Tetrahedron 72 (2016) 3863–3876

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Synthesis of wax esters and related trehalose esters from *Mycobacterium avium* and other mycobacteria

Salam G. Taher [a,†], Juma'a R. Al Dulayymi [a], H. Giresse Tima [b], Hanan M. Ali [a], Marta Romano [b], Mark S. Baird [a,*]

[a] School of Chemistry, Bangor University, Bangor, Gwynedd, Wales, LL57 2UW, UK
[b] Host-Pathogen Interactions, Scientific Service Immunology, O.D. Communicable and Infectious Diseases, Scientific Institute of Public Health (WIV-ISP), B1180 Brussels, Belgium

ARTICLE INFO

Article history:
Received 15 February 2016
Received in revised form 17 April 2016
Accepted 3 May 2016
Available online 6 May 2016

Keywords:
Wax ester
Mycobacterium avium
TDM
TMM
Trehalose dimycolate
Trehalose monomycolate
Inflammation

ABSTRACT

The synthesis of mycobacterial mycolic acid related wax esters and of their trehalose di- and mono-esters is described. The trehalose dimycolates (TDMs) synthesised activated bone marrow derived dendritic cells (BMDCs) in vitro more strongly than trehalose dibehenate or the trehalose monomycolates (TMMs). The inflammatory effects were similar to those of TDM from either a synthetic keto- or methoxy-mycolic acid, but somewhat stronger than those of a TDM from an α-mycolic acid. In vivo, the effects of one wax ester TDM were similar to those of the methoxy-MA and α-mycolic acid TDMs and trehalose dibehenate.

1. Introduction

Mycobacterial cell walls show unusually low permeability, a factor which apparently contributes to their resistance to therapeutic agents. This is linked to an exceptionally thick monolayer formed by the packing of esters of C_{60}–C_{90} fatty acids.[1–7] These 'mycolic acids' (MA), exemplified by structures **1**–**3**, contain various structural features including *cis*-cyclopropanes, α-methyl-*trans*-cyclopropanes, α-methyl-β-methoxy and α-methyl-β-keto groups,[8–14] *cis*-alkene, α-methyl-*trans*-alkene and α-methyl-*trans*-epoxy fragments,[15,16] and they are generally present as mixtures of homologues differing by two methylene-units. Each contains a common *R*,*R*-β-hydroxy acid group,[14,17–20] though less is known about the absolute stereochemistries of the other groups. There is evidence that the 1-methyl-2-methoxy unit at the distal position from the hydroxy acid in mycolic acids **2** is *S*,*S*,[13,19–21] while other reports identify a *R*-stereochemistry for the three stereocentres of the α-methyl-*trans*-epoxy unit.[15,16] [but see also Al Kremawi et al.][22] In the case of *Mycobacterium tuberculosis*, the MA are of three main types, α-MA (**1**), methoxy-MA (**2**) and keto-MA (**3**) (Scheme 1), though other mycobacteria contain different functional groups in the mero-chain. The detailed composition of the mixture can provide a fingerprint of the specific mycobacterium, or even of different strains or stages of development of a single bacterium. The fingerprint can be used directly to demonstrate infection.[23]

In the case of *Mycobacterium avium* there are no methoxy-MA, but these are replaced by so called 'wax-esters'. Over 60 years ago, Anderson, in an epic series of papers, initiated studies on mycobacterial lipids and reported the isolation of two new optically active long-chain alcohols from the neutral fraction of the saponified waxes of the so called 'timothy bacillus',[2] later classified as *Mycobacterium phlei*.[1,2,4,6] These alcohols were identified as *d*-2-eicosanol ($[\alpha]_D$ +3.5) and *d*-2-octadecanol ($[\alpha]_D$ +5.7). It was noted that the acidic fraction from these saponified waxes contained a high molecular weight component, tentatively identified

* Corresponding author. Fax: +44 1248 3702528; e-mail address: chs028@bangor.ac.uk (M.S. Baird).
† Current address: Chemistry Department, Faculty of Science and Health, Koya University, Iraq

http://dx.doi.org/10.1016/j.tet.2016.05.004
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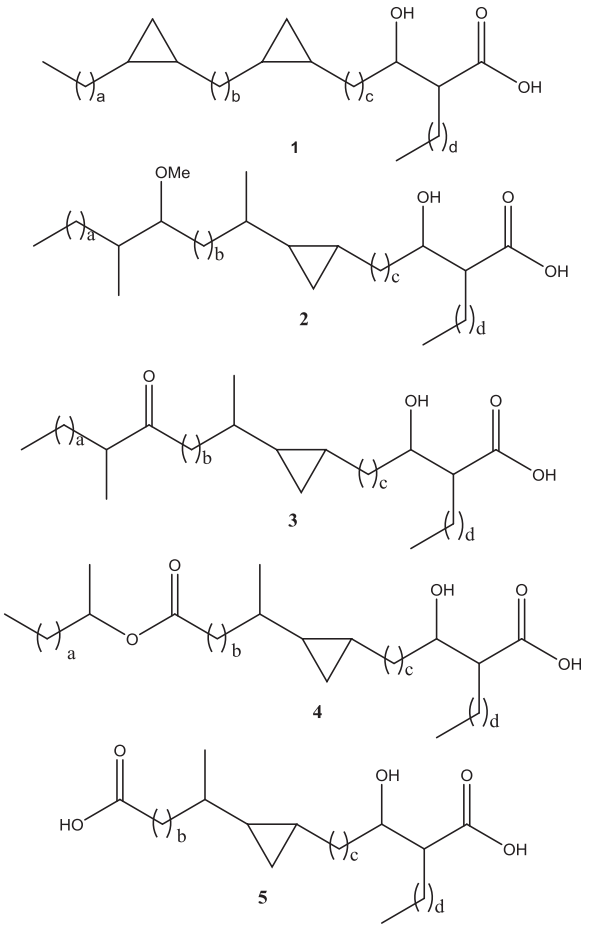


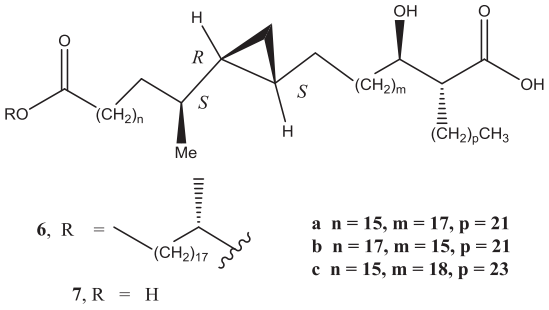
Scheme 1. Typical structures of mycolic acids **1**–**3**, and related wax esters **4** and wax dicarboxylic acid **5**.

as being dibasic.[24] A careful analysis of the firmly bound lipids from avian tubercle bacilli (*M. avium*) again yielded long-chain alcohols, with *d*-2-eicosanol as main component.[24] These lipid fractions also produced a long-chain di-acid, recognized for the first time as a mycolic acid derivative and given the title γ-mycolic acid ($[\alpha]_D$ +5.3).[24] The pioneering work of Etémadi et al.,[25–28] and of Minnikin and Polgar,[8,9] who isolated methyl avium mycolate ($[\alpha]_D$ +3.05) from *M. avium* strains Dn, 485 and 7169, and provided mass spectrometric evidence for structures and molecular weights, led to a clearer understanding of the presence of esters of these acids, apparently derived by a biological Baeyer-Villiger reaction on keto-MA.[4,13,29,31,32]

Wax esters from *Mycobacterium avium ssp. paratuberculosis* were partially characterised as **4** and the corresponding diacids **5**,[30] and found as a constituent of trehalose mycolates of *M. phlei*.[33,34] The relationship between the keto-MA and the wax ester MA in *M. phlei* and *Mycobacterium aurum* was examined by radiolabelling, which showed that the keto-MA appeared first in extractable lipids, and the wax ester MA first in wall-linked derivatives.[35] The mass relationship was probed directly by MALDI-MS.[36] In parallel studies, similar alcohols and acids were characterized from an organism claimed to be the causative agent of leprosy.[37] It is clear, however, that this bacterium was not the leprosy bacillus, as *Mycobacterium leprae* has not been cultivated to date and the mycolic acid composition of *M. leprae* is distinct.[38] The use of two dimensional TLC provided additional evidence of the presence of wax esters in other mycobacteria.[39] In this way, the distribution of various classes of MA could be characterised. Some mycobacteria, e.g., *Mycobacterium vaccae*, contain α, α′, keto and wax ester MA; others, such as *M. avium ssp. avium* and *M. avium ssp. paratuberculosis*, contain α, keto and wax ester MA. Others, such as *Mycobacterium komossense* and *Mycobacterium heckeshornense*, produce α, keto, methoxy and wax ester MA.[38,40] HPLC of MA from some strains of *Mycobacterium gordonae* showed double cluster patterns due to the presence of dicarboxy mycolates after saponification.[41] Wax esters MA are known to be a characteristic component in the *M. avium–Mycobacterium intracellulare* group and other rapidly growing bacteria.[42,43] Their presence in *Mycobacterium smegmatis* has been implied,[44] although not confirmed by a later study.[39] Usually they have been analysed as the corresponding α-methyl-alkanol and free acid after hydrolysis.[32,39,45] Some such wax esters, such as those from *M. aurum*, do not appear to contain cyclopropanes,[46] while in other cases the composition is not clear.[5,25–27,40,47–50]

The detection of intact trehalose dimycolate (TDM) containing a wax ester mycolate has been achieved by mass spectrometry. Three types of TDM were observed, one containing two α-mycolate residues, one two wax esters, and one having one residue of each type.[34] The analysis of the intact trehalose monomycolate (TMM) derivatives by MALDI-TOF mass spectrometry shows predominant ions due to C_{85} and C_{87} wax esters from *M. avium–M. intracellulare*, and C_{80}, C_{81}, C_{82} and C_{83} for *M. phlei* and *Mycobacterium flavescens*.[51] Direct observation of the corresponding wax ester trehalose dimycolate (TDM) species is also reported.[42]

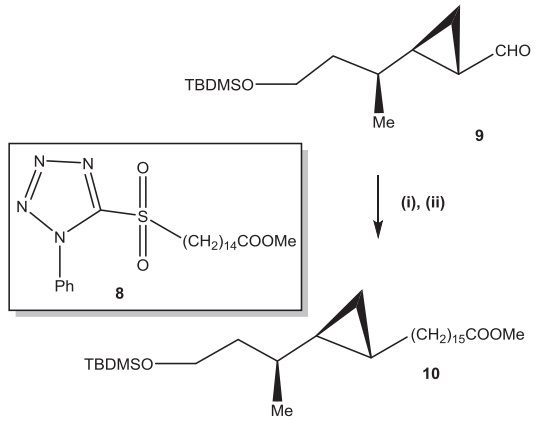
Yano et al. have reported that TDM isolated from *M. tuberculosis* is antigenic to antibodies present in the serum of patients infected with tuberculosis, and that the sub-class of TDM based on methoxy-MA is recognised more strongly than those based on α-MA or keto-MA.[52–55] Antibodies in the serum of patients infected with TB responded less strongly to TDM isolated from *M. avium* complex (MAC), while antibodies in serum of MAC infected patients showed the opposite response pattern. Disease caused by MAC is an important opportunistic pulmonary infection; the clinical symptoms of MAC pulmonary disease and TB resemble each other, but the treatment is different, so rapid distinction between the infections is desirable. The use of a serodiagnostic assay based on the selective antigenicity of TDM and TMM offers a clear opportunity to solve this problem, and considerable work has been carried out in this area, using extracts from different types of mycobacterial cell.[56–60] Although TDM from *M. tuberculosis* or *Mycobacterium bovis* induces significant resistance against influenza or *M. tuberculosis* infections when combined with muramyl dipeptide, TDM from *M. avium*, did not show the same effect. Moreover, while TDM from Mtb confers resistance to *Toxoplasma gondii* infections, that from *M. avium* does not.[61] There are major differences in toxicity and granulomatogenic activity in mice of TDMs from different mycobacteria, depending on the balance of different classes of MA and wax ester.[62] In addition to the problems in diagnosing MAC infection in humans, *M. avium ssp. paratuberculosis* is the etiologic agent of Johne's disease, one of the most widespread bacterial diseases of domestic animals.[63] Given that one major difference between the constituents of *M. tuberculosis* and *M. avium* is that the former contains methoxy-MA, but not normally wax ester MA, while the latter contains wax-ester MA but not methoxy-MA, the synthesis of both wax esters and of the corresponding sugar esters may allow a number of issues to be resolved. We have already reported the synthesis of the dicarboxylic acid fragment of a wax ester containing an α-methyl-*trans*-cyclopropane;[64] we now report the synthesis of three complete wax esters and of the derived trehalose esters. Compound **6a** (Scheme 2) is reported to be the major wax ester of *M. avium*;[43] compound **6b**, with reversed chain lengths is reported to be the major isomer in *M. gordonae*.[41] In addition, the longer chain wax ester **6c** was prepared in order to study the effects of chain length on bioactivity (Scheme 2).



Scheme 2. Typical chain lengths of some mycobacterial wax esters and di-acids.

