 [CH_2, C(3″)], 70.17 [CH_2, C(4‴)], 70.24 (CH_2), 70.5 (CH_2), 70.6 (CH_2), 70.8 (CH), 70.99 (CH_2), 71.03 (CH_2), 72.5 (CH), 72.8 (CH), 73.4 [CH_2, C(1′)], 75.0 [CH, C(3)], 123.3 [CH, C(1‴)], 145.3 [C, C(2‴)], 165.1 (C, NHC=ONH), 169.1 [C, C(1″)], 175.2 [C, C(10‴)]; MS (TOF ES+) *m/z* 1276.8 ([M + Na]$^+$, 100%), 649.9 (7), 398.1 (5); HRMS (TOF ES+) calcd for $C_{67}H_{127}N_7O_{12}SNa$ [M + Na]$^+$ 1276.9161, found 1276.9166.

Fluor 488-Labeled α-GalCer (11) (1:1 mixture of regioisomers in the label). A $CuSO_4$ solution (6 μL of a 0.5 M solution, 3 μmol) and a sodium ascorbate solution (13 μL of a 1.0 M solution, 13 μmol) were added to a solution of azide **22** (1.4 mg, 1.7 μmol) and alkyne **23** (1.0 mg, 1.7 μmol) in a *t*-BuOH/H_2O mixture (0.5 mL, 1:1) at room temperature.

The reaction mixture was heated for 10 h at 50 °C, diluted with CHCl$_3$ (5 mL), and washed with brine (1.5 mL). The phases were separated, and the aqueous layer was extracted with CHCl$_3$ (2 × 2.5 mL). The combined organic layers were dried over Na$_2$SO$_4$, and the volatiles were removed under reduced pressure. Purification of the residue by flash column chromatography (CHCl$_3$/MeOH/H$_2$O, 65:25:4) afforded triazole **11** as a red solid (1.4 mg, 59%, 1:1, mixture of regioisomers in the label): R_f = 0.32 (CHCl$_3$/MeOH/H$_2$O, 65:25:4); ^{1}H NMR (400 MHz, CDCl$_3$/CD$_3$OD, 2:1) δ 0.83 (t, J = 6.7 Hz, 3H), 0.84 (t, J = 6.7 Hz, 3H), 1.10−1.39 (stack, 44H), 1.40−1.65 (m, 1H), 1.65−1.89 (m, 1H), 1.92−2.03 (stack, 5H), 2.12−2.30 (1H, m), 2.66−2.79 (stack, 2H), 3.46−3.79 (stack, 22H), 3.86 (dd, J = 10.8, 4.8 Hz, 1H), 3.90 (br s, 1H), 4.12−4.22 (m, 1H), 4.55−4.67 (m, 2H), 4.88 (d, J = 3.6 Hz, 1H), 5.21−5.41 (stack, 5H), 6.63−6.77 (stack, 4H), 6.96−7.09 (stack, 2H), 7.31 (d, J = 9.0 Hz, 0.5H), 7.76 (br s, 0.5H), 7.92 (s, 1H), 8.13−8.24 (m, 1H), 8.31 (d, J = 9.0 Hz, 0.5H), 8.72 (br s, 0.5H); MS (TOF ES−) m/z 1396.8 ([M − H]$^-$, 10%), 1218.8 (5, [M − galactose]$^-$), 1004.6 (15), 915.6 (25), 850.7 (65), 815.7 (35), 451.5 (95), 423.5 (100), 179.1 (20, [galactose]$^-$).

Experimental procedures and characterization data for compounds **25**, **26**, **13a**, **13b**, **14**, (2S)-methyl 2-azidohexacosanoate, (S)-**15**, (S)-**17**, **18**, (S)-**10**, (rac)-**15**, **17**, **10**, **11**, **27**−**30**, and **20** can be found in the Supporting Information.

