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Synthesis of threitol ceramide and [^{14}C]threitol ceramide, non-glycosidic analogues of the potent CD1d antigen α-galactosyl ceramide

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This paper is dedicated to Professor George Fleet on the occasion of his 65th birthday and in recognition of his great contributions to carbohydrate chemistry

ABSTRACT

The synthesis of threitol ceramide, which is a non-glycosidic analogue of the potent CD1d antigen α-galactosyl ceramide, is described. The synthesis of a ^{14}C-labelled threitol ceramide analogue is also presented. This radiolabelled analogue will allow the intracellular trafficking pattern/itinerary of this iNKT-CD1d cell agonist to be studied.

1. Introduction

CD1 molecules are cell-surface glycoproteins related in structure and evolutionary origin to MHC class 1 antigen-presenting molecules.[1–3] The CD1 molecules comprise a multi-gene family, but unlike MHC class 1 molecules lack allotypic polymorphism.[1–3] Variable numbers of CD1 isoforms are found in different mammalian species. Genes encoding five distinct CD1 proteins (CD1a, -b, -c, -d and -e) are present on chromosome 1 in humans,[4–7] and two genes encoding proteins closely homologous to human CD1d (mCD1d1 and d2) are located on chromosome 3 in mice.[4–7] In common with MHC class I proteins, the CD1 proteins are expressed on the surface of cells as polypeptides associated non-covalently with β_2-microglobulin. The three-dimensional structures of three different CD1 proteins show a striking similarity in structure to MHC class I molecules.[8–10] The protein sequences of CD1 molecules suggest that they can be classified into two groups, with CD1a, -b and -c forming group 1, and CD1d defining group 2.[11] Molecules in group 1 appear to be involved in the presentation of specific antigens, which constitutes a novel antigen recognition pathway that is likely to be important for host defence against infections. In contrast, group 2 molecules, that is, CD1d, appear to have a regulatory role in innate and adaptive immune responses.[12]

α-Galactosyl ceramide (αGalCer) **1** (Fig. 1) has the ability to induce CD1d-restricted Natural Killer T (NKT) cells specifically to produce high levels of both IL-4 and IFNγ in vitro and in vivo.[13–19] NKT cells in different situations display both tolerogenic and immunostimulatory functions *in vivo* following αGalCer administration.[13–19]

The therapeutic potential of αGalCer **1** is currently being explored, but the induction of both Th1 and Th2 cytokines by this agent may limit its usefulness. Importantly, analogues of αGalCer, such as OCH **2** (Fig. 1), which differs from αGalCer in possessing truncated aliphatic chains, can induce NKT cell-derived cytokines more selectively.[20] Thus, OCH **2** strongly induces the secretion of IL-4 while having little effect on IFNγ levels.[20] Conversely, other αGalCer analogues are more effective at inducing both IFNγ and IL-4 from NKT cells than αGalCer.[21] By designing analogues that can effectively promote the appropriate functions of CD1d-restricted T-cells, it may be possible to treat diseases in which it is important to alter Th1 or Th2 polarisation. To achieve this aim, we have recently developed a non-glycosidic threitol ceramide (ThrCer) **3** analogue of αGalCer as a lead compound.[22] In this report, we describe the chemical synthesis of ThrCer **3**. We also describe the synthesis of radiolabelled Thr[^{14}C]Cer, [^{14}C]-**3**, which provides a chemical probe for examining the intracellular trafficking pattern/itinerary of this iNKT-CD1d cell agonist. Results from such studies should provide a more complete understanding of how iNKT responses are modified by such agents, which in turn should help realise the full potential of this novel therapeutic approach.

2. Results and discussion

2.1. Synthesis of threitol ceramide

The retrosynthesis of ThrCer **3** is outlined in Figure 2. Disconnection of the amide bond in ThrCer **3** provided amine **4** and the corresponding commercially available fatty acid **5**. Introducing

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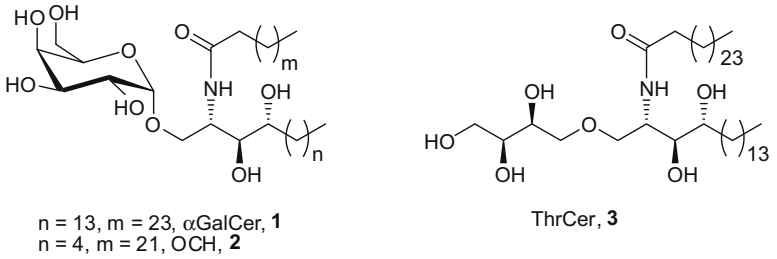


Figure 1. CD1d agonists.

the fatty acid chain in the final step in the synthesis would have a number of advantages. First, it would incorporate a late-stage point of diversity into the synthesis, allowing us to introduce a selection of fatty acids, including unsaturated derivatives, for studying the effect of how such structural changes affect the biological response.[23,24] From a more practical viewpoint, introducing the long-chain fatty acid in the final step would also minimise the manipulations of a difficult-to-handle amphiphilic dialkyl glycolipid. Amine **4** would be accessed from the corresponding azide **6**. Disconnecting the ether bond in azide **6** provided the two coupling partners for a Williamson ether synthesis, namely triflate **7**,[25] which would be prepared from commercially available benzylidene-protected L-threitol derivative **8**,[26] and alcohol **9**,

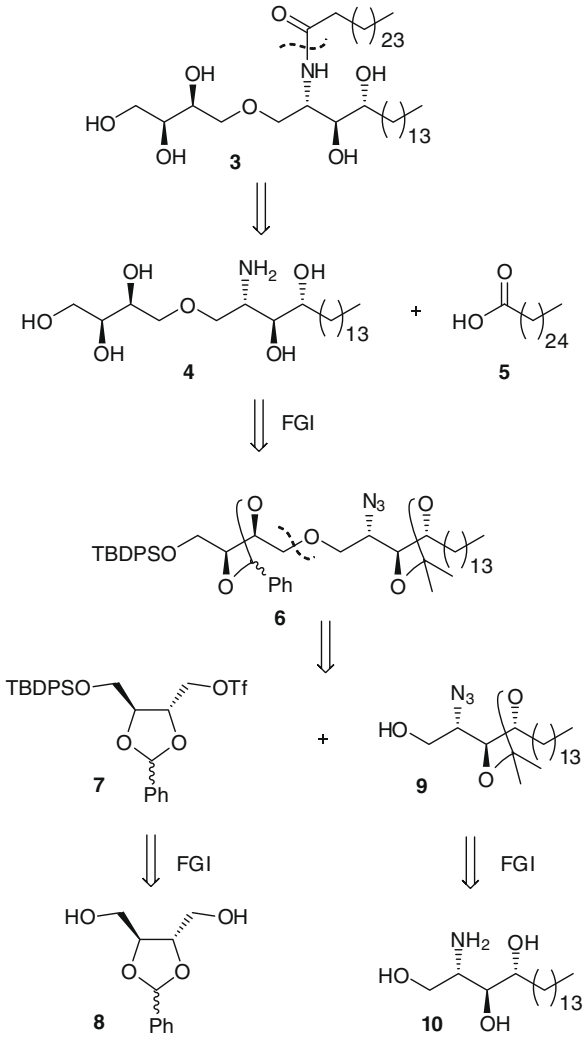
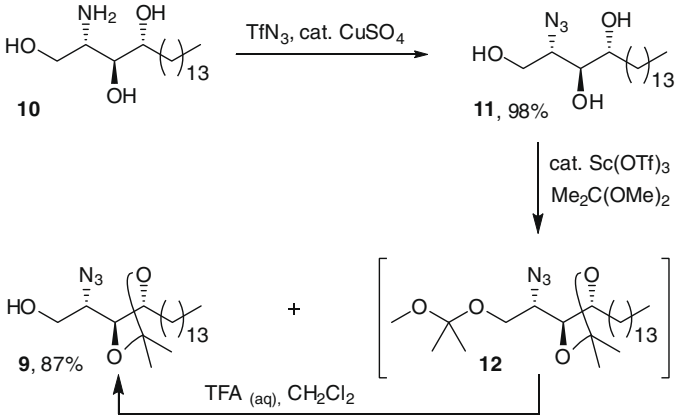