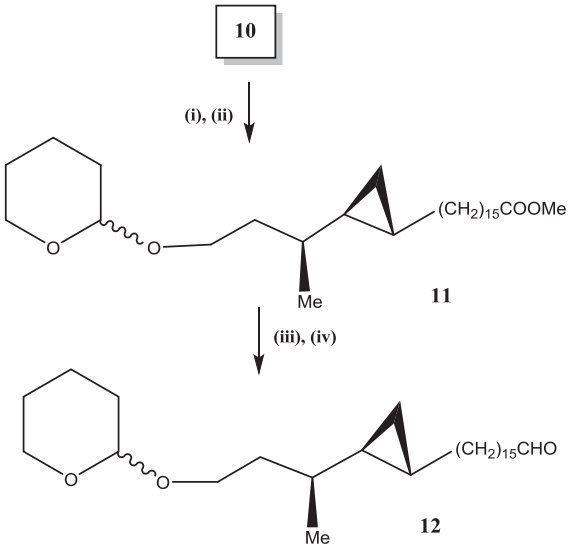
2. Results and discussion

The *trans*-cyclopropane fragment (**9**) (Scheme 3), prepared as described earlier was coupled to the sulfone (**8**) in a modified Julia-Kocienski reaction, followed by saturation of the derived mixture of *E*/*Z*-alkenes using di-imide. This led to the chain extended ester (**10**).



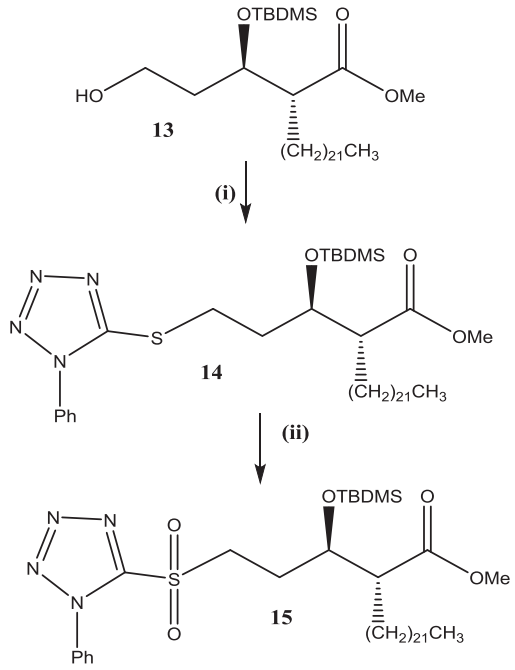
Scheme 3. (i) LHMDS, **8**, dry THF (85%); (ii) dipotassium azodicarboxylate, THF-methanol, CH_3COOH (96%).

Replacement of the silyl protecting group of (**10**) by a tetrahydropyranyl group, was followed by conversion of the ester into aldehyde (**12**) (Scheme 4).



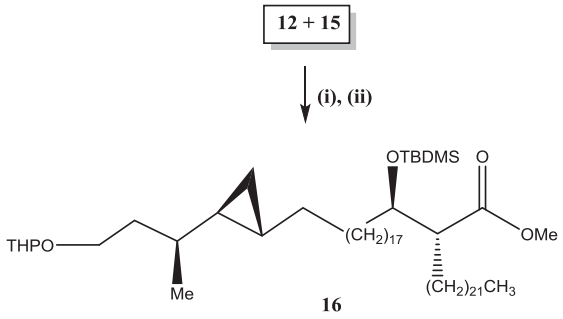
Scheme 4. (i) nBu_4NF, dry THF (96%); (ii) pyridinium-*p*-toluenesulfonate (PTSA), 3,4-dihydro-2*H*-pyran, dry CH_2Cl_2 (98%); (iii) $LiAlH_4$, dry THF (94%); (iv) PCC, CH_2Cl_2 (93%).

In order to generate the dicarboxylic acid part of the wax ester, the aldehyde (**12**) needed to be coupled to an *R*,*R*-β-hydroxyacid fragment (**15**), with a pendant C_{22} chain. This was prepared from **13**,[66] using the standard methods (Scheme 5).



Scheme 5. (i) 1-phenyl-1*H*-tetrazole-5-thiol, Ph_3P, DEAD, dry THF, (85%) (ii) MCPBA, CH_2Cl_2 (86%).

Reaction of the aldehyde (**12**) with sulfone (**15**) and base, followed by saturation of the derived alkenes provided the fragment (**16**) (Scheme 6).

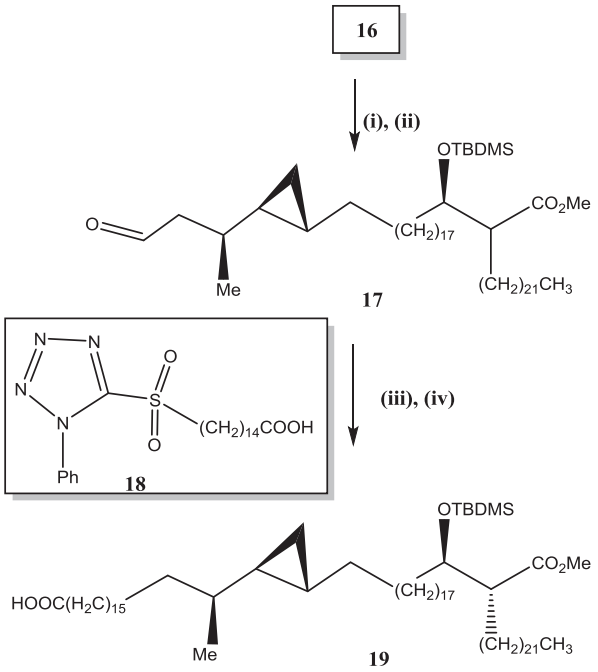


Scheme 6. (i) LHMDS, dry THF (78%); (ii) dipotassium azodicarboxylate, THF, methanol, CH_3COOH (91%).

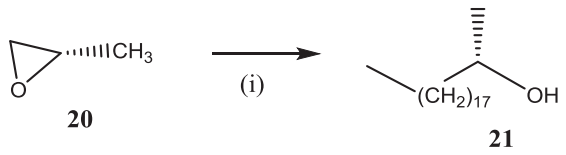
The tetrahydropyranyl protecting group was removed from ester (**16**) and the resulting alcohol was oxidised to produce aldehyde (**17**). Coupling of this with the sulfone (**18**) and base followed by saturation of the derived alkenes provided the ω-dicarboxylic acid mono-ester (**19**) (Scheme 7).

In order to generate the full wax ester, the ω-dicarboxylic acid mono-ester (**19**) needed to be coupled to (*S*)-2-eicosanol (**21**). This was synthesized by reacting the Grignard reagent from 1-bromoheptadecane with (*S*)-1-epoxypropane (**20**) to give the desired alcohol (**21**) (Scheme 8).

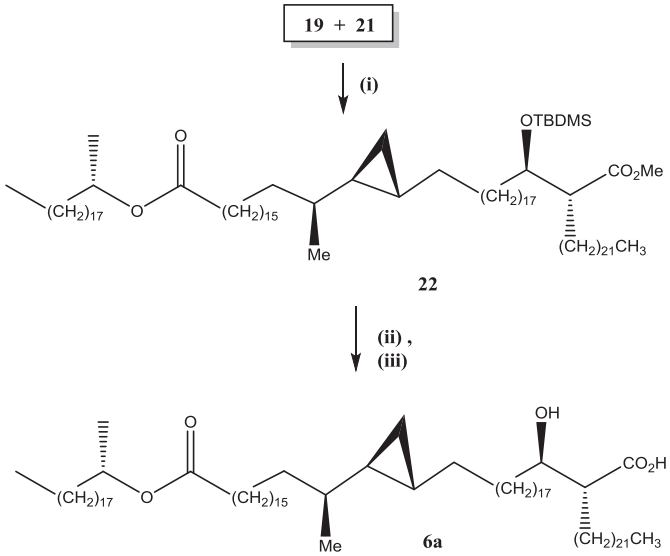
The ω-dicarboxylic acid mono-ester (**19**) was esterified with *S*-2-eicosanol (**21**) through a Steglisch reaction (Scheme 9). Removal of the silyl ether and methyl ester protection was achieved in two steps to produce the free wax ester mycolic acid (**6a**).



Scheme 7. (i) PTSA, MeOH (84%); (ii) PCC, dichloromethane (93%); (iii) LHMDS, **18**, dry THF (80%); (iv) dipotassium azodicarboxylate, THF, methanol, CH_3COOH (80%).



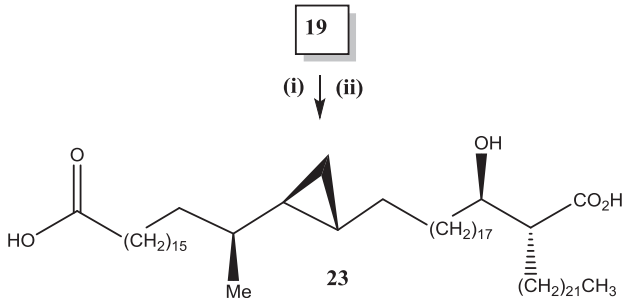
Scheme 8. (i) $CH_3(CH_2)_{16}MgBr$, CuI, dry THF (53%).



Scheme 9. (i) DCC, DMAP, dry CH_2Cl_2 (80%); (ii) HF-pyridine complex, dry THF (71%); (iii) 5% aq nBu_4NOH (60%).

The product **6a**, α_D^{21}+7.6 (*c* 0.40, $CHCl_3$), showed the characteristic apparent sextet for the hydrogen adjacent to the ester at δ 4.91 (1H, *J* 6.2 Hz), as well as the signals for the α- and β-hydrogens of the hydroxy acid at 3.72 (1H, dt, *J* 4.8, 9.2 Hz), 2.46 (1H, dt, *J* 5.3, 9.0 Hz), and a triplet for the methylene group adjacent to the ester carbonyl at 2.27 (2H, t, *J* 7.5 Hz).

The ω-dicarboxylic acid (**23**) has also been isolated from the cell wall as a component of a complex mixture.[43] Compound (**23**), α_D^{21}+5.8 (*c* 0.86, $CHCl_3$), was also synthesized starting from the ω-dicarboxylic acid mono-ester (**19**) after TBDMS deprotection and hydrolysis (Scheme 10). The acid showed the typical signals for the α-methyl-*trans*-cyclopropane in the ^{1}H NMR spectrum, together with the typical signals for the α- and β-hydrogens of the hydroxy acid.



Scheme 10. (i) HF-Pyridine, dry THF, 17 h (85%); (ii) LiOH, THF, water-MeOH, 17 h (85%).

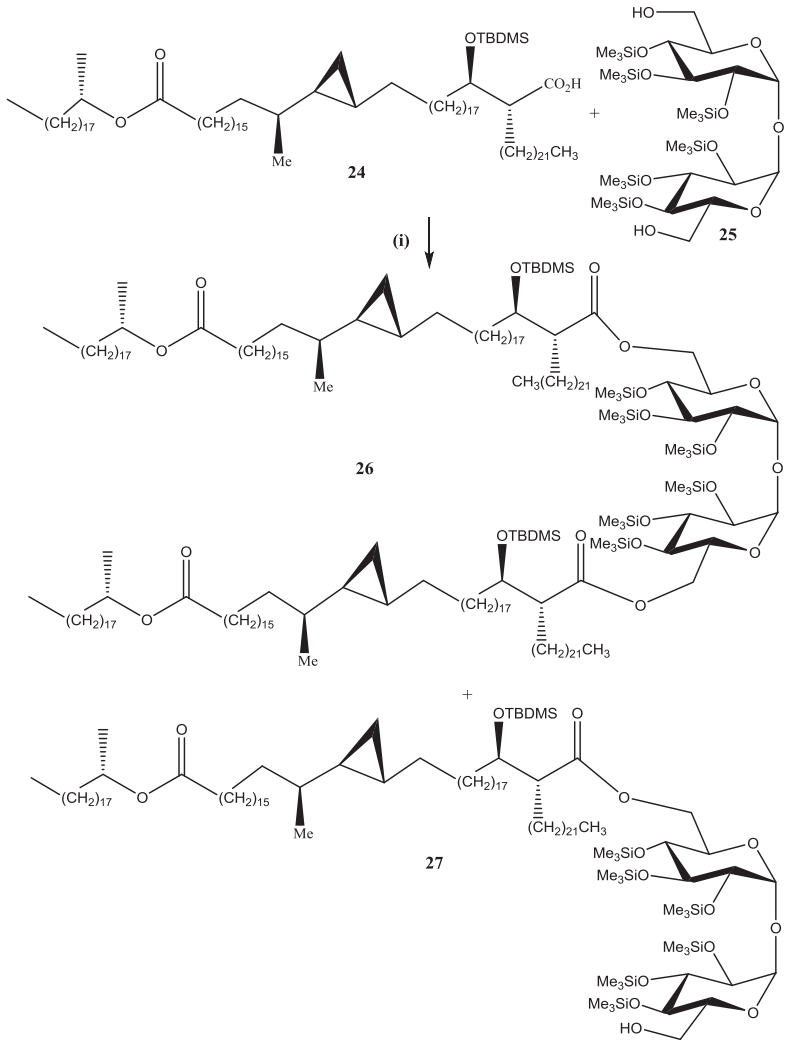
The acid **6a** was then converted into the corresponding TDM and TMM using methods described earlier.[67,68] The protected wax ester mycolate **24** was prepared from the corresponding free hydroxy wax ester **6a** by reaction with an excess of *tert*-butyldimethylsilylchloride and imidazole in the presence of 4-dimethylaminopyridine for 24 h at 70 °C, followed by hydrolysis of the TBDMS ester on the acid group by stirring in THF for 15 min with 4% aqueous tetrabutylammonium hydroxide. Compound **24** was coupled to protected trehalose (**25**) using 1-(3-dimethylaminopropyl-3-ethylcarbodiimide hydrochloride, DMAP and molecular sieves in CH_2Cl_2 (Scheme 11). This gave the protected TDM **26** (52%) and the protected TMM **27** (32%).

The TDM was de-protected in two steps to give **28** and **29**, and the TMM was deprotected in the same way to give **30** and **31** (Schemes 12 and 13).