Mice, Cell Lines, and Cultures. C57 BL/6 mice were used in accordance with the Animals (Scientific Procedures) Act 1986. iNKT cells were generated from healthy blood donors as previously described.[29,34] iNKT cell cultures were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 1% Pen/Strep, 5% human serum (Gibco), and 500 units of IL-2/mL. Functional assays and C1R CD1d lipid pulsing were performed in RPMI-1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 1% Pen/Strep (Gibco), and 10% fetal calf serum (FCS) (Sigma).

iNKT Cell TCR Tetramer Staining. The synthesis of soluble iNKT TCR heterodimers has been described previously.[29] C1R CD1d cells were pulsed with different concentrations of the indicated iNKT cell agonists for 16 h. Cells were washed with a PBS/10% FCS mixture and incubated with 0.5−1.0 μg of iNKT cell TCR tetramer or equivalent amounts of streptavidin on ice for 30 min. Cells were also stained with an antibiotin FITC-conjugated antibody (Jackson Laboratories) for 30 min on ice. Cells were washed, and samples were analyzed on a FACScalibur instrument (BD). Data analysis was performed using Flowjo (Treestar).

iNKT Cell Activation and Cytokine Detection. A polyclonal human iNKT cell line, or a mouse iNKT cell hybridoma (DN32), was incubated for 36 h with monocyte-derived DCs in the presence or absence of iNKT cell agonists and the level of cytokines released by iNKT cells measured in the supernatants. The concentrations of human IFN-γ and mouse IL-2 were measured by an enzyme-linked immunosorbent assay (ELISA) as previously described.[24] All samples were tested at the same time on a single ELISA plate for each assay. In addition, for in vitro activation of mouse iNKT cells, 5×10^5 splenocytes from C57 BL/6 mice were pulsed with various concentrations of lipids for 48 h. Supernatants were removed, and the presence of IFN-γ was determined by an ELISA.[24]

■ RESULTS AND DISCUSSION

Chemical labeling studies of α-GalCer **1** have previously introduced the label at C(6)OH of the galactose residue;[35,36]
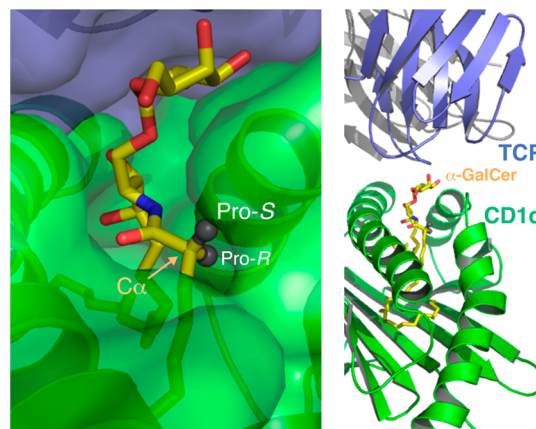


Figure 2. Substituents appended to the *pro-S* hydrogen substituent in the α position of the N-acyl chain (highlighted with the arrow) should extend beyond and away from the TCR−glycolipid−CD1d recognition site (structure taken from Protein Data Bank entry 2PO6[43]).