Figure 2. Retrosynthetic analysis of threitol ceramide.



Scheme 1. Synthesis of requisite azido alcohol.

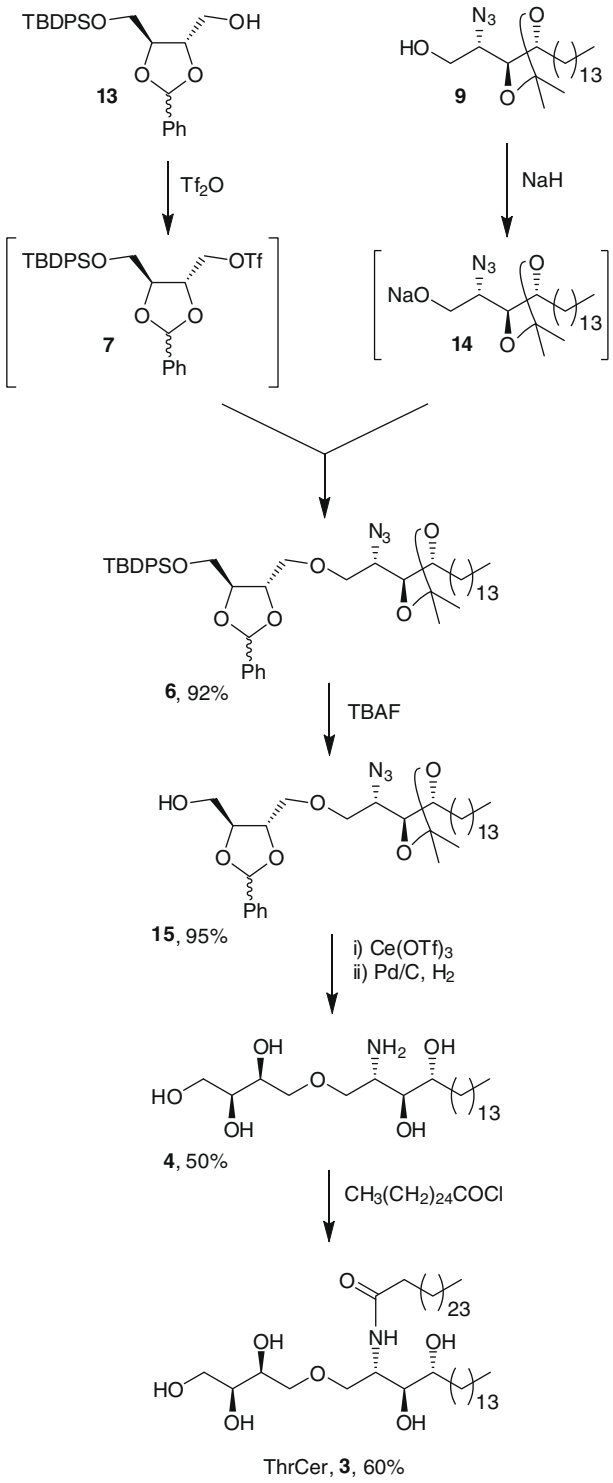
which would be accessed from phytosphingosine **10** (Fig. 2). Reversing the electrophile and nucleophile in the ether coupling would also be straightforward using this strategy should this be necessary.

The synthesis of the suitably protected azido alcohol **9** was accomplished in two steps from phytosphingosine **10** (Scheme 1). Thus, copper-catalysed diazo transfer[27,28] with a freshly prepared solution of TfN_3 yielded the corresponding azido-triol **11** in high yield.[29] Subsequent treatment of triol **11** with a 1:1 mixture of acetone and 2,2-dimethoxy propane in the presence of $Sc(OTf)_3$ provided a mixture of acetals **9** and **12**. The acyclic acetal in **12** was readily hydrolysed chemoselectively by treating the mixture briefly with TFA to provide 1,3-dioxolane **9** exclusively.[30]

The electrophilic coupling partner required for our Williamson ether synthesis of azide **6** was next prepared in two steps from diol **8**. Monoprotection of diol **8** as its TBDPS ether provided alcohol **13** in 71% yield, from which the corresponding triflate **7** was prepared by treatment with Tf_2O in pyridine (Scheme 2). Owing to its unstable nature, triflate **7** was used immediately and without purification in the subsequent etherification, which proceeded smoothly on treating **7** with the sodium alkoxide **14** in THF. From azide **6**, ThrCer was synthesised in four steps (Scheme 2). TBAF-mediated deprotection of the silyl ether provided alcohol **15**. Subsequent $Ce(OTf)_3$-mediated acetal hydrolysis[31] and hydrogenolysis of the azide functionality using Pd/C in acidic MeOH provided amine **4**, which was finally acylated with the acid chloride of hexacosanoic acid to provide our target ThrCer **3**.

2.2. Synthesis of ^{14}C -labelled threitol ceramide

We next turned our attention to a radiolabelled analogue of ThrCer **3**. Mindful of the need to minimise any manipulations involving radioactive compounds, we chose to incorporate a ^{14}C label into the fatty acid, which would be introduced at a late stage in the synthesis. Moreover, in our synthesis of non-radiolabelled



Scheme 2. Syntheis of threitol ceramide, **3**.

ThrCer **3** (Scheme 2), we had shown that the acylation of amine **4** could be performed in excellent yield even on the small scales that we would be employing in the synthesis of its radiolabelled analogue. Na^{14}CN was identified as a convenient source of ^{14}C; nucleophilic displacement of a suitable alkyl halide would provide a straightforward method for generating an alkyl nitrile, and thence the carboxylic acid upon hydrolysis.