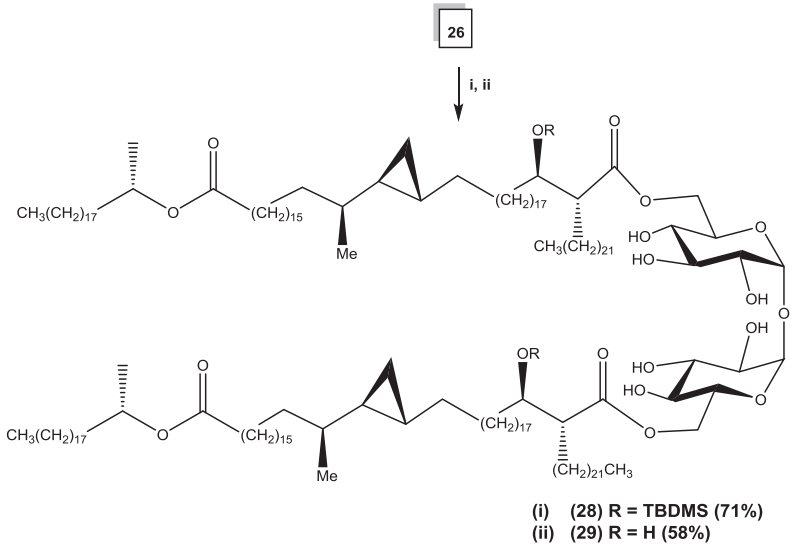
Using a similar method, the second wax ester, **6b** was prepared and converted into the corresponding TDM (**32**) and TMM (**33**) (Scheme 14), while wax ester **6c** was converted into the corresponding TDM (described in full in the Supplementary data).

3. Wax ester TDM and TMM are inflammatory in vitro and in vivo

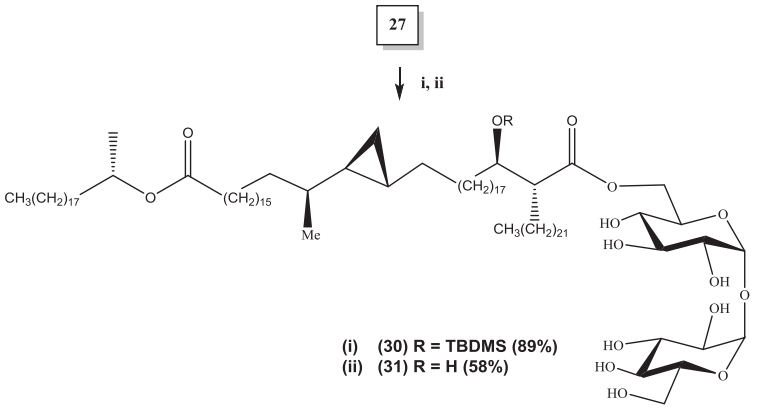
The ability of synthetic wax ester TDM and TMM to activate murine bone marrow derived dendritic cells (BMDCs) in vitro was examined. For that purpose, BMDCs were stimulated with 1 μM of the different wax esters and controls (evaporated isopropanol as a negative control and trehalose dibehenate as a positive control) for 24 h and the production of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) was analysed by ELISA in the supernatant. All the tested compounds induced the production of pro-inflammatory cytokines. The levels of TNF-α, IL-1β and IL-6 induced by wax ester TMMs **31** and **33** were significantly lower compared to the levels induced by the corresponding wax ester TDMs **29** and **32** and TDB (Fig. 1A), indicating that the number of mycolate chains bound to trehalose influences the activation of BMDCs in terms of pro-inflammatory cytokine secretion. When a dose-response analysis was performed using pairs of TMM and TDM composed of the same wax ester, we confirmed that at equimolar concentrations TDM is more inflammatory than TMM and TDB (Fig. 1B). These data are in



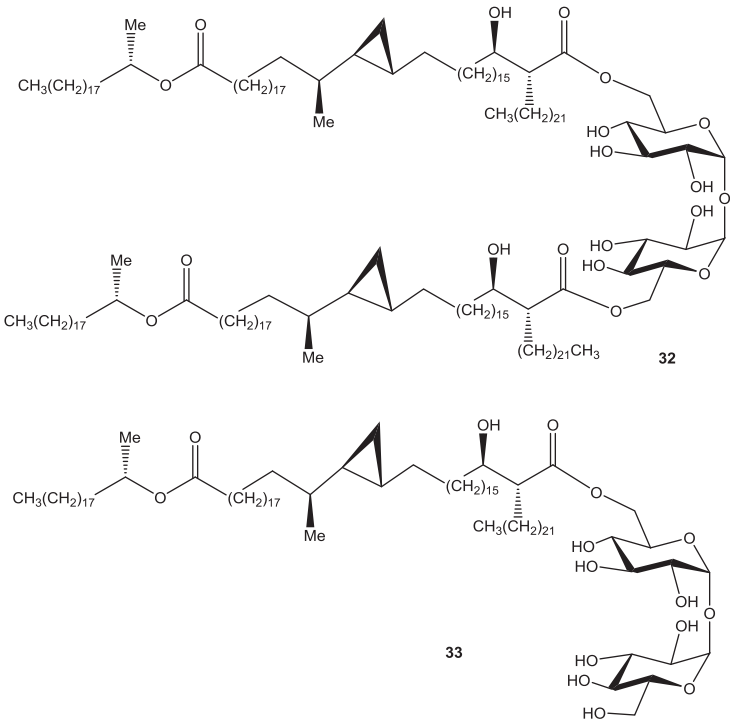
Scheme 11. EDCI, DMAP, 4 Å molecular sieves, CH_2Cl_2, 6 days at ambient temperature.



Scheme 12. (i) nBu_4NF, dry THF; (ii) HF-pyridine complex, dry THF.



Scheme 13. (i) nBu_4NF, dry THF; (ii) HF-pyridine complex, dry THF.



Scheme 14. TDM and TMM from **6b**.

accordance with our previous findings on trehalose and glucose mycolate esters.[71] Wax ester TDM **31** induced a comparable level of TNF-α to TDMs from a *cis*-cyclopropane containing methoxy-mycolic acid (Supplementary data, S1) or a *cis*-cyclopropane containing keto-mycolic acid (**S3**). These three compounds induced more TNF-α than a TDM from a di-*cis*-cyclopropane containing α-mycolic acid (**S2**) in vitro (Fig. 1C) (for structures see Supplementary data).

The in vivo inflammatory potential of synthetic wax ester TDM (**29**) was further compared to the synthetic *cis*-cyclopropane methoxy and α-TDMs. The compounds were formulated in a water in oil in water emulsion and the preparations were injected subcutaneously in the two hind footpads of C57BL/6 mice. The footpad swelling was measured with a caliper as read out for local inflammation. The data showed that all synthetic TDM compounds and TDB can induce a significant footpad swelling 3 days after injection as compared to vehicle control. The level of inflammation induced was comparable for the four compounds (Fig. 2).

4. Conclusion

We have described the first syntheses of complete mycobacterial wax esters in a process that can be adjusted to provide any required absolute stereochemistry or chain length. The wax esters are initially produced as methyl esters at the β-hydroxy-ester, but these may be selectively hydrolysed to the free β-hydroxy-acid without cleavage of the wax ester part, an eicosanol ester. Selective protection of the hydroxyl-group using trimethylsilyl allowed the coupling of the wax ester to a protected trehalose. Following

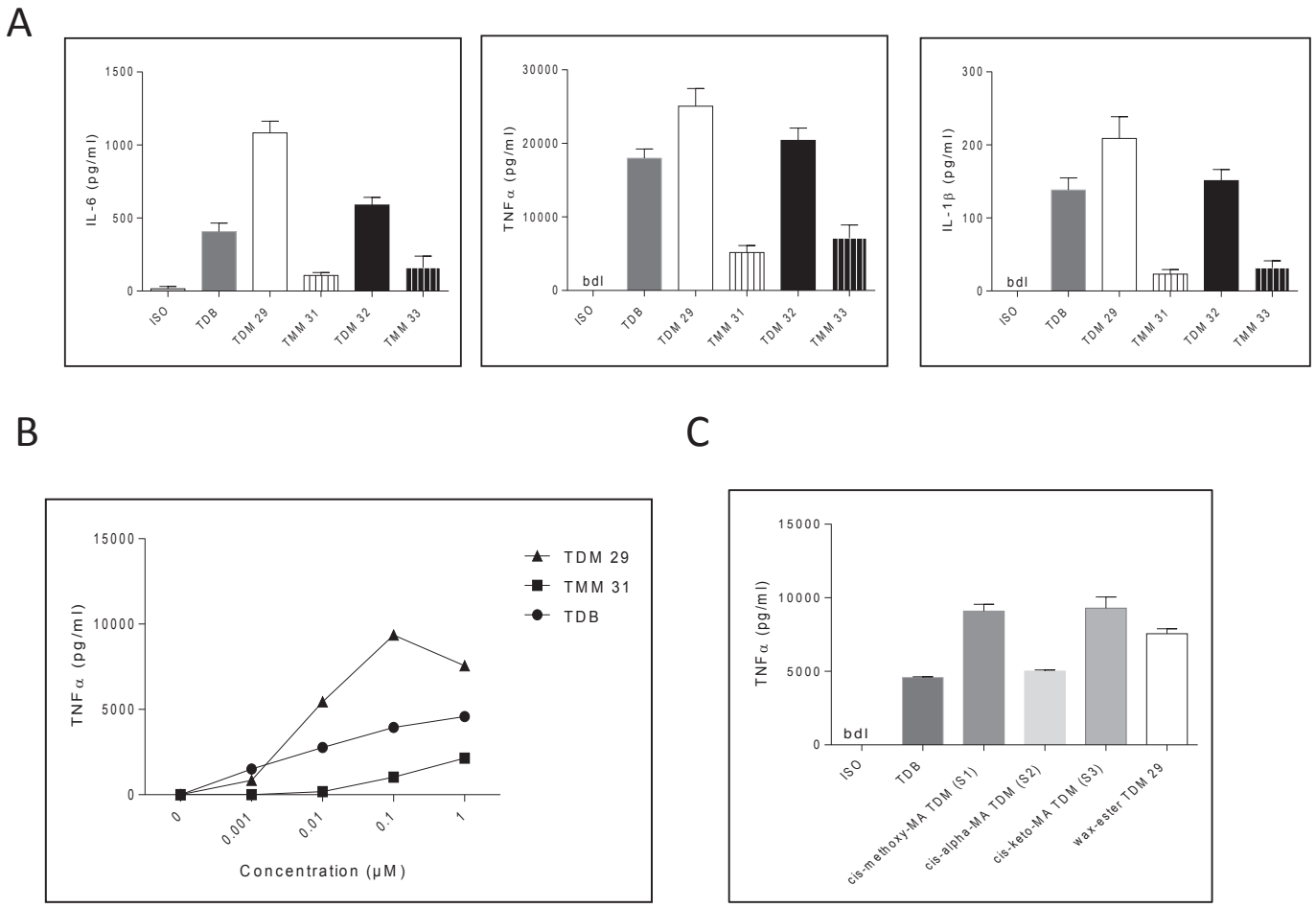


Fig. 1. Wax ester TDM and TMM activate BMDCs in vitro: BMDCs derived from C57BL/6 mice were stimulated for 24 h in triplicate with 1 μM or the indicated concentration of plate-coated synthetic wax ester TDMs **29** and **32** or TMMs **31** and **33**, synthetic TDMs containing *cis*-cyclopropane methoxy-MA (**S1**), keto-MA (**S3**) or α-mycolic acid (**S2**) (structures in Supplementary data), or controls (trehalose dibehenate (TDB) or evaporated isopropanol (ISO)). The supernatants were harvested separately and the amount of pro-inflammatory TNF-α, IL-1β and IL-6 was determined by sandwich ELISA. Results are expressed as mean pg/ml of cytokines ±SD and representative of at least three independent experiments (bdl: below detection limit).

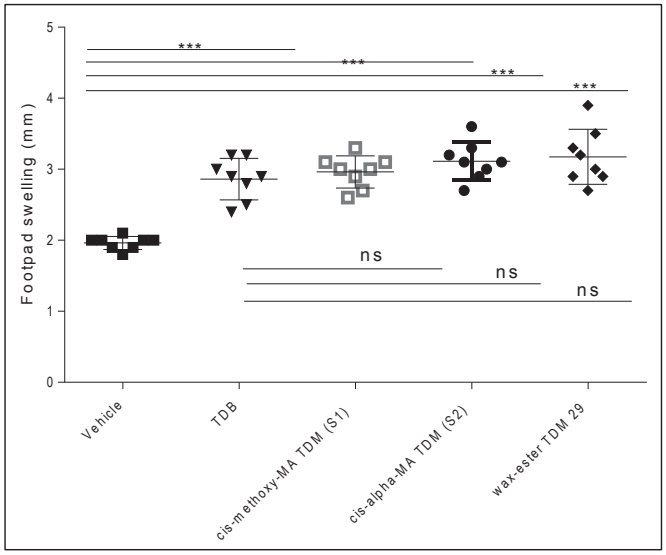


Fig. 2. Wax ester TDM induces inflammation in vivo: Groups of 4–5 C57BL/6 mice were injected s.c in the hind footpads with w/o/w emulsions composed of 3.2% Incomplete Freund's Adjuvant and 5 μg/footpad of synthetic mycolate esters **29**, **S1** and **S2**. In the emulsion of the vehicle control group no glycolipid was present, in the TDB control group 5 μg/footpad of TDB replaced synthetic glycolipid in the emulsion.

deprotection, this led to the free trehalose dimycolate and monomycolate of the wax ester.

The wax-esters are immuno-stimulatory in vitro and in vivo and the level of inflammation induced is at least comparable to that induced by TDB. The applications of the wax ester derivatives as antigens for the serodiagnosis of diseases caused by mycobacterial infections will be described elsewhere.