however, this strategy was rejected for a number of reasons. First, we desired a labeling methodology that could be employed generally for a range of CD1d agonists, including truncated sugar analogues such as ThrCer **5**, which lack a C(6) tethering site.[37] Second, crystal structures of C(6)-derivatized α-GalCer analogues reveal the functionality at C(6) can exploit a hydrophobic binding site in CD1d,[38] which is not available to α-GalCer, and that this additional binding affects the biological activity,[39] something to be avoided when trying to retain the activity of the agonist under investigation. Labels have also been appended to the terminus of the N-acyl chain of α-GalCer;[40−42] however, we reasoned that a CD1d agonist modified in this fashion would result in the label being buried deep inside the A′ pocket of the protein molecule. This might significantly alter the binding conformation of the glycolipid under study, and analogues biotinylated in this way would mean the biotin label would likely be unavailable for streptavidin or antibiotin antibody recognition. Analysis of X-ray structures of the TCR−α-GalCer−CD1d complex,[43] and similar ternary complexes involving α-GalCer analogues,[40−42] reveals the glycolipid binds to the CD1d molecule in a similar fashion, with the N-acyl chain occupying the hydrophobic A′ pocket of the protein and the ceramide base the less voluminous F′ pocket, leaving the polar sugar residue surface-exposed for TCR recognition. For our purposes, this analysis allowed us to identify the α-methylene unit of the amide chain, and specifically the *pro-S* hydrogen at this position, as being amenable to substitution because this group is directed out toward bulk solvent in the ternary complex (Figure 2). We postulated that a label incorporated into this position would protrude away from the ternary TCR−glycolipid−CD1d complex without deleteriously affecting its conformation and, at the same time, also permit recognition of a tethered reporter group such as a biotin label.
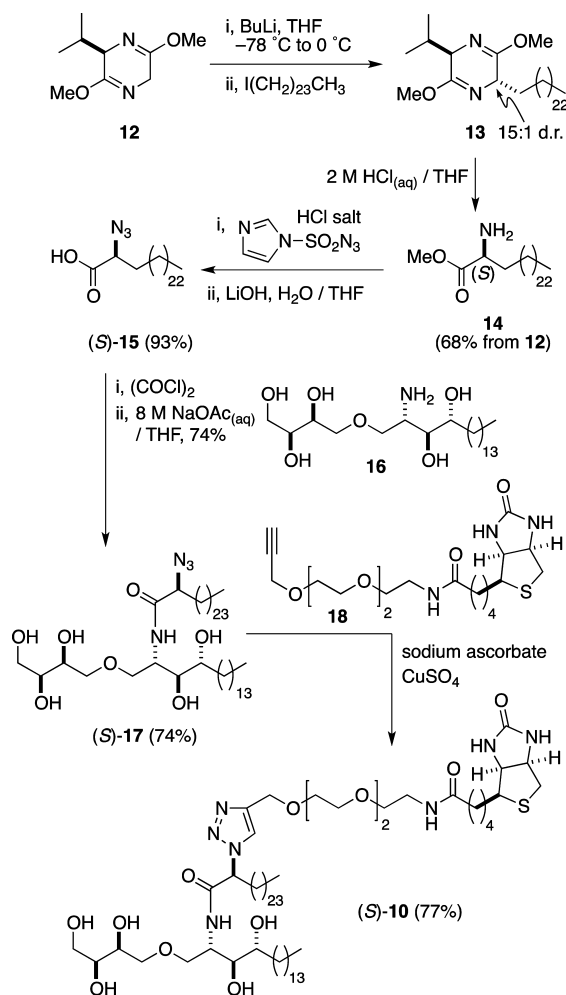
Because of the challenging practical issues associated with handling glycolipids, and the high cost of many labels, our chosen synthetic strategy for the two targets, (S)-**10** [note that the (S) label denotes the absolute configuration of the stereogenic center located α to the amide carbonyl group]
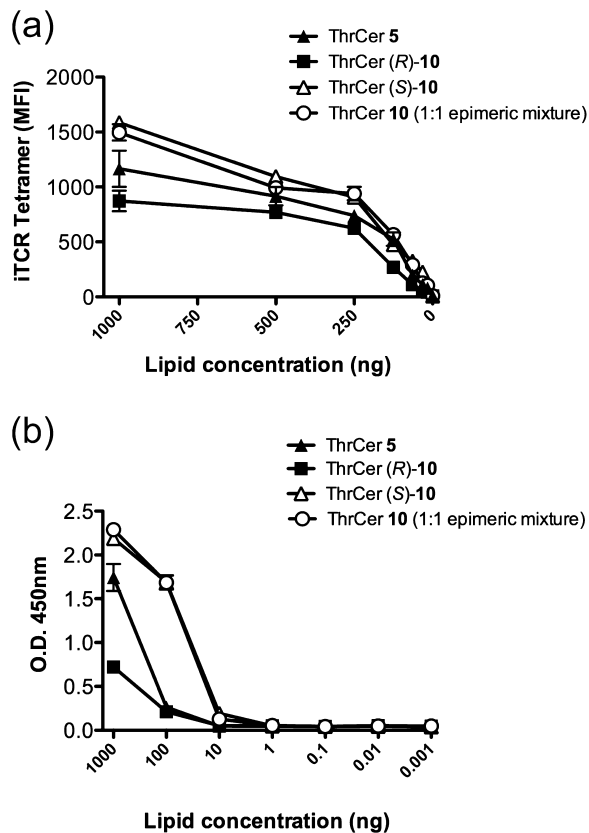
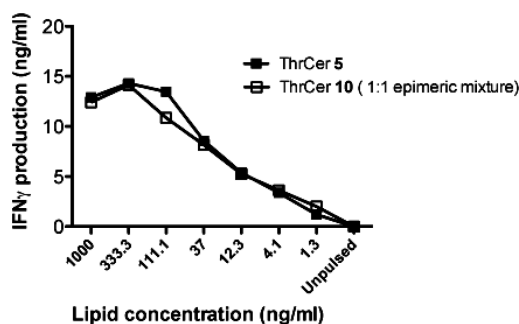**Scheme 1. General Retrosynthetic Strategy for the Assembly of Labeled Glycolipids and Target Molecules (S)-10 and 11**
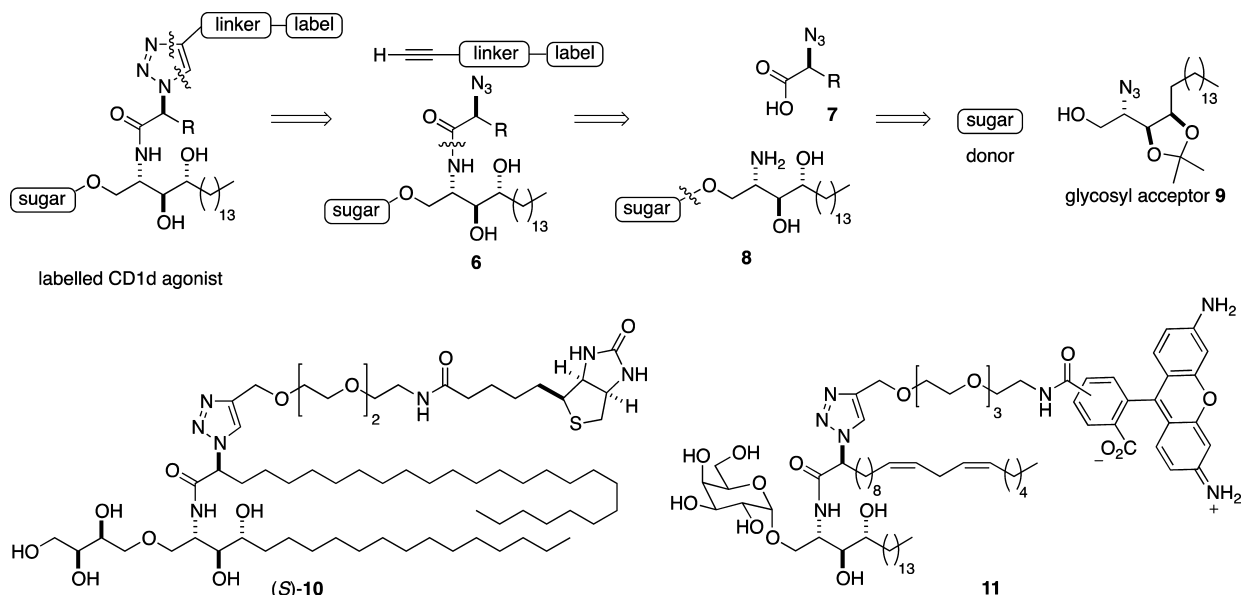
Scheme 2. Synthesis of Biotinylated ThrCer (S)-10