To this end, the required C_{25}-chain alkyl halide was assembled by coupling undecynol **16** with alkyl bromide **17** (Scheme 3). Use of 2 equiv of base to deprotonate both the alcohol and terminal alkyne in **16**, and HMPA as the solvent in the nucleophilic substitution, allowed the reaction to proceed without protection of the primary alcohol.[32] Subsequent hydrogenation of the alkyne in the substitution product **18** provided alcohol **19**, which was transformed into bromide **20** by treatment with CBr_4/PPh_3. One-carbon homologation[33] proceeded efficiently on heating a solution of bromide **20** with Na^{14}C N in DMSO at 85 °C to provide nitrile **21**.[34] Finally, base-mediated hydrolysis of nitrile **21** provided ^{14}C-labelled acid [^{14}C]-**5** in quantitative yield.[34] Following our established procedure for acylation, acid [^{14}C]-**5** was converted into the corresponding acid chloride by treatment with oxalyl chloride, and then reacted with amine **4** to provide radiolabelled Thr[^{14}C]Cer [^{14}C]-**3** (Scheme 3).

3. Conclusion

Since it lacks the glycosidic linkage found in αGalCer, ThrCer **3** represents a hydrolytically more stable analogue of this most potent CD1d agonist. We have described a short route to ThrCer, and have shown that our approach can be used to incorporate a radiolabel into the molecule. Thr[^{14}C]Cer provides a useful chemical probe for examining the intracellular trafficking pattern/itinerary of this novel iNKT-CD1d cell agonist, which will be the focus of future studies.

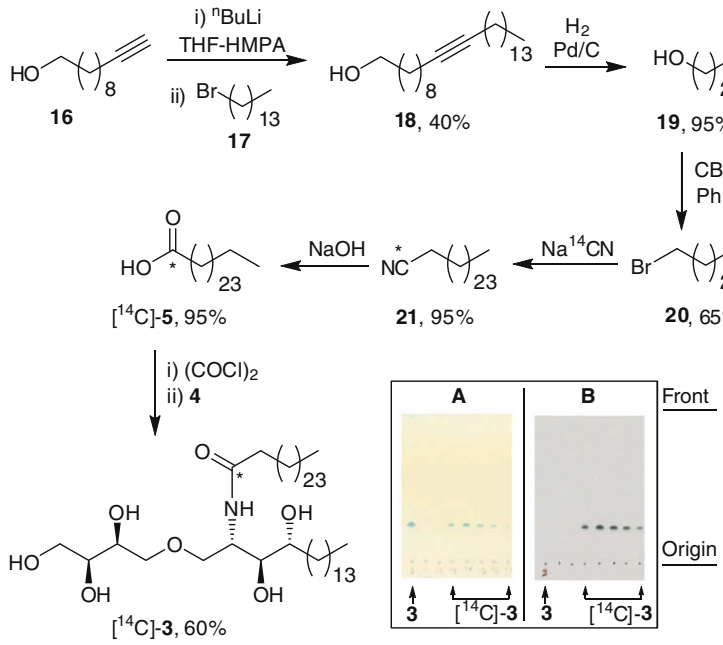
4. Experimental

4.1. General experimental procedures

Optical rotations were measured using an Optical Activity PolAAr2001 automatic polarimeter. Melting points were determined using open capillaries on a Gallenkamp MPD350 melting point apparatus, and are uncorrected. Infrared spectra were recorded either neat as thin films between NaCl discs on a Perkin Elmer 1600 FTIR spectrometer, or neat on a Perkin Elmer Spectrum 100 fitted with a universal ATR accessory. The intensity of each band is described as s (strong), m (medium) or w (weak), and with the prefix v (very) and suffix br (broad) where appropriate. ^{1}H NMR spectra were recorded at 500 MHz, 400 MHz or 300 MHz, using Bruker DRX 500, Bruker AMX 400, Bruker AV 400, Bruker AV 300 and Bruker AC 300 spectrometers. ^{13}C NMR spectra were recorded at 125 MHz, 100 MHz or 75 MHz, respectively, using Bruker DRX 500, Bruker AMX 400, Bruker AV 400, Bruker AV 300 and Bruker AC 300 spectrometers. Chemical shifts are reported as δ values (ppm) referenced to the following solvent signals: $CHCl_3$, δ_H 7.26; $CDCl_3$, δ_C 77.0; CH_3OH, δ_H 3.34; CD_3OD, δ_C 49.9. The term 'stack' is used to describe a region where resonances arising from non-equivalent nuclei are coincident, and multiplet, m, to describe a region where a resonance arises from a single nucleus (or equivalent nuclei) but where coupling constants cannot be readily assigned. Mass spectra were recorded on a Micromass LCT spectrometer utilising electrospray ionisation (and a MeOH mobile phase), and are reported as (*m/z* (%)). HRMS were recorded on a Micromass LCT spectrometer using a lock mass incorporated into the mobile phase.

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

Reactions were monitored by TLC using pre-coated aluminium-backed ICN silica plates (60A F_{254}) and visualised by UV detection (at 254 nm) and staining with 5% phosphomolybdic acid in EtOH (MPA spray). Column chromatography was performed on Merck silica gel (particle size 40–63 μm mesh) or Fluka 60 (40–60 μm



Scheme 3. Synthesis of [^{14}C]ThrCer, [^{14}C]-**3**. Panel **A**: TLC showing **3** (left-hand lane) and purified fractions of [^{14}C]-**3** after staining with phosphomolybdic acid spray; panel **B** shows the same TLC visualised using autoradiography.

mesh) silica gel. Autoradiograms were produced by 2.5 min exposure of Kodak X-Omat AR films to the TLC plates in the dark.

4.2. (2*S*,3*S*,4*R*)-2-Azido-1,3,4-octadecanetriol 11

TfN_3 was freshly prepared prior to the reaction as follows: NaN_3 (12.5 g, 192 mmol) was dissolved in a minimum volume of H_2O (35 mL, solubility of NaN_3 in H_2O is ~0.4 g mL^{-1}), and was cooled to 0 °C. CH_2Cl_2 (35 mL) was added, followed by dropwise addition of Tf_2O over 15 min (15.92 mL, 96 mmol) with vigorous stirring of the solution. The flask was stoppered and stirring continued at 0 °C. After 2 h, $NaHCO_3$ solution (30 mL) was carefully added, while stirring was continued until gas evolution had ceased. The reaction contents were then transferred to a separating funnel, and the phases were separated. The aqueous phase was washed with CH_2Cl_2 (2 × 25 mL). The combined organic phases were washed with $NaHCO_3$ solution (1 × 30 mL).