5. Experimental section

5.1. General

Chemicals used were obtained from commercial suppliers (Sigma, Aldrich, and Alfa Aeser) or prepared from them by the methods described. Solvents which were required to be dry, e.g., ether, THF were dried over sodium wire and benzophenone under nitrogen, while dichloromethane and HMPA were dried over calcium hydride. All reagents and solvents used were of reagent grade unless otherwise stated. Silica gel (Merck 7736) and silica gel plates used for column chromatography and thin layer chromatography were obtained from Aldrich; separated components were detected using variously UV light, I_2 or phosphomolybdic acid solution in IMS followed by charring. Anhydrous magnesium sulfate was used to dry organic solutions. Infra-red (IR) spectra were carried out on a Perkin–Elmer 1600 FTIR spectrometer as liquid films or KBr disc (solid). Melting points were measured using a Gallenkamp melting

point apparatus. NMR spectra were carried out on a Bruker Avance 400 or 500 spectrometer. Specific rotations were recorded in $CHCl_3$ on a POLAAR 2001 Optical Activity Polarimeter. Mass spectra were recorded on a Bruker matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) values are given plus sodium to an accuracy of 1 d.p.; accurate mass values obtained in Bangor were run on a Bruker LC-MS and those using MALDI-TOF MS were determined by Dr. Paul Gates in Bristol University.

5.2. Methyl 16-((1*S*,2*R*)-2-((*S*)-4-((*tert*-butyldiphenylsilyl)oxy) butan-2-yl)cyclopropyl)hexadecanoate (10)

LHMDS (17.8 mL, 18.9 mmol, 1.06 M) was added dropwise to a stirred solution of (1*S*,2*R*)-2-[(*S*)-3-(*tert*-butyldiphenylsilanyloxy)-1-methylpropyl]cyclopropanecarbaldehyde **9** (4.01 g, 10.5 mmol)[64] with 16-(1-phenyl-1*H*-tetrazole-5-sulfonyl)-pentadecanoic acid methyl ester **8** (5.86 g, 12.6 mmol)[65] in dry THF (75 mL) under nitrogen at −15 °C. The mixture was stirred for 1 h at room temperature, then cooled to 0 °C and quenched with satd aq ammonium chloride (30 mL). The organic layer was treated with petrol/ethyl acetate 10:1 (50 mL), and the aqueous layer was re-extracted with petrol/ethyl acetate 10:1 (2×25 mL). The combined organic layers were dried and evaporated; the product was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give a colourless oil, methyl (*E*/*Z*)-16-((1*R*,2*S*)-2-(4-((*tert*-butyldiphenylsilyl)oxy)-2-methylbutan-2-yl)cyclopropyl) hexadec-15-enoate as a 2:1 mixture (5.6 g, 85%). Dipotassium azodicarboxylate (5.03 g, 26.2 mmol) was added to a stirred solution of the above alkenes (5.55 g, 8.98 mmol) in THF (75 mL) and methanol (5 mL) at 5 °C. Glacial acetic acid (5 mL) in THF (5 mL) was added dropwise over 72 h. The mixture was poured into satd aq sodium bicarbonate, and then extracted with ethyl acetate (3×150 mL). The combined organic layers were dried and evaporated to give a thick oil; column chromatography eluting with petrol/ethyl acetate (20:1) gave compound **10** as a colourless oil (5.4 g, 96%), α_D^{21}+7.9 (*c* 0.91, $CHCl_3$) [Found $(M)^+$: 620.4645, $C_{40}H_{64}O_3Si$ requires: 620.4625]; δ_H (400 MHz, $CDCl_3$): 7.68–7.66 (4H, m), 7.42–7.36 (6H, m), 3.76–3.71 (2H, m), 3.67 (3H, s), 2.30 (2H, t, *J* 7.6 Hz), 1.7–1.48 (6H, m), 1.43–1.26 (26H, br m), 1.05 (9H, s), 0.89 (3H, s), 0.42 (1H, m), 0.13 (2H, m); δ_C (101 MHz, $CDCl_3$): 174.2, 135.9, 134.2, 129.57, 127.5, 62.4, 51.4, 40.2, 34.8, 34.4, 34.1, 29.7, 29.63, 29.6, 29.4, 29.3, 29.2, 26.9, 25.9, 25.0, 19.9, 18.6, 10.6; ν_{max}/cm^{-1}: 2925, 2854, 1742, 1428, 1111.

5.3. Methyl 16-((1*S*,2*R*)-2-((2*S*)-4-((tetrahydro-2*H*-pyran-2-yl) oxy)butan-2-yl)cyclopropyl)hexa-decanoate (11)

(i) Tetra-*n*-butylammonium fluoride (10.2 mL, 10.2 mmol) was added to a stirred solution of ester **10** (5.30 g, 8.54 mmol) in dry THF (70 mL) at 0 °C under nitrogen. The mixture was allowed to reach room temperature and stirred for 5 h, then cooled to 5 °C and quenched with satd aq ammonium chloride (30 mL). The product was extracted with ethyl acetate (3×150 mL), then the combined organic layers were washed with brine (100 mL), dried and evaporated to give a crude product, which was purified by column chromatography eluting with petrol: ethyl acetate (5:1) to give methyl 16-((1*S*,2*R*)-2-((*S*)-4-hydroxybutan-2-yl)cyclopropyl)hexadecanoate as a colourless oil (3.1 g, 96%), α_D^{21}+14 (*c* 0.93, $CHCl_3$) [Found $(M)^+$: 382.3467, $C_{24}H_{46}O_3$ requires: 382.3447]; δ_H (400 MHz, $CDCl_3$): 3.78–3.69 (2H, m), 3.67 (3H, s), 2.30 (2H, t, *J* 7.6 Hz), 1.76–1.67 (1H, m), 1.63–1.60 (2H, m), 1.59–1.52 (1H, m), 1.37–1.20 (26H, m), 1.18–1.11 (1H, m), 0.96 (3H, d, *J* 6.6 Hz), 0.90–0.81 (1H, m), 0.51–0.44 (1H, m), 0.25–0.13 (3H, m); δ_C (101 MHz, $CDCl_3$): 174.8, 61.4, 51.4, 40.4, 35.0, 34.4, 34.1, 29.7, 29.63, 29.6, 29.5, 29.3, 29.2, 26.6, 25.9, 25.0, 19.9, 18.8, 16.9, 10.6, 10.0; ν_{max}/cm^{-1}: 3369, 2923, 2853, 1742, 1459, 1170.

(ii) Pyridinium-*p*-toluenesulfonate (1.01 g, 4.01 mmol) was added to a stirred solution of the above alcohol (3.05 g, 7.98 mmol) and 3,4-dihydro-2*H*-pyran (1.67 g, 2.96 mmol) in dry CH_2Cl_2 (40 mL) under nitrogen at rt. The reaction was stirred for 30 min, then quenched with satd aq $NaHCO_3$ (20 mL). The product was extracted with CH_2Cl_2 (2×75 mL) and dried. The solvent was evaporated and the product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **11** as a colourless oil, a mixture of diastereoisomers (3.7 g, 98%), α_D^{21}+19 (*c* 0.75, $CHCl_3$) [Found $(M)^+$: 466.4011, $C_{29}H_{54}O_4$ requires: 466.4022]; δ_H (400 MHz, $CDCl_3$): 4.57 (1H, br m), 3.88–3.79 (2H, m), 3.68 (3H, s), 3.48 (2H, tdd, *J* 5.8, 10.2, 16.1 Hz), 2.31 (2H, t, *J* 7.6 Hz), 1.87–1.71 (1H, m), 1.69–1.64 (2H, m), 1.58–1.54 (7H, m), 1.37–1.20 (25H, m), 1.18–1.11 (1H, m), 0.95 (3H, d, *J* 6.8 Hz), 0.90–0.80 (1H, m), 0.51–0.43 (1H, m), 0.18–0.14 (3H, m); δ_C (101 MHz, $CDCl_3$): 174.4, 99.0, 98.9, 66.2, 65.9, 62.4, 51.4, 37.2, 37.1, 35.3, 35.2, 34.4, 34.1, 30.9, 29.74, 29.7, 29.62, 29.6, 29.5, 29.3, 29.2, 26.0, 25.9, 25.5, 25.0, 19.83, 19.8, 18.62, 18.6, 10.6; ν_{max}/cm^{-1}: 2924, 2853, 1743, 1454.

5.4. 16-((1*S*,2*R*)-2-((2*S*)-4-((Tetrahydro-2*H*-pyran-2-yl)oxy)butan-2-yl)cyclopropyl)-hexadecanal (12)

(i) The ester **11** (3.63 g, 7.78 mmol) in THF (10 mL) was added to a stirred suspension of $LiAlH_4$ (0.440 g, 11.7 mmol) in THF (60 mL) at −10 °C under nitrogen and then refluxed for 1 h, then quenched with satd aq sodium sulfate decahydrate at −10 °C until a white precipitate had formed. The mixture was stirred at rt for 30 min, filtered through a pad of Celite and the solvent was evaporated to give the product which was purified by column chromatography eluting with petrol/ethyl acetate (3:1) to give 16-((1*S*,2*R*)-2-((2*S*)-4-((tetrahydro-2*H*-pyran-2-yl)oxy)butan-2-yl)cyclopropyl)hexadecan-1-ol as a colourless oil, a mixture of diastereoisomers (3.4 g, 94%), α_D^{21}+19 (*c* 0.94, $CHCl_3$) [Found $(M)^+$: 438.4084, $C_{28}H_{54}O_3$ requires: 438.4073]; δ_H (400 MHz, $CDCl_3$): 4.57 (1H, br m), 3.94–3.77 (2H, m), 3.64 (2H, t, *J* 6.6 Hz), 3.56–3.39 (2H, m), 1.92–1.64 (3H, m), 1.59–1.54 (7H, br m), 1.34–1.26 (28H, br m), 1.17 (1H, m), 0.96 (3H, d, *J* 6.7 Hz), 0.90–0.77 (1H, m), 0.58–0.40 (1H, m), 0.28–0.07 (3H, m); δ_C (101 MHz, $CDCl_3$): 99.0, 98.9, 67.7, 66.2, 65.9, 63.0, 62.4, 37.2, 37.1, 35.3, 35.2, 34.4, 32.8, 30.9, 30.8, 29.7, 29.65, 29.62, 29.6, 29.4, 26.0, 25.9, 25.8, 25.5, 19.82, 19.81, 19.8, 18.64, 18.6, 10.6; ν_{max}/cm^{-1}: 3392, 2923, 2852, 1468.

(ii) The above alcohol (3.25 g, 7.41 mmol) in CH_2Cl_2 (10 mL) was added to stirred suspension of PCC (3.99 g, 18.6 mmol) in CH_2Cl_2 (80 mL) and stirred for 2 h, then diluted with petrol/ethyl acetate 10:1 (50 mL), and filtered through a pad of silica gel and Celite. The solvent was evaporated and the product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **12** as a colourless oil, a mixture of diastereoisomers (3.0 g, 93%); α_D^{21}+5.23 (*c* 1.01, $CHCl_3$) [Found $(M)^+$: 436.3911, $C_{28}H_{52}O_3$ requires: 436.3916]; δ_H (400 MHz, $CDCl_3$): 9.77 (1H, t, *J* 1.9 Hz), 4.61–4.53 (1H, br m), 3.95–3.77 (2H, m), 3.56–3.40 (2H, m), 2.42 (2H, td, *J* 7.4, 11 Hz), 1.80–1.75 (3H, m), 1.59–1.53 (7H, br m), 1.34–1.26 (26H, br m), 0.96 (3H, d, *J* 6.9 Hz), 0.91–0.78 (1H, m), 0.59–0.41 (1H, m), 0.29–0.10 (3H, m); δ_C (101 MHz, $CDCl_3$): 203.0, 99.0, 98.9, 66.2, 65.9, 62.4, 37.2, 37.0, 35.3, 35.2, 34.4, 30.9, 30.8, 29.73, 29.7, 29.65, 29.62, 29.6, 29.4, 25.9, 25.5, 19.82, 19.8, 18.61, 18.6, 10.6; ν_{max}/cm^{-1}: 2923, 2852, 1728, 1455.

5.5. Methyl (*R*)-2-((*R*)-1-((*tert*-butyldimethylsilyl)oxy)-3-((1-phenyl-1*H*-tetrazol-5-yl)-thio)propyl)tetra-cosanoate (14)

Diethyl azodicarboxylate (DEAD) (2.38 g, 13.7 mmol) in dry THF (8 mL) was added to a stirred solution of methyl (*R*)-2-((*R*)-1-((*tert*-

butyldimethylsilyl)oxy)-3-hydroxypropyl)tetracosanoate **13** [66] (6.0 g, 10.5 mmol), triphenylphosphine (3.58 g, 13.7 mmol) and 1-phenyl-1*H*-tetrazole-5-thiol (2.43 g, 13.9 mmol) in dry THF (70 mL) at 0 °C under nitrogen atmosphere. The mixture was allowed to reach rt and then stirred for 3 h. The solvent was evaporated and the residue was stirred with petrol/ethyl acetate (10:1, 150 mL) for 30 min and then filtered through a pad of Celite. The filtrate was evaporated and the crude product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **14** as a thick pale yellow oil (6.5 g, 85%), α_D^{21}−13.2 (*c* 1.06, $CHCl_3$) [Found $(M-Bu^t)^+$: 673.4535 calculated for $C_{37}H_{65}N_4O_3SiS$: 673.4547]; δ_H (400 MHz, $CDCl_3$): 7.59–7.52 (5H, m), 4.07 (1H, m), 3.67 (3H, s), 3.51–3.44 (1H, m), 3.40–3.34 (1H, m), 2.59 (1H, ddd, *J* 4.8, 6.9, 11.4 Hz), 2.16–2.09 (1H, m), 2.01–1.93 (1H, m), 1.59–1.21 (42H, br m), 0.90–0.87 (12H, m, including t at 0.87, *J* 6.9 Hz), 0.08 (3H, s), 0.06 (3H, s); δ_C (101 MHz, $CDCl_3$): 174.4, 154.1, 130.1, 129.8, 123.8, 72.0, 51.5, 51.48, 33.1, 31.9, 29.7, 29.66, 29.6, 29.5, 29.4, 28.6, 27.9, 27.1, 25.7, 22.7, 14.1, −4.4, −4.9; ν_{max}/cm^{-1}: 2918, 2852, 1741, 1498, 1459, 1161.