Figure 3. *i*NKT cell recognition of ThrCer **5** and biotinylated ThrCer analogues, tested as single epimers (S)-**10** and (R)-**10** and as a 1:1 mixture. (a) Recognition of ThrCer and biotinylated analogues by human *i*NKT TCR assessed by FACS analysis following co-incubation of fluorescent human *i*NKT cell TCR and C1R cells loaded with indicated lipids. (b) Activation of *i*NKT cell hybridoma following overnight culture with dendritic cells loaded with ThrCer and biotinylated analogues as determined by IL-2 production in the supernatant. Data presented are means of triplicate wells and are representative of three independent experiments.

and **11**, would incorporate the label at a late stage of the synthesis and use Click chemistry to assemble the components, ideally using an already fully deprotected glycolipid (Scheme 1). An oligo(ethylene glycol) spacer unit would be employed as

Figure 4. In vitro activation of human *i*NKT cells by ThrCer **5** and biotinylated ThrCer **10** (epimeric mixture). A human *i*NKT cell line was incubated with C1R CD1d cells pulsed either with ThrCer **5** (■) or with biotinylated ThrCer **10** (epimeric mixture) (□). IFN-γ secretion was analyzed after 36 h by an ELISA. The results are representative of three separate experiments.

a linker,[44] to ensure the label extends sufficiently from the TCR recognition site so as not to interrupt antigen presentation. We expected such a linker might also impart favorable solubility properties on the final products.[45,46] Further disconnection of amide **6** revealed α-azido acid **7**, which would be coupled selectively with the amino functionality embedded in **8**. Amine **8** would be assembled from phytosphingosine-derived glycosyl acceptor **9**,[25] and an appropriate glycosyl donor.

The synthesis of our first target, biotinylated ThrCer (*S*)-**10**, is detailed in Scheme 2. Enantiopure (*S*)-2-azidohexacosanoic acid (*S*)-**15** was readily accessed from Schöllkopf's auxiliary

12;[47−49] thus, treatment of bis-lactim **12** with BuLi, followed by reaction of the resulting lithiated intermediate with 1-iodotetracosane,[50] afforded the alkylation product as a separable 15:1 mixture of diastereoisomers. The desired major diastereoisomer **13** was then hydrolyzed to afford α-amino ester **14**.[49] Subsequent diazo transfer[51] and ester hydrolysis[49] afforded α-azido acid (*S*)-**15**, which was converted into the corresponding acid chloride and coupled with amine **16** (see the Supporting Information) under biphasic reaction conditions to afford amide (*S*)-**17** [note that the (*S*) label denotes the absolute configuration of the stereogenic center located α to the amide carbonyl group] in good yield. While there is the potential for racemization of the α-azido acid chloride coupling partner, a comparison of the ^{13}C NMR data of (*S*)-**17** with those of (*R/S*)-**17** [note that the (*R/S*) label denotes a 1:1 mixture of diastereoisomers epimeric at the stereogenic center located α to the amide carbonyl group in **17**] showed that no epimerization had occurred at the α-stereogenic center under the acylation reaction conditions (see the Supporting Information). In the final step, Huisgen [3+2] dipolar cycloaddition[52,53] of the azide in (*S*)-**17** with alkyne **18** (see the Supporting Information) provided our target, biotinylated ThrCer (*S*)-**10**, in 77% isolated yield.