The resulting solution of TfN_3 in CH_2Cl_2 was used in the azidation step without further purification as follows: Amine **10** (10.0 g, 31.5 mmol) and $CuSO_4{\cdot}5H_2O$ (32 mg, 0.13 mmol) were dissolved in the same volume of H_2O as the volume of TfN_3 solution to be employed (85 mL). The CH_2Cl_2 solution of TfN_3 was then added with vigorous stirring. MeOH (570 mL) was then added over 5 min. After 18 h, the reaction mixture was diluted with H_2O (260 mL) and extracted with EtOAc (3 × 260 mL). The combined organic phases were filtered through a silica plug and washed with EtOAc until complete elution of the product. Removal of the solvent under reduced pressure afforded azide **11** as a white solid (10.6 g, 98%): R_f = 0.7 (eluent: EtOAc); mp 92–94 °C (lit. 91–92 °C);[35] $[\alpha]_D^{22} = +9.6$ (*c* 1.0, $CHCl_3$) (lit. $[\alpha]_D^{25} = +10$ (*c* 1, $CHCl_3$);[29] ν_{max} (film)/cm^{-1} 3683m (OH), 3390m (OH)) 3328m (OH), 3020s, 2918m, 2847m, 2108m (N_3), 1602w, 1521m, 1462w, 1410m, 1355m, 1215s, 1148m, 1071w, 1014w, 929m, 849w; δ_H (300 MHz, $CDCl_3/CD_3OD$, 2:1) 0.66 (3H, t, *J* 6.5, CH_3CH_2), 0.95–1.22 (24H, stack, CH_2), 1.37–1.42 (2H, stack, CH(OH)CH_2CH_2), 3.34–3.39 (3H, stack), 3.58 (1H, dd, *J* 6.1, 5.7), 3.72 (1H, dd, *J* 3.9, 3.7); δ_C (75 MHz, CD_3OD) 14.4 (CH_3, CH_2CH_3), 23.7 (CH_2), 26.7 (CH_2), 30.5 (CH_2), 30.7 (CH_2), 30.8 (CH_2), 33.1 (CH_2), 33.9 (CH_2), 62.5 (CH_2, CH_2OH), 66.6 (CH, CHN_3), 72.9 (CH, CHOH), 76.0 (CH, CHOH), some overlap in the alkyl resonances; *m/z* (TOF ES+) 366.2 ([M+Na]$^+$, 100%); HRMS *m/z* (TOF ES+) 366.2728 ([M+Na]$^+$. $C_{18}H_{37}N_3O_3Na$ requires 366.2733).

4.3. (2*S*,3*S*,4*R*)-2-Azido-3,4-*O*-isopropylidene-1,3,4-octadecanetriol 9

$Sc(OTf)_3$ (286 mg, 0.58 mmol) was added to a suspension of triol **11** (2.00 g, 5.82 mmol) in a 1:1 mixture of acetone and 2,2-dimethoxy propane (50 mL) at 0 °C, resulting in complete dissolution of the solid. The reaction mixture was stirred at this temperature for 20 min, and then neutralised with Et_3N. The solvent was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 (50 mL). The solution was washed with $NaHCO_3$ solution (20 mL), and the aqueous phase was extracted with CH_2Cl_2 (3 × 20 mL). The organic fractions were combined, dried (Na_2SO_4) and filtered. The solvent was evaporated under reduced pressure to provide a mixture of acetals **9** and **12**, which was dissolved in CH_2Cl_2/H_2O (10:1; 40 mL) and treated with 50% TFA in H_2O (432 μL, 5.82 mmol). After 10 min, TLC analysis showed that the acyclic acetal in **12** had been hydrolysed leaving exclusively acetal **9**. The reaction mixture was neutralised with Et_3N and diluted with $NaHCO_3$ solution (20 mL). The phases were separated, and the aqueous phase was extracted with CH_2Cl_2 (2 × 20 mL). The combined organic phases were dried ($MgSO_4$) and filtered, and the solvent was removed under reduced pressure. Purification of the residue by flash column chromatography (eluent: 20% EtOAc in hexanes) afforded the acetonide **9** as a white, low-melting point, amorphous solid (1.95 g, 87%): R_f = 0.3 (eluent: 25% EtOAc in hexanes); $[\alpha]_D^{22} = +23$ (*c* 1.0, $CHCl_3$); ν_{max}(film)/cm^{-1} 3440br (OH), 2925s, 2854s, 2100s (N_3), 1465m, 1370m, 1247m, 1217s, 1169m, 1063br, 870w, 758s; δ_H (300 MHz, $CDCl_3$) 0.88 (3H, t, *J* 6.0, CH_3CH_2), 1.26–1.30 (23H, stack), 1.34 (3H, s, 1 × C(CH_3)$_2$), 1.43 (3H, s, 1 × C(CH_3)$_2$), 1.50–1.63 (3H, stack), 2.08 (1H, t, *J* 6.0, CH_2OH), 3.44–3.50 (1H, m), 3.83–3.91 (1H, m), 3.94–4.03 (2H, stack), 4.15–4.21 (1H, m); δ_C (75 MHz, $CDCl_3$) 14.1 (CH_3, CH_3CH_2), 25.5 (CH_3, 1 × C(CH_3)$_2$), 26.5 (CH_2), 28.0 (CH_3, 1 × C(CH_3)$_2$), 29.35 (CH_2), 29.41 (CH_2), 29.53 (CH_2), 29.58 (CH_2), 29.59 (CH_2), 29.65 (CH_2), 29.68 (CH_2), 29.69 (CH_2), 31.9 (CH_2), 61.2 (CH, CHN_3), 63.9

(CH_2, CH_2OH), 76.6 (CH, CHO), 77.7 (CH, CHO), 108 (quat. C, $C(CH_3)_2$), some overlap in the alkyl resonances; *m/z* (TOF ES+) 406.2 ($[M+Na]^+$, 100%); HRMS *m/z* (TOF ES+) 406.3033 ($[M+Na]^+$. $C_{21}H_{41}N_3O_3Na$ requires 406.3046).