5.6. Methyl (*R*)-2-((*R*)-1-((*tert*-butyldimethylsilyl)oxy)-3-((1-phenyl-1*H*-tetrazol-5-yl)-sulfonyl)propyl)-tetracosanoate (15)

m-Chloroperoxybenzoic acid (7.87 g, 32.1 mmol) was added to a stirred solution of ester **14** (7.80 g, 10.7 mmol) in CH_2Cl_2 (50 mL), followed by addition of $NaHCO_3$ (3.59 g, 42.7 mmol). The mixture was stirred for 24 h at rt, then poured into sat.aq $NaHCO_3$ (150 mL), and stirred for 2 h. The product was extracted with CH_2Cl_2 (3×100 mL), and the combined organic layers were dried and evaporated to give a crude product which was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **15** as a white solid (5.0 g, 86%), α_D^{21}−14.4 (*c* 1.12, $CHCl_3$) [Found $(M-Bu^t)^+$: 705.4431; calculated for $C_{37}H_{65}N_4O_5SiS$: 705.4445]; δ_H (400 MHz, $CDCl_3$): 7.72–7.7 (2H, m), 7.65–7.61 (3H, m), 4.15 (1H, m), 3.81 (2H, m), 3.69 (3H, s), 2.53 (1H, ddd, *J* 4.0, 7.4, 11.0 Hz), 2.16–2.09 (2H, m), 1.59–1.21 (42H, br m), 0.90–0.87 (12H, m, including t at 0.87, *J* 6.9 Hz), 0.08 (3H, s), 0.06 (3H, s); δ_C (101 MHz, $CDCl_3$): 174.1, 153.2, 133, 131.5, 129.7, 125.0, 70.9, 51.8, 51.6, 51.4, 31.9, 29.7, 29.66, 29.62, 29.6, 29.5, 29.4, 29.3, 27.7, 27.4, 26.2, 25.7, 22.70, 14.1, −4.5, −5.1; ν_{max}/cm^{-1}: 2920, 2852, 1729, 1497, 1467, 1155.

5.7. Methyl (2*R*)-2-((1*R*)-1-((*tert*-butyldimethylsilyl)oxy)-19-((1*S*,2*R*)-2-((2*S*)-4-((tetra-hydro-2*H*-pyran-2-yl)oxy)butan-2-yl)cyclopropyl)nonadecyl)tetracosanoate (16)

LHMDS (11.1 mL, 11.7 mmol, 1.06 M) was added dropwise to a stirred solution of sulfone **15** (5.97 g, 7.84 mmol) and aldehyde **12** (2.85 g, 6.53 mmol) in dry THF (50 mL) under nitrogen at −15 °C. The mixture was stirred for 1 h at room temperature, then cooled to 0 °C and quenched with satd aq ammonium chloride (30 mL). The organic layer was extracted with petrol/ethyl acetate 10:1 (50 mL), and the aqueous layer was re-extracted with petrol/ethyl acetate (10:1, 2×25 mL). The combined organic layers were dried and evaporated to give a crude product, which was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give methyl (*E*/*Z*)-(2*R*)-2-((1*R*,*E*)-1-((*tert*-butyldimethylsilyl)oxy)-19-((1*S*,2*R*)-2-((2*S*)-4-((tetrahydro-2*H*-pyran-2-yl)oxy)butan-2-yl)cyclopropyl)-nonadec-3-en-1-yl)tetracosanoate as a diastereomeric mixture in ratio 2:1 (5.0 g, 78%). Dipotassium azodicarboxylate (2.52 g, 13.2 mmol) was added to a stirred solution of the above alkene mixture (4.95 g, 5.09 mmol) in THF (75 mL) and methanol (5 mL) at 5 °C. Glacial acetic acid (5 mL) in THF (5 mL) was added dropwise over 72 h. The mixture was poured into satd aq sodium bicarbonate solution, and extracted with ethyl acetate (3×100 mL). The combined organic layers were dried and evaporated to give a thick oil residue, which was purified by column chromatography eluting with petrol/ethyl acetate (20:1) to give the *title* compound **16** as a mixture of diastereoisomers (4.6 g, 91%), α_D^{21}−8.6 (*c* 0.91, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 997.8949, $C_{62}H_{122}NaO_5Si$ requires: 997.8954], δ_H (400 MHz, $CDCl_3$): 4.58 (1H, br m), 3.97–3.76 (3H, m), 3.67 (3H, s), 3.56–3.40 (2H, m), 2.53 (1H, ddd, *J* 3.7, 7.1, 10.8 Hz), 1.75–1.64 (3H, m), 1.61–1.06 (84H, br m), 0.96 (3H, d, *J* 6.6 Hz), 0.90–0.87 (12H, m, including t at 0.87 with *J* 6.9 Hz), 0.57–0.40 (1H, m), 0.27–0.11 (3H, m), 0.05 (3H, s), 0.02 (3H, s); δ_C (101 MHz, $CDCl_3$): 175.2, 99.0, 98.9, 73.2, 66.2, 65.9, 62.4, 51.5, 51.4, 37.4, 37.2, 37.1, 35.3, 35.2, 34.4, 32.8, 31.9, 30.8, 29.8, 29.7, 29.63, 29.60, 29.5, 29.4, 29.3, 27.9, 27.7, 27.6, 25.9, 25.7, 25.5, 23.7, 22.7, 22.6, 20.4, 19.8, 11.2, 10.0, −4.4, −4.9; ν_{max}/cm^{-1}:, 2924, 2853, 1741, 1464.

5.8. Methyl (*R*)-2-((*R*)-1-((*tert*-butyldimethylsilyl)oxy)-19-((1*S*,2*R*)-2-((*S*)-4-oxobutan-2-yl)cyclo-propyl)nonadecyl)tetracosanoate (17)

(a) Pyridinium-*p*-toluenesulfonate (0.58 g, 2.3 mmol) was added to a stirred solution of methyl tetrahydropyranyl acetal **16** (4.51 g, 4.62 mmol) in THF (50 mL), methanol (10 mL) and stirred at 45 °C for 6 h. Satd aq $NaHCO_3$ (20 mL) and water (20 mL) were added and extracted with ethyl acetate (3×50 mL). The combined organic layers were dried and the solvent was evaporated. Column chromatography eluting with petrol/ethyl acetate (12:1) gave *methyl* (*R*)-2-((*R*)-1-((*tert-butyldimethylsilyl*)*oxy*)-19-((1*S*,2*R*)-2-((*S*)-4-*hy-droxybutan*-2-*yl*)*cyclopropyl*)*nonadecyl*)*tetracosanoate* (3.5 g, 84%), α_D^{21}+7.9 (*c* 1.1, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 913.8369; $C_{57}H_{114}NaO_4Si$ requires 913.8384]; δ_H (400 MHz, $CDCl_3$); 3.92 (1H, ddd, *J* 4.9, 7.6, 11.8 Hz), 3.80–3.68 (2H, m), 3.66 (3H, s), 2.53 (1H, ddd, *J* 3.8, 7.1, 10.9 Hz), 1.72 (1H, td, *J* 6.8, 13.5 Hz), 1.54–1.26 (81H, br m), 0.96 (3H, d, *J* 6.5 Hz), 0.91–0.84 (12H, m, including t at 0.87 with *J* 6.9 Hz), 0.55–0.44 (1H, m), 0.29–0.13 (3H, m), 0.05 (3H, s), 0.03 (3H, s); δ_C (10 MHz, $CDCl_3$): 175.2, 73.2, 61.4, 51.6, 51.2, 40.4, 35.0, 34.4, 33.7, 31.9, 31.6, 29.8, 29.72, 29.7, 29.63, 29.6, 29.59, 29.5, 29.4, 29.1, 27.8, 27.7, 27.65, 27.5, 25.9, 25.8, 25.3, 23.7, 22.7, 22.66, 21.0, 20.5, 19.8, 18.8, 17.9, −4.4, −4.9; ν_{max}/cm^{-1}: 3584, 2924, 2853, 1740, 1463, 1066.

(b) The above alcohol (3.25 g, 3.65 mmol) in CH_2Cl_2 (20 mL) was added to a stirred suspension of PCC (2.36 g, 10.9 mmol) in CH_2Cl_2 (120 mL), stirred for 2 h, then diluted with petrol/ethyl acetate (10:1, 100 mL), and filtered through a pad of silica gel and Celite. The solvent was evaporated and the product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **17** as a colourless oil (3.0 g, 93%), α_D^{21}+1.6 (*c* 1.06, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 912.2; $C_{57}H_{112}NaO_4Si$ requires 911.8]; δ_H (400 MHz, $CDCl_3$): 9.79 (1H, t, *J* 2.5 Hz), 3.92 (1H, dt, *J* 5.9, 7.9 Hz), 3.66 (3H, s), 2.50–2.48 (2H, m), 2.38 (1H, ddd, *J* 2.5, 7.7, 15.7 Hz), 1.54–1.26 (79H, br m), 1.03 (3H, d, *J* 6.8 Hz), 0.92–0.80 (12H, m, including t at 0.87 with *J* 6.9 Hz), 0.50–0.48 (1H, m), 0.38–0.19 (3H, m), 0.05 (3H, s), 0.02 (3H, s); δ_C (101 MHz, $CDCl_3$): 202.9, 73.2, 51.6, 51.5, 34.1, 33.9, 33.7, 31.9, 29.8, 29.73, 29.7, 29.6, 29.5, 29.4, 27.8, 27.72, 27.7, 27.5, 25.9, 25.8, 25.3, 23.7, 22.7, 22.66, 21.0, 20.5, 19.8, 18.82, 14.1, 11.4, −4.4, −4.9; ν_{max}/cm^{-1}: 2924, 2853, 1738, 1464.

5.9. 15-((1-Phenyl-1*H*-tetrazol-5-yl)sulfonyl)pentadecanoic acid (18)

(a) Aq sodium hydroxide (8M) 100 mL was added with stirring to methyl 15-((1-phenyl-1*H*-tetrazol-5-yl)thio)pentadecanoate (10.5 g, 93.8 mmol)[69] in THF (100 mL), followed by addition of MeOH (15 mL). The mixture was refluxed for 30 min, then the solvent was evaporated, and the residue was dissolved in water (50 mL), and then acidified with aq HCl (2M) to pH 2. The aqueous layer was extracted with ethyl acetate (3×100 mL), and the combined organic layers were dried and evaporated to give crude product. Re-crystalization from ethyl acetate gave 15-((1-

phenyl-1*H*-*tetrazol*-5-*yl*)*thio*)*pentadecanoic acid* as a white solid (9.0 g, 89%), mp 78–80 °C {[MALDI-Found] $(M+Na)^+$: 441.4; $C_{22}H_{34}N_4O_2S$ requires 441.2}; δ_H (400 MHz, $CDCl_3$): 7.74–7.59 (5H, m), 3.40 (2H, t, *J* 7.4 Hz), 2.36 (2H, t, *J* 7.5 Hz), 1.82 (2H, quintet, *J* 7.4 Hz), 1.66–1.64 (2H, quintet, *J* 7.4 Hz), 1.48–1.41 (2H, quintet, *J* 7.4 Hz), 1.30–1.26 (19H, m); δ_C (101 Hz, $CDCl_3$): 130.1, 129.8, 123.9, 33.7, 33.4, 29.6, 29.5, 29.49, 29.4, 29.3, 29.28, 29.1, 29.04, 28.6, 24.7; ν_{max}/cm^{-1}: 3436, 2918, 2849, 1725, 1594, 1496, 1463, 1413, 1381, 1172.

(b) A solution of ammonium molybdate (VI) tetrahydrate (7.60 g, 6.14 mmol) in 35% H_2O_2 (15 mL) was prepared and cooled in an ice bath, it was added to a stirred solution of the above acid (5.01 g, 12.3 mmol) in THF (30 mL) and IMS (30 mL) at 10 °C, and stirred at rt for 2 h. A further solution of ammonium molybdate (VI) tetrahydrate (3.80 g, 3.07 mmol) in 35% H_2O_2 (8 mL) was added and the mixture was stirred at rt for 18 h. The mixture was poured into water (250 mL) and extracted with ethyl acetate (3×75 mL). The combined organic layers were dried and the solvent was evaporated. The product was purified by recrystallisation from MeOH/acetone (1:1) to give the *title* compound **18** as a white solid (4.2 g, 78%), mp 88–89 °C [MALDI-Found $(M+Na)^+$: 472.9; $C_{22}H_{34}N_4O_4S$ requires 473.2]; δ_H (400 MHz, $CDCL_3$): 7.74–7.70 (2H, m), 7.66–7.61 (3H, m), 3.74 (2H, t, *J* 8.2 Hz), 2.36 (2H, t, *J* 7.5 Hz), 1.96 (2H, quintet, *J* 7.4 Hz), 1.66 (2H, quintet, *J* 7.4 Hz), 1.48 (2H, quintet, *J* 7.4 Hz), 1.30–1.26 (19H, m); δ_C (101 MHz, $CDCl_3$): 131.5, 129.7, 125.1, 56.1, 33.8, 29.5, 29.42, 29.4, 29.2, 29.17, 29.0, 28.9, 28.1, 24.7, 22.0; ν_{max}/cm^{-1}: 3467, 2921, 2851, 1694, 1597, 1466, 1359, 1152.