Before progressing with biological analysis of biotinylated ThrCer (*S*)-**10** and to assess the importance of obtaining the correct stereochemistry at the tethering site, we used racemic 2-azidohexacosanoic acid in the synthetic sequence summarized in Scheme 2 to access the diastereoisomeric end product (*R*)-**10** [epimeric at the tethering site; the (*R*) label denotes the
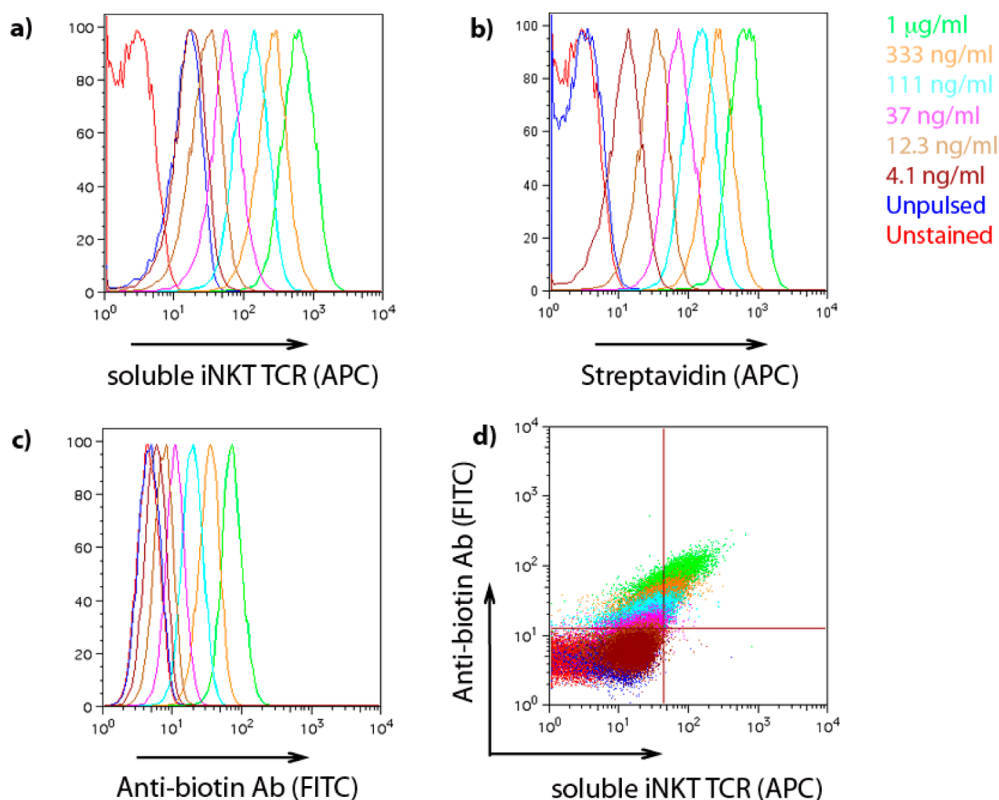
Figure 5. Detection of biotinylated ThrCer **10** (1:1 epimeric mixture) on the surface of APCs. C1R CD1d cells were pulsed with the indicated concentrations of biotinylated ThrCer for 16 h and then stained with (a) soluble *i*NKT cell TCR (APC), (b) fluorescent streptavidin (APC), or (c) the antibiotin antibody [labeled with fluorescein isothiocyanate (FITC)]. Panels a−c depict histogram overlays of the indicated fluorescence intensities. The dot plot shown in panel d depicts a double staining with the soluble *i*NKT cell TCR (APC) and antibiotin antibody (FITC). The results are representative of two separate experiments.

Scheme 3. Synthesis of Fluor 488-Labeled α-GalCer C20:2 11
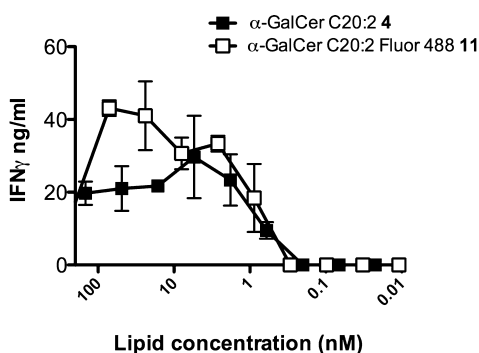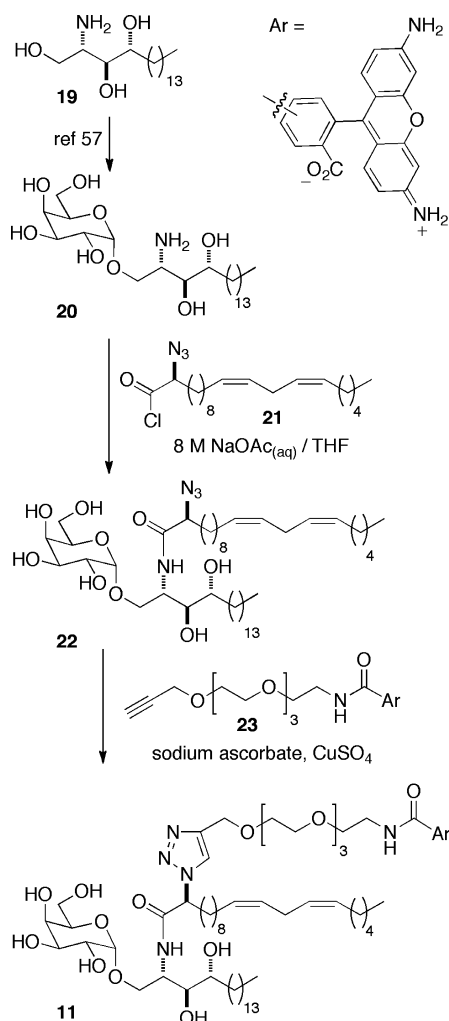
Figure 6. In vitro activation of *i*NKT cells by Fluor 488-labeled α-GalCer C20:2 **11**. Splenocytes from C57 BL/6 mice were cultured in the presence of various concentrations of either unlabeled **4** or Fluor 488-labeled α-GalCer C20:2 **11** for 48 h. The supernatants were then analyzed for the presence of IFN-γ by an ELISA. Data are means of duplicate wells and are representative of two independent experiments.