4.4. 1-*O*-(*tert*-Butyldiphenylsilyl)-2,3-*O*-benzylidene-L-threitol 13

A solution of (−)-2,3-*O*-benzylidene-L-threitol (1.00 g, 2.23 mmol) in THF (7 mL) was added dropwise over 5 min to a suspension of NaH (60% dispersion in mineral oil, 98 mg, 2.45 mmol) in THF (10 mL) at 0 °C. After 30 min, a solution of $^tBuPh_2SiCl$ (674 mg, 2.45 mmol) in THF (7 mL) was added dropwise over 15 min. After stirring at rt for 12 h, the reaction was quenched by the sequential addition of MeOH (2 mL) and $NaHCO_3$ solution (20 mL). The phases were separated, and the aqueous phase was washed with CH_2Cl_2 (3 × 20 mL). The combined organic phases were washed with brine (20 mL), dried ($MgSO_4$), filtered, and the solvent was evaporated under reduced pressure. The residue was purified by silica gel flash column chromatography (eluent: 20% EtOAc in hexanes) to provide silyl ether **13** as a colourless oil (1:1 mixture of diastereoisomers, 919 mg, 71%); Data on diastereoisomeric mixture: R_f = 0.25 (20% EtOAc in hexanes); ν_{max}(film)/cm^{-1} 3432br (OH), 3070m, 2930s, 2858s, 1654w, 1589w, 1471m, 1427m, 1390m, 1361m, 1310m, 1218m, 1112s, 1027m, 998m, 917m; δ_H (300 MHz, $CDCl_3$) 1.07 (4.5H, s, $(CH_3)_3CSi$), 1.09 (4.5H, s, $(CH_3)_3CSi$), 1.96–2.05 (1H, stack, CH_2OH), 3.70–3.96 (4H, stack), 4.11–4.25 (1.5H, stack), 4.32–4.37 (0.5H, m), 5.96 (0.5H, s, PhC*H*), 5.99 (0.5H, s, PhC*H*), 7.35–7.50 (11H, stack, Ph), 7.65–7.72 (4H, stack, Ph); δ_C (75 MHz, $CDCl_3$) [19.1 (quat. C, $(CH_3)_3CSi$), 19.2 (quat. C, $(CH_3)_3CSi$)], [26.72 (CH_3, $(CH_3)_3C$), 26.75 (CH_3, $(CH_3)_3C$)], [62.7 (CH_2, CH_2O), 62.8 (CH_2, CH_2O)], [63.9 (CH_2, CH_2O), 64.0 (CH_2, CH_2O)], [77.8 (CH, CHO), 78.7 (CH, CHO), 79.6 (CH, CHO), 79.9 (CH, CHO)], [103.7 (CH, PhCH), 104.1 (CH, PhCH)], [126.52 (CH, Ph), 126.58 (CH, Ph), 127.73 (CH, Ph), 127.77 (CH, Ph), 128.26 (CH, Ph), 128.37 (CH, Ph), 129.3 (CH, Ph), 129.5 (CH, Ph), 129.79 (CH, Ph), 129.81 (CH, Ph), 129.82 (CH, Ph), 129.85 (CH, Ph)], [132.81 (quat. C, *ipso*Ph), 132.85 (quat. C, *ipso*Ph)], [135.49 (CH, Ph), 135.53 (CH, Ph)], [137.2 (quat. C, *ipso*Ph), 137.4 (quat. C, *ipso*Ph)] some overlap in aromatic resonances; *m/z* (TOF ES+) 471 ($[M+Na]^+$, 100%); HRMS *m/z* (TOF ES+) 471.1972 ($[M+Na]^+$. $C_{27}H_{32}O_4SiNa$ requires 471.1968).

4.5. 1-*O*-[1′-*O*-*tert*-Butyldiphenylsilyl-2′,3′-*O*-benzylidene-L-threitol]-2-azido-3,4-*O*-isopropylidene-1,3,4-D-ribo-octadecanetriol 6

Tf_2O (329 μL, 1.96 mmol) was added dropwise over 10 min to a solution of alcohol **13** (878 mg, 1.96 mmol) and 2,6-di-*tert*-butylpyridine (484 μL, 2.16 mmol) in CH_2Cl_2 (20 mL) at 0 °C. After 1 h, the reaction mixture was diluted with CH_2Cl_2 (20 mL), and the resulting solution washed sequentially with cold H_2O (2 × 50 mL) and brine (10 mL), dried ($MgSO_4$) and filtered. Removal of the solvent under reduced pressure and purification of the residue by flash column chromatography (eluent: 5% EtOAc in hexanes containing drops of Et_3N) yielded triflate **7** as a colourless oil (1:1 mixture of diastereoisomers, 870 mg, 85%). The triflate was unstable and used immediately. Selected data on diastereoisomeric mixture: R_f = 0.3 (5% EtOAc in hexanes); δ_H (300 MHz, $CDCl_3$) 1.07 (4.5H, s, $(CH_3)_3CSi$), 1.09 (4.5H, s, $(CH_3)_3CSi$), 3.81–3.99 (2H, stack), 4.11–4.17 (1H, stack), 4.41–4.45 (0.5H, m), 4.52–4.61 (1.5H, stack), 4.69–4.75 (1H, stack), 5.95 (0.5H, s, PhC*H*), 6.03 (0.5H, s, PhC*H*), 7.36–7.48 (11H, stack, Ph), 7.63–7.70 (4H, stack, Ph). Alcohol **9** (575 mg, 1.5 mmol) in THF (10 mL) was treated with NaH (60% in mineral oil, 64 mg, 1.6 mmol) at 0 °C. After 1 h, a solution of triflate **7** (870 mg, 1.5 mmol) in THF (5 mL) was added dropwise over 5 min. The resulting solution was stirred at this temperature for 1 h, and then at rt for 12 h. The reaction was then quenched by the addition of MeOH (2 mL) followed by $NaHCO_3$ solution (10 mL). The phases were separated, and the aqueous phase was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic fractions were washed with brine (15 mL) and dried ($MgSO_4$), filtered, and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography (eluent: 5% EtOAc in hexanes) to provide ether **6** as a colourless oil (1:1 mixture of diastereoisomers, 1.13 g, 92%). Data on diastereoisomeric mixture: R_f = 0.35 (5% EtOAc in hexanes); ν_{max}(film)/cm^{-1} 3071w, 2926s, 2855s, 2098s (N_3), 1589w, 1461m, 1427m, 1379m, 1220m, 1112s, 874w; δ_H (300 MHz, $CDCl_3$) 0.85 (3H, t, *J* 6.6, CH_3CH_2), 1.06 (4.5H, s, $(CH_3)_3CSi$), 1.09 (4.5H, s, $(CH_3)_3CSi$), 1.24–1.41 (32H, stack), 3.55–3.63 (1H, stack), 3.67–4.01 (7H, stack), 4.09–4.17 (1.5H, stack), 4.23–4.33 (1H, stack), 4.42–4.46 (0.5H, m), 5.99 (0.5H, s, PhC*H*), 6.00 (0.5H, s, PhC*H*), 7.33–7.51 (11H, stack, Ph), 7.66–7.73 (4H, stack, Ph); δ_C (75 MHz, $CDCl_3$) 14.1 (CH_3, CH_3CH_2), 19.2 (quat. C, $(CH_3)_3CSi$), 22.7 (CH_2), [25.6 (CH_3, 1 × $C(CH_3)_2$), 25.7 (CH_3, 1 × $C(CH_3)_2$], 26.4 (CH_2), [26.82 (CH_3, $C(CH_3)_3$, 26.86 (CH_3, $C(CH_3)_3$], [28.1 (CH_3, 1 × $C(CH_3)_2$, 28.2 (CH_3, 1 × $C(CH_3)_2$], 29.3 (CH_2), 29.41 (CH_2), 29.45 (CH_2), 29.53 (CH_2), 29.57 (CH_2), 29.59 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 31.9 (CH_2), [59.8 (CH, CHN_3), 59.9 (CH, CHN_3)], [64.2 (CH_2, CH_2O), 64.3 (CH_2, CH_2O)], [72.0 (CH_2, CH_2O), 72.2 (CH_2, CH_2O)], [72.89 (CH_2, CH_2O), 72.94 (CH_2, CH_2O)], 75.6 (CH, CHO), [77.7 (CH, CHO), 77.8 (CH, CHO)], [77.9 (CH, CHO), 78.0 (CH, CHO)], [78.7 (CH, CHO), 78.8 (CH, CHO)], [104.0 (CH, PhCH), 104.3 (CH, PhCH)], [108.2 (quat. C, $C(CH_3)_2$), 108.3 (quat. C, $C(CH_3)_2$)], [126.7 (CH, Ph), 126.8 (CH, Ph)], [127.70 (CH, Ph), 127.74 (CH, Ph)], [128.2 (CH, Ph), 128.3 (CH, Ph)], [129.25 (CH, Ph), 129.34 (CH, Ph)], 129.73 (CH, Ph), 129.74 (CH, Ph), 129.77 (CH, Ph), 133.02 (quat. C, *ipso*Ph), 133.05 (quat. C, *ipso*Ph), 133.06 (quat. C, *ipso*Ph), 133.09 (quat. C, *ipso*Ph), 135.6 (CH, Ph), [137.4 (quat. C, *ipso*Ph), 137.5 (quat. C, *ipso*Ph), some overlap in the alkyl chain resonances; *m/z* (TOF ES+) 836.3 ($[M+Na]^+$, 100%); HRMS *m/z* (TOF ES+) 836.5041 ($[M+Na]^+$. $C_{48}H_{71}N_3O_6SiNa$ requires 836.5010).