5.10. (*S*)-18-((1*R*,2*S*)-2-((19*R*,20*R*)-19-((*tert*-Butyldimethylsilyl)oxy)-20-(methoxycarbonyl)dotetracontyl)cyclopropyl)-nonadecanoic acid (19)

Lithium *bis*(trimethylsilyl)amide (9.40 mL, 9.97 mmol, 1.06 M) was added dropwise to a stirred solution of ester **17** (2.95 g, 3.32 mmol) and sulfone **18** (1.79 g, 3.98 mmol) in dry THF (40 mL) under nitrogen at −15 °C. The mixture was stirred for 1 h at room temperature, then cooled to 0 °C and quenched with satd aq ammonium chloride (30 mL). The organic layer was extracted with petrol/ethyl acetate (10:1, 40 mL), and the aqueous layer was re-extracted with petrol/ethyl acetate (10:1, 2×20 mL). The combined organic layers were dried and evaporated; the product was purified by column chromatography eluting with 20:1 petrol/ethyl acetate to give (*E*/*Z*)-18-((1*R*,2*S*)-2-((19*R*,20*R*)-19-((*tert*-butyldimethylsilyl)-oxy)-20-(methoxycarbonyl)dotetracontyl)cyclopropyl)-nonadec-15-enoic acid (3.0 g, 80%) as a mixture in ratio (2.3:1); dipotassium azodicarboxylate (3.75 g, 19.5 mmol) was added to a stirred solution of the above alkene (2.9 g, 2.6 mmol) in THF (60 mL) and methanol (5 mL) at 5 °C. Glacial acetic acid (5 mL) in THF (5 mL) was added dropwise over 72 h. The mixture was poured in to satd aq sodium bicarbonate, then extracted with ethyl acetate (3×100 mL). The combined organic layers were dried and evaporated. The product was purified by column chromatography eluting with petrol/ethyl acetate (10:1) to give the *title* compound **19** as a semi-solid (2.6 g, 80%), α_D^{21}+4.1 (*c* 0.48 $CHCl_3$) {[MALDI-Found] $(M+Na)^+$: 1138.0530; $C_{72}H_{142}NaO_5Si$ requires 1138.0519}; δ_H (400 MHz, $CDCl_3$): 3.92 (1H, dt, *J* 4.8, 7.1 Hz), 3.66 (3H, s), 2.53 (1H, ddd, *J* 3.8, 7.2, 10.9 Hz), 2.36 (2H, t, *J* 7.5 Hz), 1.64 (3H, m), 1.57–1.03 (106H, br m), 0.94–0.83 (15H, m, including t at 0.87 with *J* 6.9 Hz), 0.73–0.62 (1H, m), 0.51–0.40 (1H, m), 0.26–0.07 (3H, m), 0.05 (3H, s), 0.02 (3H, s); δ_C (101 MHz, $CDCl_3$): 178.8, 175.2, 73.2, 68.2, 60.4, 51.6, 51.2, 38.7, 38.1, 37.4, 33.9, 33.7, 32.7, 31.9, 29.7, 29.6, 29.4, 29.3, 27.6, 27.5, 27.3, 25.8, 25.7, 23.7, 22.7, 21.0, 19.3, 19.2, 18.6, 18.0, 14.10, 14.0, 11.4, 10.5, 10.3, −4.4, −4.9; ν_{max}/cm^{-1}: 2924, 2853, 1743, 1711, 1464.

5.11. (*S*)-Eicosan-2-ol (21)

Bromoheptadecane (5.01 g, 15.6 mmol) was dissolved in dry THF (5 mL) and then added slowly to a stirred suspension of magnesium turnings (2.26 g, 94.1 mmol) in dry THF (8 mL) and warmed gently until the Grignard reagent started to form. The mixture was refluxed for 1 h. The Grignard reagent was added slowly to a stirred suspension of copper iodide (0.980 g, 5.17 mmol) in dry THF (40 mL) at −30 °C and stirred for 30 min. (*S*)-1-Epoxypropane (0.40 g, 6.89 mmol) in dry THF (5 mL) was added dropwise at −30 °C. The mixture allowed to reach room temperature and stirred for 16 h, then quenched with ammonium chloride and then extracted with ethyl acetate (3×100 mL). The combined organic layers were dried and evaporated to give the crude product; column chromatography eluting with petrol/ethyl acetate (10:1) gave (*S*)-eicosan-2-ol **21** (1.1 g, 53%). Mp 61–63 °C, α_D^{21}+3.8 (*c* 1.01, $CHCl_3$); δ_H (400 MHZ, $CDCl_3$): 3.80 (1H, sextet, *J* 6 Hz), 1.48 (2H, m), 1.38–1.25 (32H, br m), 1.2 (3H, d, *J* 6.2 Hz), 0.88 (3H, t, *J* 6.7 Hz); δ_C (101 MHz, $CDCl_3$): 68.2, 39.4, 31.9, 29.7, 29.66, 29.63, 29.6, 29.4, 25.8, 23.5, 22.5, 14.1; ν_{max}/cm^{-1}: 3386, 2924, 2853, 1464.

5.12. Methyl (*R*)-2-((*R*)-1-((*tert*-butyldimethylsilyl)oxy)-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxoneonadecan-2-yl)cyclopropyl)nonadecyl)tetracosanoate (22)

Acid **19** (2.41 g, 2.16 mmol), (*S*)-eicosan-2-ol **21** (0.67 g, 2.22 mmol) and DMAP (0.39 g, 3.22 mmol) were dissolved in dry CH_2Cl_2 (15 mL) under nitrogen at room temperature. DCC (0.88 g, 4.3 mmol) in dry CH_2Cl_2 (7 mL) was added dropwise over 15 min and stirred at rt for 3 h, then diluted with DCM, filtered and evaporated. Column chromatography eluting with petrol/ethyl acetate (20:1) gave the *title* compound **22** as a colourless oil (2.4 g, 80%), α_D^{21}+6.1 (*c* 0.90, $CHCl_3$) [Found $(M+Na)^+$: 1418.3659; $C_{92}H_{182}NaO_5Si$ requires 1418.3654]; δ_H (400 MHz, $CDCl_3$): 4.91 (1H, sextet, *J* 6.2 Hz), 3.92 (1H, dt, *J* 4.8, 7.1 Hz), 3.66 (3H, s), 2.53 (1H, ddd, *J* 3.8, 7.1, 10.9 Hz), 2.27 (2H, t, *J* 7.5), 1.54–1.26 (142H, br m), 1.20 (3H, d, *J* 6.2 Hz), 0.91 (3H, d, *J* 6.8 Hz), 0.91–0.87 (15H, m, including two t at 0.87 with *J* 7 Hz), 0.71–0.63 (1H, m), 0.49–0.41 (1H, m), 0.22–0.08 (3H, m), 0.05 (3H, s), 0.02 (3H, s); δ_C (101 MHz, $CDCl_3$): 175.2, 173.6, 73.2, 70.7, 51.6, 51.2, 38.1, 37.4, 36.0, 34.8, 34.5, 33.7, 31.9, 30.1, 29.83, 29.8, 29.75, 29.73, 29.7, 29.6, 29.57, 29.52, 29.5, 29.4, 29.3, 29.2, 27.8, 27.5, 27.2, 26.2, 25.8, 25.4, 25.1, 23.8, 23.7, 22.7, 20.0, 19.7, 18.6, 18.0, 11.0, 10.5, −4.4, −4.9; ν_{max}/cm^{-1}: 2924, 2853, 1738, 1464.

5.13. (*R*)-2-((*R*)-1-Hydroxy-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxononadecan-2-yl)cyclo-propyl)nonadecyl)tetracosanoic acid (6a)

(a) Ester **22** (2.30 g, 1.79 mmol) was dissolved in dry THF (30 mL) in a dry polyethylene vial equipped with a rubber septum, followed by addition of pyridine (0.7 mL) at rt under nitrogen. The mixture was cooled to 10 °C, and then HF-pyridine complex as ~70% (4.0 mL) was added dropwise. The mixture was stirred at 43 °C for 17 h, then neutralized by pouring slowly into satd aq $NaHCO_3$ until no more CO_2 was liberated. The product was extracted with petrol/ethyl acetate (5:1, 3×30 mL), then the combined organic layers were dried and evaporated. Chromatography eluting with petrol/ethyl acetate (10:1) gave *methyl* (*R*)-2-((*R*)-1-*hydroxy*-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-*eicosan*-2-*yloxy*)-19-*oxononadecan*-2-*yl*)*cyclopropyl*)-*nonadecyl*)*tetracosanoate* as a colourless thick oil (1.5 g, 71%), α_D^{21}+7.3 (*c* 0.11, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 1304.2782; $C_{86}H_{168}O_5Na$ requires 1304.2784]; δ_H (400 MHz, $CDCl_3$): 4.91 (1H, sextet, *J* 6.2 Hz), 3.71 (3H, s), 3.66 (1H, m), 2.53 (1H, m), 2.27 (2H, t, *J* 7.5 Hz), 1.54–1.26 (143H, br m), 1.20 (3H, d, *J* 6.2 Hz), 0.90 (3H, d, *J* 6.8 Hz), 0.91–0.87 (6H, t, including two t with *J* 6.9 Hz), 0.71–0.63

(1H, m), 0.49–0.41 (1H, m), 0.22–0.08 (3H, m); δ_C (101 MHz, $CDCl_3$):176.2, 173.6, 72.3, 70.7, 51.6, 51.2, 38.1, 37.4, 36.0, 34.8, 34.5, 33.7, 31.9, 30.1, 29.6, 29.5, 27.8, 27.5, 27.2, 26.1, 25.8, 25.4, 25.1, 23.8, 23.7, 22.7, 20.1, 19.7, 18.6, 18.0, 14.1, 10.5; ν_{max}/cm^{-1}: 3441, 2918, 2850, 1734, 1732, 1466.

(b) The above alcohol (1.42 g, 1.11 mmol) was dissolved in aq nBu_4NOH (64 mL, 5%) and refluxed at 100 °C for 24 h. The product was extracted with petroleum/ether (5:2, 3×75 mL), dried and evaporated. Chromatography eluting with petrol/ethyl acetate (5:1) gave the *title* compound **6a** as a white solid (0.84 g, 60%), α_D^{21}+7.6 (*c* 0.40, $CHCl_3$), mp 55–56 °C [MALDI-Found $(M+Na)^+$: 1290.2617; $C_{85}H_{166}O_5Na$ requires 1290.2627]; δ_H (400 MHz, $CDCl_3$): 4.91 (1H, sextet, *J* 6.2 Hz), 3.72 (1H, dt, *J* 4.8, 9.2 Hz), 2.46 (1H, dt, *J* 5.3, 9.0 Hz), 2.27 (2H, t, *J* 7.5 Hz), 1.81–0.95 (144H, br m), 1.20 (3H, d, *J* 6.2 Hz), 0.90 (3H, d, *J* 6.8 Hz), 0.91–0.87 (6H, t, including two t with *J* 6.9 Hz), 0.72–0.63 (1H, m), 0.50–0.41 (1H, m), 0.22–0.02 (3H, m); δ_C (101 MHz, $CDCl_3$): 179.6, 173.5, 72.1, 70.8, 50.8, 38.1, 37.4, 36.0, 35.5, 34.8, 34.5, 31.9, 30.1, 29.7, 29.54, 29.5, 29.43, 29.4, 29.3, 29.2, 27.32, 27.3, 26.1, 25.7, 25.4, 25.1, 22.7, 20.0, 19.7, 18.6, 14.1, 10.5; ν_{max}/cm^{-1}: 3432, 2918, 2850, 1721, 1712, 1470.

5.14. (*R*)-2-((*R*)-19-((1*S*,2*R*)-2-((*S*)-18-Carboxyoctadecan-2-yl)-cyclopropyl)-1-hydroxynonadecyl)tetracosanoic acid (23)

(a) (*S*)-18-((1*R*,2*S*)-2-((19*R*,20*R*)-19-((*tert*-Butyldimethylsilyl) oxy)-20-(methoxycarbonyl)dotetracontyl)-cyclopropyl)nonadecanoic acid (0.10 g, 0.09 mmol) was dissolved in dry THF (5 mL) in a dry polyethylene vial equipped with a rubber septum, and followed by addition of pyridine (0.1 mL) at rt under nitrogen. The mixture was cooled to 10 °C, and then HF-pyridine complex (~70%, 0.4 mL) was added dropwise. The mixture was stirred at 43 °C for 17 h, then poured slowly into satd aq sodium bicarbonate until no more CO_2 was liberated. The product was extracted with petrol/ethyl acetate (5:1, 3×10 mL), dried and evaporated; chromatography eluting with petrol/ethyl acetate (4:1) gave (*S*)-18-((1*R*,2*S*)-2-((19*R*,20*R*)-19-*Hydroxy*-20-(*methoxycarbonyl*)*dotetracontyl*)-*cyclopropyl*)*nona-decanoic acid* as a viscous oil (75 mg, 85%), α_D^{21}+7.8 (*c* 1.6, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 1024.6; $C_{66}H_{128}NaO_5$ requires 1023.9]; δ_H (400 MHz, $CDCl_3$): 3.71 (3H, s), 3.68–3.65 (1H, m), 2.53 (1H, dt, *J* 4.7, 9.8 Hz), 2.36 (2H, t, *J* 7.5 Hz), 1.74–1.62 (4H, m), 1.55–1.26 (106 H, m), 0.91–0.86 (6H, d, *J* 5.4 Hz, including t with *J* 5.7 Hz), 0.69–0.62 (1H, m), 0.49–0.41 (1H, m), 0.22–0.08 (3H, m); δ_C (101 MHz, $CDCl_3$): 178.6, 176.3, 72.3, 60.4, 51.6, 51.0, 38.2, 37.4, 35.7, 34.5, 33.9, 31.9, 30.1, 29.76, 29.73, 29.7, 29.63, 29.6, 29.58, 29.54, 29.5, 29.47, 29.43, 29.4, 27.3, 29.1, 27.4, 27.2, 25.7, 24.7, 22.7, 21.1, 19.7, 18.6, 14.2; ν_{max}/cm^{-1}: 2917, 2849, 1733, 1701, 1464.