absolute configuration of the stereogenic center located α to the amide carbonyl group], which could be separated from its epimer (*S*)-**10** by careful column chromatography.

A 1:1 epimeric mixture (*S*/*R*)-**10** and the single epimers of biotinylated ThrCer, (*S*)-**10** and (*R*)-**10**, were next tested separately for their ability to be loaded onto human CD1d

molecules as defined by staining with tetrameric human *i*NKT cell TCR[29,54] (Figure 3a) and recognized by murine *i*NKT cells (Figure 3b).[23] In the first experiment, C1R-human CD1d cells were loaded with ThrCer **5** and the corresponding biotinylated analogues and then incubated with fluorescent human *i*NKT cell TCR. Subsequent fluorescence-activated cell sorting (FACS) analysis revealed that (*S*)-**10** and the epimeric mixture behaved like unlabeled ThrCer **5** in their ability to be recognized by the *i*NKT cell TCR, while the activity of the (*R*)-epimer, (*R*)-**10**, used on its own, was slightly attenuated (Figure 3a). In a second assay, C1R-mouse CD1d cells were first loaded with ThrCer **5** and the corresponding biotinylated analogues and then cultured overnight in the presence of *i*NKT cell hybridoma (DN32). *i*NKT cell activation was assessed by measuring interleukin-2 (IL-2) production by an ELISA.[55,56] Once again, and in line with our predictions, the two epimeric biotinylated ThrCer analogues behaved differently in their ability to activate *i*NKT cells, and in accord with our hypothesis, the labeled analogue, possessing the (*S*)-configuration at the tethering site, behaved significantly better than its (*R*)-epimer (Figure 3b). In this experiment, the more active labeled epimer, (*S*)-**10**, appears to show greater activity than unlabeled ThrCer itself at high lipid concentrations. Modifying the structure of any agonist is likely to have some impact on the activity, and in this experiment, our labeled analogue is no different. The greater activity compared with that of its unlabeled analogue (ThrCer) in this assay is interesting, and we postulate that differences in lipid processing (i.e., uptake and presentation on CD1d molecules) of murine and human APCs and the subsequent *i*NKT cell TCR may explain these observations. Although there was a difference in activity between the two epimers, the biological results using a 1:1 epimeric mixture (in which the concentration of the more active epimer is halved) proved to be similar to those using the single epimer (*S*)-**10**, and for this reason, all subsequent experiments described below were performed with the more readily accessed epimeric mixture.

Further experiments using human *i*NKT cells confirmed that the presence of the biotin label does not affect optimal loading onto CD1d molecules and presentation to human *i*NKT cells. To this end, C1R-hCD1d cells were pulsed with labeled (**10**) and unlabeled ThrCer (**5**), and their ability to be recognized by *i*NKT cells was assessed by measuring the release of IFN-γ by an ELISA after the cells had been cultured for 36 h (Figure 4). The similar response profile observed with ThrCer **5** and its biotinylated analogue (**10**) indicated that human *i*NKT cells are sensitized to a similar level, and that the biotin label does not affect significantly CD1d loading or *i*NKT cell presentation.