4.6. 1-*O*-[2′,3′-*O*-Benzylidene-L-threitol]-2-azido-3,4-*O*-isopropylidene-1,3,4-D-ribo-octadecanetriol 15

TBAF (1 M solution in THF, 1.4 mL, 1.4 mmol) was added to a solution of silyl ether **6** (1.10 g, 1.35 mmol) in THF (15 mL) at rt. After 4 h, NH_4Cl solution (10 mL) was added. The phases were separated, and the aqueous phase was extracted with CH_2Cl_2 (3 × 10 mL). The solvent was removed under reduced pressure, and the residue was purified by flash column chromatography (eluent: 25% EtOAc in hexanes) to provide alcohol **15** as a colourless oil (1:1 mixture of diastereoisomers, 740 mg, 95%). Data on diastereoisomeric mixture: R_f = 0.35 (30% EtOAc in hexanes); ν_{max}(film)/cm^{-1} 3463br (OH), 2924s, 2853s, 2099s (N_3), 1459m, 1379m, 1246m, 1220m, 1092m, 1065m, 1027m, 869w; δ_H (300 MHz, $CDCl_3$) 0.88 (3H, t, *J* 6.6, CH_3CH_2), 1.23–1.31 (28H, stack), 1.35–1.41 (3H, stack), 1.45–1.60 (1H, stack), 1.96–2.04 (1H, stack, O*H*), 3.53–3.60 (1H, stack), 3.67–3.98 (7H, stack), 4.08–4.31 (3H, stack), 5.97 (0.5H, s, PhC*H*), 6.00 (0.5H, s, PhC*H*), 7.37–7.40 (3H, stack, Ph), 7.48–7.51 (2H, stack, Ph); δ_C (75 MHz, $CDCl_3$) 14.0 (CH_3, CH_2CH_3), 22.6 (CH_2), [25.5 (CH_3, 1 × $C(CH_3)_2$, 25.6 (CH_3, 1 × $C(CH_3)_2$], 26.3 (CH_2), [28.0 (CH_3, 1 × $C(CH_3)_2$, 28.1 (CH_3, 1 × $C(CH_3)_2$], 29.27 (CH_2), 29.33 (CH_2), 29.35 (CH_2), 29.46 (CH_2), 29.51 (CH_2), 29.57 (CH_2), 29.6 (CH_2), 31.8 (CH_2), [59.8 (CH, CHN_3), 59.9 (CH, CHN_3)], 62.5 (CH_2, CH_2O), [71.5 (CH_2, CH_2O), 71.6 (CH_2, CH_2O)], [72.78 (CH_2, CH_2O), 72.84 (CH_2, CH_2O)], 75.5 (CH, CHO), 76.4 (CH, CHO), 77.2 (CH, CHO), [77.68 (CH, CHO), 77.70 (CH, CHO)], [79.7 (CH, CHO), 79.9 (CH, CHO)], [103.8 (CH, PhCH), 104.0 (CH, PhCH)], [108.20 (quat. C, $(CH_3)_2C$), 108.23 (quat. C, $(CH_3)_2C$)], 126.6 (CH, Ph), [128.2 (CH, Ph), 128.3 (CH, Ph)], [129.3 (CH, Ph), 129.4 (CH, Ph)], [137.2 (quat. C, *ipso*Ph), 137.4 (quat. C,

*ipso*Ph), some overlap in alkyl chain region; *m/z* (TOF ES+) 598.2 ([M+Na]$^+$, 100%); HRMS *m/z* (TOF ES+) 598.3805 ([M+Na]$^+$. $C_{32}H_{53}N_3O_6Na$ requires 598.3832).

4.7. 1-*O*-[L-Threitol]-2-amino-1,3,4-D-ribo-octadecantriol 4

$Ce(OTf)_3$ (117 mg, 0.20 mmol) was added to a vigorously stirred solution of acetal **15** (200 mg, 0.32 mmol) in $MeNO_2$ (saturated with H_2O, 3 mL) and CH_2Cl_2 (2 mL). After 4 h at rt, the reaction mixture was diluted with CH_2Cl_2 (5 mL), and $NaHCO_3$ solution (10 mL) was added. The aqueous phase was extracted with CH_2Cl_2 (10 mL), and the organic phases were combined and washed with brine (5 mL), and then dried (Na_2SO_4). The solvent was removed under reduced pressure. The residue (yellow solid) was dissolved in MeOH (5 mL), and Pd/C (30 mg, 32 μmol) and AcOH (40 μL, 0.65 mmol) were added. The reaction vessel was evacuated, and then placed under an atmosphere of H_2. The suspension was stirred overnight. The reaction mixture was filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography (eluent: 10% MeOH in $CHCl_3$ to 50% MeOH in $CHCl_3$) to afford amine **4** as a white foam (72 mg, 50%); $[\alpha]_D$ the insolubility of this amphiphilic compound at rt prevented us from obtaining reliable optical rotation data; R_f = 0.1 (20% MeOH in $CHCl_3$); δ_H (300 MHz, $CDCl_3$) 0.79 (3H, t, *J* 6.7, CH_2CH_3), 1.08–1.32 (23H, stack, alkyl chain), 1.38–1.50 (1H, m), 1.55–1.69 (2H, m), 3.14–3.21 (1H, m), 3.25–3.65 (9H, stack), 3.68–3.75 (1H, m), exchangeable protons were exchanged with deuterium from CD_3OD for clarity prior to running the ^{1}H NMR spectrum; δ_C (75 MHz, $CDCl_3$) 13.9 (CH_3, CH_2CH_3), 22.5 (CH_2), 25.6 (CH_2), 29.2 (CH_2), 29.6 (CH_2), 31.8 (CH_2), 33.5 (CH_2), 52.9 (CH), 63.4 (CH_2), 70.1 (CH), 71.0 (CH_2), 71.6 (CH), 72.5 (CH_2), 73.2 (CH), 73.9 (CH), some overlap in alkyl chain resonances; *m/z* (TOF ES+) 422.3 ([M+H]$^+$, 100%); HRMS *m/z* (TOF ES+) 422.3489 ([M+H]$^+$. $C_{22}H_{48}NO_6$, requires 422.3482).