(b) The above acid (0.071 g, 0.072 mmol) was added to a stirred solution of THF (8 mL), water (1 mL) and MeOH (0.8 mL), followed by the addition of lithium hydroxide monohydrate (0.044 g, 1.073 mmol, 15 mol eq.). The mixture was heated at 45 °C for 16 h, then diluted with petrol/ethyl acetate (5:1, 4 mL), and acidified to pH 2 using satd aq $KHSO_4$. The aqueous layer was extracted with petrol/ethyl acetate (5:1, 3×5 mL), and the combined organic layers were dried and evaporated to give a crude product; chromatography eluting with petrol/ethyl acetate (1:3) gave the *title* compound **23** as a white solid (59 mg, 85%), mp 76–78 °C, α_D^{21}+5.8 (*c* 0.86, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 1009.9520; $C_{65}H_{126}NaO_5$ requires 1009.9497]; δ_H (400 MHz, $CDCl_3$): 3.74–3.70 (1H, m), 2.53 (1H, dt, *J* 4.3, 9.8 Hz), 2.35 (2H, t, *J* 7.5 Hz), 1.77–1.62 (4H, m), 1.59–1.26 (107 H, m), 0.91–0.86 (6H, d, *J* 5.4 Hz, including t with *J* 5.7 Hz), 0.73–0.68 (1H, m), 0.50–0.44 (1H, m), 0.24–0.10 (3H, m); δ_C (101 MHz, $CDCl_3$): 180.1, 179.1, 72.2, 38.1, 37.5, 35.6, 34.5, 34.0, 31.9, 30.8, 29.7, 29.6, 29.5, 29.4, 29.1, 27.4, 27.3, 26.2, 25.7, 24.7, 22.7, 19.7, 18.6, 14.0, 10.5; ν_{max}/cm^{-1}: 2917, 2849, 1733, 1701, 1464.

5.15. (*R*)-2-((*R*)-1-((*tert*-Butyldimethylsilyl)oxy)-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxo-nonadecan-2-yl)cyclopropyl)nonadecyl)tetracosanoic acid (24)

Imidazole (0.302 g, 4.507 mmol) was added to a stirred solution of acid **6a** (0.560 g, 0.448 mmol) in dry DMF (3.5 mL) and dry toluene (5 mL) at rt followed by the addition of TBDMSCl (0.675 g, 4.482 mmol) and DMAP (0.055 g, 0.448 mmol). The mixture was stirred at 70 °C for 18 h, then the solvent was removed under high vacuum and the residue was diluted with petrol/ethyl acetate (5:1, 30 mL) and water (20 mL). The organic layer separated and the aqueous layer was re-extracted with petrol/ethyl acetate (5:1, 2×20 mL). The combined organic layers were washed with water, dried and evaporated. The residue was dissolved in THF (8 mL), to this was added aq nBu_4NOH (4.5 mL, 4%). The mixture was stirred for 15 min at room temperature, and then diluted with water (5 mL) and petrol/ethyl acetate (2:1, 20 mL). The organic layer was separated and the aqueous layer was re-extracted with petrol/ethyl acetate (2×20 mL). The combined organic layers were dried and evaporated to give a crude product; chromatography eluting with petrol/ethyl acetate (20:1) gave the *title* compound **24** as a syrup (0.53 g, 87%), α_D^{21}+23 (*c* 0.50, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 1404.8; $C_{91}H_{180}NaO_5Si$ requires 1404.3]; δ_H (400 MHz, $CDCl_3$): 4.92 (1H, sextet, *J* 6.2 Hz), 3.83 (1H, m), 2.53 (1H, ddd, *J* 2.8, 6, 9.1 Hz), 2.27 (2H, t, *J* 7.4 Hz), 1.81–0.95 (143H, br m), 1.20 (3H, d, *J* 6.2 Hz), 0.993 (9H, s), 0.90 (3H, d, *J* 6.8 Hz), 0.91–0.87 (6H, t, including two t with *J* 6.9 Hz), 0.70–0.40 (1H, m), 0.48–0.41(1H, m), 0.15 (3H,s), 0.12 (3H, s), 0.22–0.06 (3H, m); δ_C (101 MHz, $CDCl_3$): 179.7, 173.6, 73.7, 70.7, 60.4, 50.1, 38.1, 37.4, 36.0, 35.5, 34.8, 34.5, 31.9, 30.1, 29.7, 29.5, 29.45, 29.4, 29.36, 29.3, 29.2, 27.3, 27.26, 26.1, 25.8, 25.4, 25.1, 22.7, 22.3, 20.0, 19.7, 18.6, 17.9, 14.1, 14.05, 10.5, −4.2, −4.9; ν_{max}/cm^{-1}: 3432, 2918, 2850, 1721, 1712, 1470.

5.16. 6-*O*-[(*R*)-2-((*R*)-1-Hydroxy-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxononadecan-2-yl)-cyclopropyl)nonadecyl)tetracosanoate]-α-D-glucopyranosyl-(1-1′)-6′-*O*-[(*R*)-2-((*R*)-1-hydroxy-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxononadecan-2-yl)cyclopropyl)nonadecyl)tetracosanoate]-α-D-glucopyranoside (29) and 6-*O*-[(*R*)-2-((*R*)-1-hydroxy)-19-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-19-oxononadecan-2-yl)*cyclopropyl*)nonadecyl)tetracosanoate]-α-D-glucopyranosyl-(1-1′)-α-D-glucopyranoside (31)

(i) 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (242 mg, 1.26 mmol) and DMAP (154 mg, 1.26 mmol) were added to a stirred solution of acid **24** (500 mg, 0.360 mmol), protected trehalose **25** (141 mg, 0.18 mmol)[67,70] and powdered 4 Å molecular sieves in dry CH_2Cl_2 (4 mL) at rt under nitrogen. The mixture was stirred for 5 days then three spatulas of silica gel was added and the solvent was evaporated under reduced pressure to give a residue; chromatography on silica eluting with petroleum ether/ethyl acetate (25:1) gave first compound **26** (0.33 g, 52%), α_D^{21}+21 (*c* 0.50, $CHCl_3$) [MALDI-Found $(M+Na)^+$ 3524.0; $C_{212}H_{426}NaO_{19}Si_8$ requires 3524.0]; δ_H (400 MHz, $CDCl_3$): 4.92 (2H, sextet, *J* 6.3 Hz), 4.85 (2H, d, *J* 3.0 Hz), 4.38 (2H, br, d, *J* 10.0 Hz), 4.04–3.98 (4H, m), 4.0–3.96 (2H, m) 3.93 (2H, m), 3.52 (2H, t, *J* 8.9 Hz), 3.38 (2H, dd, *J* 2.9, 9.3 Hz), 2.55 (2H, ddd, *J* 3.5, 4.8, 10.1 Hz), 2.26 (4H, t, *J* 7.5 Hz), 1.56–1.21 (284H, m), 1.20 (6H, d, *J* 6.2 Hz), 0.88 (18H, s), 0.90 (6H, d, *J* 6.8 Hz), 0.91–0.87 (12H, t, including two t with *J* 6.9 Hz), 0.72–0.65 (2H, m), 0.48–0.41 (2H, m), 0.22–0.06 (6H, m), 0.16 (18H, s), 0.145 (18H, s), 0.138 (18H, s), 0.062 (12H, s); δ_C (101 MHz, $CDCl_3$): 173.8, 173.6, 94.8, 73.5, 73.4, 72.8, 71.8, 70.7, 62.4, 60.4, 51.9, 41.3, 38.1, 37.4, 36.0, 34.5, 33.4, 31.9, 29.8, 29.5, 29.44, 29.42, 29.4, 29.3, 29.2, 27.3, 27.2, 26.2, 25.8, 25.4, 25.2, 22.7, 22.3, 20.0, 19.7, 18.6, 18.0, 14.3, 14.2, 14.1, 11.4, 10.5, 1.1, 1.0, 0.15, −4.5, −4.7; ν_{max}/cm^{-1}: 2924, 2854, 1733, 1465, 1375, 1254, 1215,

836, 760. The second fraction was glucopyranoside **27** (0.114 g, 32%), α_D^{21}+28 (*c* 0.50, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 2160.5; $C_{121}H_{248}O_{15}Si_7Na$ requires 2160.6]; δ_H (400 MHz, $CDCl_3$): 4.92 (2H, d, *J* 3 Hz), 4.91 (1H, sextet, *J* 6.2 Hz), 4.85 (1H, d, *J* 2.9 Hz), 4.36 (1H, dd, *J* 2, 11.6 Hz), 4.08 (1H, dd, *J* 4.0, 11.7 Hz), 4.04–3.98 (4H, m), 3.85 (1H, dt, *J* 3.2, 9.5 Hz), 3.69 (2H, m), 3.5 (2H, dt, *J* 4.6, 9.1 Hz), 3.41 (1H, dd, *J* 3.1, 9.3 Hz), 3.38 (1H, dd, *J* 2.8, 9.1 Hz), 2.55 (1H, ddd, *J* 3.4, 5.4, 10.3 Hz), 2.27 (2H, t, *J* 7.4 Hz), 1.73 (1H, br m), 1.68–1.21 (141H, m), 1.20 (3H, d, *J* 6.2 Hz), 0.88 (9H, s), 0.90 (3H, d, *J* 6.8 Hz), 0.91–0.87 (6H, t, including two t with *J* 6.9 Hz), 0.70–0.40 (1H, m), 0.48–0.41 (1H, m), 0.22–0.06 (3H, m), 0.17 (18H, s), 0.15 (18H, s), 0.14 (18H, s), 0.060 (3H, s), 0.052 (3H, s); δ_C (101 MHz, $CDCl_3$): 174.5, 173.6, 94.5, 94.4, 73.4, 73.3, 72.9, 72.8, 72.7, 72.0, 71.4, 70.7, 62.4, 61.7, 51.8, 38.1, 37.4, 36.0, 34.8, 34.4, 34.1, 33.4, 31.9, 30.1, 29.72, 29.7, 29.68, 29.65, 29.63, 29.6, 29.5, 29.45, 29.4, 28.1, 27.3, 26.3, 26.1, 25.8, 25.4, 25.1, 22.62, 22.6, 22.3, 20.0, 19.7, 18.7, 18.0, 14.1, 14.0, 10.5, 1.1, 1.05, 1.004, 1.0, 0.9, 0.8, 0.2, 0.03, −4.5, −4.7; ν_{max}/cm-1: 2924, 2853, 1743, 1464.9, 1251.6, 1163, 1099, 872, 839.

(ii) Tetrabutylammonium fluoride (0.334 mL, 0.334 mmol, 1M) was added to a stirred solution of glucopyranoside **26** (0.305 g, 0.087 mmol) in dry THF (25 mL) at 5 °C under nitrogen. The mixture was allowed to reach rt and stirred for 30 min, then the solvent was evaporated and the residue was purified by column chromatography eluting with $CHCl_3$/MeOH (10:1) to give glucopyranoside **28** as a viscous oil (0.19 g, 71%), α_D^{21}+26 (*c* 0.13, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 3091.8; $C_{194}H_{378}O_{19}Si_2Na$ requires 3091.8]; δ_H (400 MHz, $CDCl_3$+few drops of CD_3OD): 5.05 (2H, d, *J* 3.2 Hz), 4.92 (2H, sextet, *J* 6.3 Hz), 4.37 (2H, br, dd, *J* 3.5, 11.3 Hz), 4.21 (2H, br d, *J* 11.1 Hz), 3.93–3.85 (4H, m), 3.77 (2H, t, *J* 9 Hz), 3.51 (2H, dd, *J* 3.4, 9.4 Hz), 3.36 (2H, m), 3.30 (2H, t, *J* 9.4 Hz), 2.55 (2H, br m), 2.22 (4H, t, *J* 7.4 Hz), 1.61–1.24 (288H, m), 1.18 (6H, d, *J* 6.3 Hz), 0.88 (18H, s), 0.90 (6H, d, *J* 6.8 Hz), 0.89–0.87 (12H, t, including two t with *J* 6.9 Hz), 0.68–0.58 (2H, m), 0.49–0.38 (2H, m), 0.20–0.06 (6H, m), 0.02 (6H, s), 0.007 (6H, s); δ_C (101 MHz, $CDCl_3$+few drops of CD_3OD): 175.2, 173.8, 93.5, 73.2, 72.8, 70.8, 70.2, 70.1, 67.8, 62.9, 51.6, 50.2, 49.7, 49.5, 49.3, 49.1, 38.0, 37.3, 34.7, 34.4, 33.8, 32.0, 29.7, 29.6, 29.5, 29.42, 29.4, 29.3, 29.2, 26.3, 25.8, 25.4, 25.1, 22.7, 22.4, 20.0, 19.7, 18.6, 18.2, 14.0, 10.4, −4.6, −5.0; ν_{max}/cm^{-1}: 3421, 2922, 2853, 1732, 1728, 1465, 1375, 1253, 836, 721.