To further investigate the availability of the biotin on the cell surface of the APC, C1R CD1d cells were pulsed with biotinylated ThrCer **10** for 16 h before being stained with fluorescent streptavidin (Figure 5b), the antibiotin antibody (Figure 5), or soluble *i*NKT cell TCR (Figure 5a).[29] These results demonstrate that (i) the label can be detected by these two commonly used detection methods, opening up the possibility of using these as staining reagents to identify biotinylated ThrCer-pulsed cells, (ii) the TCR recognizes the labeled glycolipid in the context of CD1d molecules, and (iii) we are able to perform double staining using both soluble *i*NKT cell TCR and antibiotin antibody showing that the glycolipid–CD1d complex and label can be detected at the same time (Figure 5d).

Next, to assess the generality of our glycolipid labeling strategy, we chose to introduce a fluorescent label into a second CD1d agonist, namely the Th2 cytokine-biasing CD1d agonist α-GalCer C20:2 (**4**). The synthesis of labeled α-GalCer C20:2, **11**, is summarized in Scheme 3. α-Galactoside **20**, prepared in six steps from phytosphingosine **19** using our recently developed methodology for accessing 6″-azido-6″-deoxy-α-GalCer analogues,[57] was coupled with enantiomerically pure α-azido acid chloride **21**, accessed from the Schöllkopf auxiliary and linoleyl bromide (see the Supporting Information), to provide amide **22** as an advanced intermediate in excellent yield. Subsequent Click reaction with alkyne **23** afforded our target, fluorescently labeled α-GalCer C20:2, **11**.

The ability of α-GalCer C20:2 (**4**) and labeled α-GalCer C20:2 (**11**) to activate murine *i*NKT cells was assessed (Figure 6).[24] To this end, mouse splenocytes were cultured separately with various concentrations of α-GalCer C20:2 **4**, and the corresponding Fluor 488-labeled analogue **11**, and then the presence of IFN-γ was detected in the culture supernatant by an ELISA. As for the biotinylated ThrCer analogue, these results showed that the label does not significantly affect the behavior of α-GalCer C20:2 in its ability to activate murine *i*NKT cells, especially at low biologically relevant concentrations of the glycolipid, opening up the possibility of using this labeled analogue to study the biology of the important Th2 cytokine-biasing CD1d agonist α-GalCer C20:2 **4**.

CONCLUSIONS

In summary, we have shown that the *pro-S* hydrogen site in the α-methylene of the *N*-acyl chain of two important CD1d agonists, namely, ThrCer **5** and α-GalCer C20:2 **4**, can be used to append a label. In the two examples targeted, the functional activity of the labeled molecules was comparable in the human and mouse systems to that displayed by the unlabeled molecules, which should allow us to use these labeled analogues to study their trafficking behavior in vivo. The synthesis of both epimers of the biotinylated ThrCer analogue confirmed the importance of choosing the correct configuration at the tethering site, which was in accord with our predictions based on X-ray crystallographic structure analyses of the TCR−α-GalCer−CD1d ternary complex. Moreover, we were able to show for the first time that a biotin label attached to ThrCer remains accessible even when the soluble *i*NKT TCR is bound to the CD1d−lipid complex, as demonstrated by the double staining with soluble *i*NKT TCR and an antibiotin antibody. Because our synthetic strategy allows the incorporation of any sugar headgroup and fatty acid acyl chain and involves the late-stage introduction of the label from an advanced intermediate, we expect other CD1d agonists (and potentially other bioactive glycolipids) can be labeled using this approach.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures and full characterization data for compounds **25**, **26**, **13a**, **13b**, **14**, (2*S*)-methyl 2-azidohexacosanoate, (*S*)-**15**, (*S*)-**17**, **18**, (*S*)-**10**, (*rac*)-**15**, **17**, **10**, **11**, **27**−**30**, and **20**; statistical analysis of biological data summarized in Figure 3; and scanned copies of ¹H NMR and ¹³C NMR spectra for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

APC, antigen-presenting cell; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyante; α-GalCer, α-galactosyl ceramide; IFN, interferon; IL, interleukin; *i*NKT, invariant natural killer T; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; OD, optical density; PBS, phosphate-buffered saline; TCR, T cell receptor; ThrCer, threitol ceramide; Th1, type 1 helper T cell; Th2, type 2 helper T cell

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