4.8. 1-*O*-[L-Threitol]-2-hexacosanoylamino-1,3,4-D-ribo-octadecantriol 3

Hexacosanoic acid (37.6 mg, 94.9 μmol) was placed in $(COCl)_2$ (1.0 mL) and stirred at 70 °C for 2 h after which time, the solution was cooled to rt, and the $(COCl)_2$ was removed under a stream of dry argon. The residual volatiles were removed under reduced pressure. The resulting crude acyl chloride was dissolved in dry THF (0.5 mL) and added with vigorous stirring to a solution of amine **4** (20 mg, 47.4 μmol) in THF / NaOAc(aq) (8 M) (1:1, 0.8 mL). Vigorous stirring was maintained for 2 h, after which time the mixture was left to stand and the phases were separated. The aqueous phase was extracted with THF (2 × 1.0 mL), and the combined organic phases were evaporated under reduced pressure. Purification of the residue by column chromatography (gradient from $CHCl_3$ to 15% MeOH in $CHCl_3$) afforded threitol ceramide **3** as a white solid (22.5 mg, 60%); R_f = 0.3 (8% MeOH in $CHCl_3$); $[\alpha]_D$ the insolubility of this amphiphilic compound at rt prevented us from obtaining reliable optical rotation data; mp 107–109 °C; ν_{max}(neat disk)/cm^{-1} 3308br m (OH, NH), 2915s, 2849s, 2098w, 1634m (C=O), 1540m, 1471m, 1108m, 1070m, 1026m, 718m; δ_H (500 MHz, THF-d_8, 45 °C) 0.89 (6H, app t, *J* 6.7, 2 × CH_2CH_3), 1.25–1.63 (72H, stack, alkyl chain), 2.12 (2H, t, *J* 7.7, C(O)CH_2), 3.40–3.47 (2H, stack, C(3′)*H*, C(4′)*H*), 3.47–3.57 (5H, stack, C(1)H_2, C(4)H_2, C(2)*H* or C(3)*H*), 3.59–3.63 (1H, m, C(1′)H_aH_b) 3.67–3.72 (2H, stack, C(1′)H_aH_b, C(3)*H* or C(2)*H*), 4.14–4.18 (1H, m, C(2′)*H*), 6.93–6.96 (1H, m, N*H*); δ_C (125 MHz, THF-d_8, 45 °C) 14.3 (CH_3, 2 × CH_2CH_3), [23.5 (CH_2), 26.6 (CH_2), 26.8 (CH_2), 29.3 (CH_2), 30.3 (CH_2), 30.5 (CH_2), 30.59 (CH_2), 30.64 (CH_2), 30.7 (CH_2), 30.8 (CH_2), 32.8 (CH_2), 34.1 (CH_2) alkyl chain resonances, some overlap], 36.9 (CH_2, C(O)CH_2), 51.8 (CH, C(2′)), 64.6 (CH_2, C(4)), 71.3 (CH, C(2) or C(3)), 71.5 (CH_2, C(1′)), 73.0 (CH, C(4′)), 73.1 (CH, C(3) or C(2)), 74.1 (CH_2, C(1)), 76.7 (CH, C(3′)), 173.0 (quat. C, C=O); *m/z* (TOF ES+) 822.7 ([M+Na]$^+$, 100%); HRMS *m/z* (TOF ES+) 822.7175 ([M+Na]$^+$. $C_{48}H_{97}NO_7Na$ requires 822.7163).

4.9. Pentacos-10-yn-1-ol 18

^{n}BuLi (2.5 M solution in hexanes, 8.72 mL, 21.8 mmol) was added dropwise over 40 min to a solution of 10-undecyn-1-ol **16** (1.75 g, 10.4 mmol) in THF (21 mL) containing HMPA (7.68 mL, 41.6 mmol) at −78 °C. After 15 min, a solution of 1-bromotetradecane **17** (3.17 mg, 11.4 mmol) in THF (2 mL) was added. The reaction mixture was stirred at −78 °C for 1 h, and then left to warm to rt overnight. The mixture was then diluted with Et_2O (20 mL) and quenched by slow addition of H_2O (20 mL). The phases were separated, and the aqueous phase was extracted with Et_2O (2 × 20 mL). The combined organic phases were dried with $MgSO_4$, and the volatiles were removed under reduced pressure. The residue was purified by column chromatography (eluent: hexanes/Et_2O, 2:1) to afford the alkyne **18** as a white solid (1.57 g, 40%): R_f = 0.15 (5% EtOAc in hexanes); ν_{max}(neat)/cm^{-1} 3357w (OH), 2919s, 2848s, 1459m, 1130w, 1057m, 1036m, 1013m, 975w, 725s; δ_H (300 MHz, $CDCl_3$) 0.86 (3H, t, *J* 6.8, CH_3CH_2), 1.15–1.60 (38H, stack, alkyl chain), 2.12 (4H, app. t, *J* 7.1, $CH_2C{\equiv}CCH_2$), 6.63 (2H, app. q, *J* 5.5, CH_2OH), resonance for O*H* not visible; δ_C (75 MHz, $CDCl_3$) 14.1 (CH_3, CH_3CH_2), 18.8 (CH_2), 22.7 (CH_2), 25.7 (CH_2), 28.9 (CH_2), 29.2 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 29.7 (CH_2), 32.0 (CH_2), 32.8 (CH_2), 63.1 (CH_2, CH_2OH), 80.2 (quat. C, C≡C), 80.3 (quat. C, C≡C), some overlap in the alkyl chain resonances; *m/z* (TOF ES+) 471.3 ([M+Ag]$^+$, 100%); HRMS *m/z* (TOF ES+) 471.2745 ([M+Ag]$^+$. $C_{25}H_{48}OAg$ requires 471.2756).