(iii) Tetrabutylammonium fluoride (0.144 mL, 0.144 mmol, 1M) was added to a stirred solution of glucopyranoside **27** (0.103 g, 0.0480 mmol) in dry THF (13 mL) at 5 °C under nitrogen. The mixture was allowed to reach rt and stirred for 20 min, then the solvent was evaporated and the residue was purified by column chromatography eluting with $CHCl_3$/MeOH (5:1) to give glucopyranoside **30** as a semi-solid (0.073 g, 89%), α_D^{21}+17 (*c* 0.78, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 1728.3; $C_{103}H_{200}O_{15}SiNa$ requires 1728.4]; δ_H (400 MHz, $CDCl_3$+few drops of CD_3OD): 5.05 (2H, d, *J* 3.4 Hz), 4.85 (1H, sextet, *J* 6.2 Hz), 4.32–4.22 (2H, m), 3.92 (1H, br d, *J* 9.6 Hz), 3.86–3.78 (4H, m), 3.67 (1H, m), 3.5 (2H, dd, *J* 3.4, 9.7 Hz), 3.37–3.25 (3H, m), 2.52 (1H, ddd, *J* 3.4, 5.4, 10.3 Hz), 2.22 (2H, t, *J* 7.5 Hz), 1.58–1.50 (4H, m), 1.49–1.21 (145H, m), 1.16 (3H, d, *J* 6.3 Hz), 0.88 (9H, s), 0.7 (3H, d, *J* 6.8 Hz), 0.91–0.87 (6H, t, including two t with *J* 6.9 Hz), 0.66–0.57 (1H, m), 0.44–0.36 (1H, m), 0.10–0.03 (3H, m), 0.007 (3H, s), 0.015 (3H, s); δ_C (101 MHz, $CDCl_3$+few drops of CD_3OD): 175.1, 173.9, 93.5, 93.4, 73.2, 73.0, 72.6, 72.1, 71.5, 70.9, 70.7, 70.2, 69.9, 67.9, 62.4, 61.7, 51.6, 38.0, 37.3, 35.8, 34.7, 33.5, 31.8, 30.0, 29.72, 29.7, 29.6, 29.5, 29.4, 29.39, 29.3, 29.2, 29.0, 27.6, 26.9, 26.1, 25.6, 25.3, 25.0, 24.2, 22.62, 22.6, 19.8, 19.6, 18.5, 17.8, 14.6, 14.0, 10.4, −4.6, −5.0; ν_{max}/cm^{-1}: 3428, 2924, 2852, 1732, 1465, 1375, 1251, 835, 759.

(iv) A dry polyethylene vial equipped with an acid-proof rubber septum was charged with glucopyranoside **28** (0.180 gm, 0.058 mmol) and pyridine (0.07 mL) in dry THF (20 mL) and stirred at rt under nitrogen. The mixture was cooled to 10 °C, and then HF-pyridine complex (~70%, 1.35 mL) was added dropwise. The mixture was stirred at 43 °C for 17 h, then diluted with THF (5 mL). The excess of the HF was neutralized with triethylamine (2 mL), and the solvent was evaporated under high vacuum; column chromatography eluting with $CHCl_3$/MeOH 10:1 gave the glucopyranoside **29** (0.095 g, 58%), α_D^{21}+31 (*c* 0.50, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 2863.6340; $C_{182}H_{350}NaO_{19}$ requires 2863.6314]; δ_H (400 MHz, $CDCl_3$+few drops of CD_3OD): 4.98 (2H, d, *J* 3.4 Hz), 4.87 (2H, sextet, *J* 6.4 Hz), 4.68 (2H, br d, *J* 10.8 Hz), 4.24 (2H, t, *J* 9.3 Hz), 3.93 (2H, dd, *J* 3.8, 10.9 Hz), 3.73 (2H, t, *J* 9.2 Hz), 3.66–3.62 (2H, m), 3.49 (2H, dd, *J* 3.7, 9.8 Hz), 3.19 (2H, t, *J* 9.5 Hz), 2.40 (2H, br m), 2.23 (4H, t, *J* 7.4 Hz), 1.58–1.53 (8H, m), 1.52–1.22 (284 H, m), 1.16 (6H, d, *J* 6.2 Hz), 0.86 (6H, d, *J* 6.8 Hz), 0.86–0.82 (12H, t, including two t with *J* 6.9 Hz), 0.66–0.59 (2H, m), 0.43–0.37 (2H, m), 0.18–0.04 (6H, m); δ_C (101 MHz, $CDCl_3$+few drops of CD_3OD): 175.7, 173.6, 95.2, 72.5, 71.1, 70.9, 70.7, 69.8, 64.7, 52.1, 38.0, 37.3, 34.7, 34.4, 33.8, 32.0, 29.7, 29.6, 29.5, 29.42, 29.4, 29.3, 29.2, 29.0, 27.2, 26.0, 25.3, 25.0, 22.7, 22.6, 19.9, 19.5, 18.5, 14.0, 10.4; ν_{max}/cm^{-1}: 3432, 2918, 2850, 1721, 1712, 1470.

(v) A dry polyethylene vial equipped with an acid-proof rubber septum was charged with glucopyranoside **30** (0.062 gm, 0.038 mmol) and pyridine (0.06 mL) in dry THF (10 mL) and stirred at rt under nitrogen. The mixture was cooled to 10 °C, and then HF-pyridine complex (~70%, 0.5 mL) was added dropwise. The mixture was stirred at 43 °C for 17 h then worked up and purified as above, eluting with $CHCl_3$/MeOH (5:1) to give the title glucopyranoside **31** as a white solid (0.035 mg, 58%), α_D^{21}+26 (*c* 1.12, $CHCl_3$), mp 116–117 °C [MALDI-Found $(M+Na)^+$: 1614.3698; $C_{97}H_{187}NaO_{15}$ requires 1614.3684]; δ_H (400 MHz, $CDCl_3$+few drops of CD_3OD): 5.08 (1H, d, *J* 2.8 Hz), 5.02 (1H, d, *J* 3.0 Hz), 4.88 (1H, sextet, *J* 6.3 Hz), 4.69 (1H, d, *J* 11.3 Hz), 4.22 (1H, t, *J* 9.1 Hz), 3.99–3.90 (2H, m), 3.88–3.78 (3H, m), 3.63–3.54 (3H, m), 3.5 (1H, dd, *J* 2.9, 10 Hz), 3.28 (1H, t, *J* 9.4 Hz), 3.21 (1H, t, *J* 9.4 Hz), 2.38 (1H, m), 2.23 (2H, t, *J* 7.5 Hz), 1.58–1.50 (4H, m), 1.49–1.21 (147H, m), 1.17 (3H, d, *J* 6.2 Hz), 0.86 (3H, d, *J* 6.8 Hz), 0.86–0.83 (6H, t, including two t with *J* 6.9 Hz), 0.66–0.59 (1H, m), 0.44–0.36 (1H, m), 0.18–0.04 (3H, m); δ_C (101 MHz, $CDCl_3$+few drops of CD_3OD): 175.4, 173.9, 94.32, 94.3, 74.4, 73.3, 72.5, 71.4, 71.3, 71.2, 70.7, 64.0, 62.9, 52.4, 38.0, 37.2, 35.7, 34.5, 34.3, 31.7, 30.1, 29.9, 29.5, 29.3, 29.2, 29.1, 28.1, 27.3, 25.3, 25.1, 24.9, 22.6, 19.8, 19.5, 18.7, 14.0, 10.3; ν_{max}/cm^{-1}: 3357, 2919, 2851, 1730, 1467, 1375, 759, 721.

5.17. 6-*O*-[(*R*)-2-((*R*)-1-Hydroxy-17-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-21-oxo-nonadecan-2-yl)-cyclopropyl)nonadecyl)tetracosanoate]-α-D-glucopyranosyl-(1-1′)-6′-*O*-[(R)-2-((*R*)-1-hydroxy-17-((1S,2R)-2-((S)-19-((*S*)-eicosan-2-yloxy)-21-oxononadecan-2-yl)cyclopropyl)nonadecyl)tetracosanoate]-α-D-glucopyranoside (32) and 6-*O*-[(*R*)-2-((*R*)-1-hydroxy-17-((1*S*,2*R*)-2-((*S*)-19-((*S*)-eicosan-2-yloxy)-21-oxononadecan-2-yl)cyclo-propyl)nonadecyl)tetracosanoate]-α-D-glucopyranosyl-(1-1′)-α-D-gluco-pyranoside (33)

Compound **32**, a waxy colourless solid, was prepared as described in detail in the supplementary section. It showed α_D^{21}+34.2 (*c* 1.32, $CHCl_3$) [MALDI-Found $(M+Na)^+$: 2863.6353, $C_{182}H_{350}NaO_{19}$ requires: 2863.6314]; δ_H ($CDCl_3$+few drops of CD_3OD): 4.98 (2H, d, *J* 3.4 Hz), 4.87 (2H, sext, *J* 6.2 Hz), 4.68 (2H, br d, *J* 10.9 Hz), 4.24 (2H, t, *J* 8.8 Hz), 3.93 (2H, m), 3.73 (2H, t, *J* 9.2 Hz), 3.66–3.62 (2H, m), 3.49 (2H, dd, *J* 3.4, 9.7 Hz), 3.19 (2H, t, *J* 9.5 Hz), 2.40 (2H, br m), 2.23 (4H, t, *J* 7.5 Hz), 1.58–1.53 (8H, m), 1.52–1.22 (284H, m), 1.16 (6H, d, *J* 6.2 Hz), 0.86 (6H, d, *J* 6.8 Hz), 0.86–0.82 (12H, br t, *J* 6.9 Hz), 0.66–0.59 (2H, m), 0.43–0.38 (2H, m), 0.18–0.05 (6H, m); δ_C ($CDCl_3$+few drops of CD_3OD): 175.5, 174.1, 94.9, 72.5, 71.8, 71.3, 71.0, 70.6, 69.8, 64.7, 52.1, 38.0, 37.3, 34.7, 34.4, 33.8, 31.9, 29.7, 29.6, 29.5, 29.42, 29.4, 29.3, 29.2, 29.0, 27.3, 26.0, 25.3, 25.0, 22.7, 22.6, 19.8, 19.5, 18.5, 14.0, 10.5; ν_{max}/cm^{-1}: 3371, 2918, 2850, 1732, 1467, 758, 723.

Compound **33**, a colourless waxy solid, showed α_D^{21}+33 (*c* 1.3, $CHCl_3$), mp 115–117 °C [MALDI-Found $(M+Na)^+$: 1614.3674, $C_{97}H_{187}NaO_{15}$ requires: 1614.3684]; δ_H ($CDCl_3$+few drops of CD_3OD): 5.07 (1H, d, *J* 3.1 Hz), 5.02 (1H, d, *J* 3.2 Hz), 4.88 (1H, sext, *J* 6.2 Hz), 4.69 (1H, d, *J* 11.3 Hz), 4.22 (1H, t, *J* 9.1 Hz), 3.99–3.90 (2H, m), 3.88–3.78 (3H, m), 3.63–3.54 (3H, m), 3.5 (1H, dd, *J* 2.9, 10 Hz), 3.28 (1H, t, *J* 9.4 Hz), 3.21 (1H, t, *J* 9.3 Hz), 2.38 (1H, m), 2.23 (2H, t, *J* 7.5 Hz), 1.58–1.50 (4H, m), 1.49–1.21 (147H, m), 1.17 (3H, d, *J* 6.2 Hz), 0.86 (3H, d, *J* 6.8 Hz), 0.86–0.83 (6H, br t, *J* 6.9 Hz), 0.67–0.59 (1H, m), 0.44–0.37 (1H, m), 0.18–0.04 (3H, m); δ_C ($CDCl_3$+few drops of CD_3OD): 175.5, 173.9, 94.3, 94.2, 74.5, 73.3, 72.5, 71.5, 71.3, 71.2, 70.6, 63.9, 62.9, 52.4, 38.0, 37.2, 35.7, 34.5, 34.4, 33.4, 31.7, 30.0, 29.9, 29.6, 29.3, 29.2, 29.1, 28.1, 27.3, 25.9, 25.2, 25.0, 24.9, 22.5, 19.8, 18.8, 18.4, 14.0, 10.3; ν_{max}/cm^{-1}: 3369, 2922, 2852, 1732, 1466, 1373, 759.

5.18. In vitro stimulation

BMDCs were generated as previously described.[72] Briefly, murine bone marrow from femur and tibia was flushed with PBS and red blood cells were lysed with Sigma's lysing buffer. After lysis, cells were cultured in a T75 flask 5% CO_2 at 37 °C in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Greiner), 10 ng/mL recombinant murine GM-CSF (Immunotools), 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 μg/mL gentamycin (GIBCO) and non-essential amino acids (Thermo Fisher Scientific) for 7 days. Synthetic glycolipids were coated on flat bottomed culture plates (Greiner). BMDCs were harvested after 7 days and seeded in glycolipid-coated plates at a concentration of 10^6 cells/mL for 24 h. The level of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β were evaluated by ELISA (eBiosciences).

5.19. In vivo assay

Water in oil in water emulsions (w/o/w) were prepared according to a previously described protocol.[73] Briefly, glycolipids were dissolved in 3.2% of Incomplete Freund's Adjuvant and vigorously vortexed. Next, PBS containing 0.2% of Tween 80 was added and the mixture was again vortexed. Groups of 4–5 mice were injected subcutaneously in both hind footpads with 25 μL of w/o/w containing 5 μg of glycolipids. Footpad swelling in individual mice was measured after 3 days with a caliper.

Acknowledgements

SGT wished to thank the Ministry of Higher Education in Kurdistan Region-Iraq and HMA the Government of Iraq for the award of PhD studentships. We wish to thank Dr. Paul Gates of Bristol University for carrying out accurate mass MALDI determinations. HGT holds a FRIA PhD fellowship. This research was partially funded by the Interuniversity Attraction Pole Programme IAP 7/32 and by TBVAC2020 under grant agreement no. H2020-PHC-643381.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tet.2016.05.004.

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