4.10. 1-Bromo-pentacosane 20

Pd/C (29 mg, 27 μmol) was added to a solution of alkyne **18** (1.00 mg, 2.70 mmol) in Et_2O (10 mL). The reaction vessel was evacuated and replaced with a H_2 atmosphere, and then the mixture was stirred overnight. The slurry was then filtered, washing the residue with hot THF (2 × 5 mL). The solvent was evaporated under reduced pressure to afford alcohol **19** as an oil (943 mg, 95%): R_f = 0.15 (5% EtOAc in hexanes); ν_{max}(neat)/cm^{-1} 3278br (OH), 2917s, 2849s, 1473m, 1452m, 1062m, 731m, 719m; δ_H (300 MHz, $CDCl_3$) 0.87 (3H, t, *J* 6.5, CH_3CH_2), 1.20–1.37 (44H, stack, alkyl chain), 1.50–1.60 (2H, m, CH_2CH_2OH), 3.63 (2H, t, *J* 6.4, CH_2OH), resonance for O*H* not visible; δ_C (75 MHz, $CDCl_3$) 14.1 (CH_3, CH_2CH_3), 22.7 (CH_2), 25.7 (CH_2), 29.38 (CH_2), 29.45 (CH_2), 29.7 (CH_2), 31.9 (CH_2), 32.8 (CH_2), 63.1 (CH_2, CH_2OH), some overlap in alkyl chain resonances. Satisfactory mass spectral analysis could not be obtained on alcohol **19**. Alcohol **19** was used without further purification in the next step: PPh_3 (531 mg, 2.02 mmol) and CBr_4 (492 mg, 1.48 mmol) were added sequentially to a solution of alcohol **19** (500 mg, 1.35 mmol) in CH_2Cl_2 (10 mL) at 0 °C. After 1 h, hexanes (10 mL) were added slowly, and the resulting precipitate was filtered. The filtrate was diluted with CH_2Cl_2 (20 mL) and washed with H_2O (10 mL). The phases were separated, and the organic phase was dried (Na_2SO_4). The volatiles were evaporated under reduced pressure to afford a residue, which was purified by column chromatography (eluent: 2% Et_2O in hexanes) to afford alkyl bromide **20** as a white solid (378 mg, 65%): R_f = 0.4 (hexanes); δ_H (300 MHz, $CDCl_3$) 0.88 (3H, t, *J* 6.6, CH_2CH_3), 1.20–1.31 (43H, stack, alkyl chain), 1.39–1.48 (1H, m), 1.79–1.87 (2H, m), 3.40 (2H, t, *J* 6.9, CH_2Br); δ_C (75 MHz, $CDCl_3$) 14.1 (CH_3, CH_2CH_3), 22.7 (CH_2), 28.2 (CH_2), 28.8 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.7 (CH_2), 31.9 (CH_2), 32.8 (CH_2), 34.0 (CH_2), some overlap in alkyl chain resonances. Satisfactory mass spectral analysis could not be obtained on bromide **20**.

4.11. [^{14}C]Hexacosan-1-nitrile 21

Alkyl bromide **20** (13 mg, 30 μmol) was added to a solution of $Na^{14}CN$ (1 mg, 20 μmol, 53 mCi mmol^{-1} (1.961 GBq mmol^{-1})) in DMSO (1 mL). The mixture was heated at 85 °C for 72 h, and then cooled to rt. Et_2O (1 mL) was added to the mixture followed by H_2O (1 mL). The phases were separated, and the aqueous phase was extracted with Et_2O (3 × 1 mL). The combined organic phases were dried with $MgSO_4$, and the volatiles were removed under reduced pressure. The residue was purified by column chromatography (eluent: 5% EtOAc in hexanes) to provide nitrile **21** (7 mg, 95%): Characterisation data for non-radiolabelled nitrile,[33] which was prepared as described above: R_f = 0.35 (5% EtOAc in hexanes); ν_{max}(film)/cm^{-1} 2914s (br), 2848s, 2241w (CN), 1633w, 1471s, 1424w, 1377w, 730w, 716s; δ_H (300 MHz, $CDCl_3$) 0.86 (3H, t, *J* 7.0, CH_2CH_3), 1.90–1.32 (42H, stack, alkyl chain), 1.35–1.49 (2H, m), 1.60–1.72 (2H, m), 2.31 (2H, t, *J* 6.7, CH_2CN); δ_C (75 MHz, $CDCl_3$) 14.1 (CH_3, CH_2CH_3), 17.1 (CH_2), 22.7 (CH_2), 25.4 (CH_2), 28.7 (CH_2), 28.8 (CH_2), 29.3 (CH_2), 29.4 (CH_2), 29.5 (CH_2), 29.7 (CH_2), 30.0 (CH_2), 31.9 (CH_2), some overlap in the alkyl chain resonances; nitrile resonance not observed; *m/z* (TOF ES+) 400.3 ([M+Na]$^+$, 100%); HRMS *m/z* (TOF ES+) 400.3932 ([M+Na]$^+$. $C_{26}H_{51}NNa$ requires 400.3919).

4.12. [^{14}C]Hexacosanoic acid [^{14}C]-5

A solution of NaOH (220 mg, 5.5 mmol) in EtOH (2.4 mL)/H_2O (0.3 mL) was prepared. [^{14}C]Nitrile **21** (7 mg, 20 μmol) was placed in 1 mL of this solution, and the mixture heated at 70 °C for 6 d, after which time TLC indicated that the alkyl nitrile had been consumed. The mixture was diluted with H_2O (2 mL), and the pH reduced to 3 with concd hydrochloric acid. The solution was extracted with Et_2O (3 × 2 mL). The phases were separated, and the organic fraction was dried ($MgSO_4$). The volatiles were evaporated under reduced pressure to afford the fatty acid [^{14}C]-**5** as a white solid (7.0 mg, 95%): Characterisation data for non-radiolabelled acid,[33] which was prepared as described above: R_f = 0.2 (20% EtOAc in hexanes); δ_H (300 MHz, $CDCl_3$) 0.88 (3H, t, *J* 6.7, CH_2CH_3), 1.25–1.35 (44H, stack, alkyl chain), 1.60–1.69 (2H, m), 2.35 (2H, t, *J* 6.9, CH_2CO_2H), carboxylic acid resonance not visible; spectroscopic data in agreement with those reported in the literature.[36] *m/z* (TOF ES−) 395.3 ([M−H]$^-$, 100%); HRMS *m/z* (TOF ES−) 395.3908 ([M−H]$^-$ $C_{26}H_{51}O_2$ requires 395.3889).

4.13. [^{14}C]-1-*O*-[L-Threitol]-2-hexacosanoylamino-1,3,4-D-ribo-octadecantriol [^{14}C]-3

A solution of [^{14}C]hexacosanoic acid [^{14}C]-**5** (7.0 mg, 20 μmol) in $(COCl)_2$ (0.5 mL) was stirred at 70 °C for 2 h after which time, the solution was cooled to rt, and the $(COCl)_2$ was removed under a stream of dry argon. The residual volatiles were removed under reduced pressure. The resulting crude acyl chloride (20 μmol, assuming 100% conversion) was dissolved in dry THF (0.5 mL) and added with vigorous stirring to a solution of amine **4** (16.8 mg, 40 μmol) in THF/NaOAc(aq) (8 M) (1:1, 0.8 mL). Vigorous stirring was maintained for 2 h, after which time TLC showed that the fatty acid had been consumed. The mixture was let to stand, and the phases were separated. The aqueous phase was extracted with THF (2 × 1.0 mL), and the combined organic phases were evaporated under reduced pressure. Purification of the residue by column chromatography (gradient from $CHCl_3$ to 15% MeOH in $CHCl_3$) afforded threitol ceramide [^{14}C]-**3** as a white solid[33] (13.6 mg, 85%).

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