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Preparation of the tri-arabino di-mycolate fragment of mycobacterial arabinogalactan from defined synthetic mycolic acids

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1. Introduction:

Tuberculosis (TB) is one of the world’s deadliest diseases and is caused by Mycobacterium tuberculosis, which has a single known host (man) [1,2]. The air-borne nature of this bacterium makes the disease highly infectious, and the World Health Organization (WHO) has reported that one third of the world’s population is probably infected with latent TB, and that there is a new case every second [3]. The mycobacterial cell wall has a complex structure made up of lipids, glycolipids, polysaccharides and proteins [4]. There has been growing interest in the construction of Darabinofuranosyl-arabinogalactan (mAG) complexes related to mycobacterial cell wall components, arabinogalactan, lipoarabinomannan and arabinomannan from M. tuberculosis [5]. The mycolylarabinogalactan (mAG) complex is the largest component structure and forms from cross bonding between both Darabinofuranosyl (Araf) and galactofuranosyl (Galf) with a long-chain α-alkyl-branched β-hydroxylated fatty acid, ‘mycolic acid’ (MA) [6]. The complex lipids and polysaccharides within the cell wall of M. tuberculosis are assumed to be the cause of its characteristic pathogenesis [7].

Anderson and Geiger in 1937 reported the first extraction of arabinomycolate from the cell wall of Mycobacterium bovis using organic solvent [8]. Azuma et al. reported the isolation of arabinosylarabinate under acidic conditions [9]. The structure of the mycolyl-arabinogalactan complex of M. tuberculosis was reinterpreted using mass spectrometry and NMR spectroscopy [10,11]. Synthetic arabinofuranosyl oligosaccharides, including a branched pentasaccharide were reported in 1998 [12,13]. In 2005, the preparation of a tetramycolylpentarabinose (1) (Fig. 1) using a complex natural mixture of MAs was described [14]. However, only in 2010 were structural studies of the composition of the arabinosyl mycolates of the cell wall of M. bovis reported [15]. Ishiwata et al. reported the synthesis of a series of mono- (3), di- and tetra-arabinomycoclates (1) found at the terminal position of the cell wall skeleton of BCG from M. bovis, using natural MA mixtures extracted from cells. All of the compounds showed strong TNF-α inducing activity in vitro. Such arabinose mycolates have also been reported to show anti-cancer properties [6]. The mechanism of the activity of arabinomycolates is not clear [14].

Although a methoxy trisaccharide of arabinofuranose 2 (Fig. 1) has been prepared and used in an acylation reaction with fatty acids such as behenic acid, palmitic acid and butyric acid [16], there are no examples of the synthesis of arabinofuranose oligosaccharides esterified with structurally defined complete synthetic mycolic acids (MAs) which are the main components of the cell wall of M. tuberculosis [6]. MAs 4 are β-hydroxy fatty acids with a long α-alkyl side chain (Fig. 2) [17,18].

Fig. 1: Structure of targets of mycoloyl-arabinans.

Fig. 2: Generalised MA structure and arabinose building blocks

MAs in M. tuberculosis have 80-90 carbons, compared to those from corynebacterium (30-36 carbons), Rhodococcus (34-38 carbons) and Nocardia (46-60 carbons) [19,20]. Natural mixtures extracted from mycobacteria consist of many individual MA containing a range of functional groups, X and Y, including cis- and trans-cyclopropanes, cis- and transalkenes, methoxy and keto fragments, and different chain lengths [21].

The aim of this study was to synthesize a series of dimycolyl triarabinoses (2), comprising single defined synthetic mycolic acids, in order that the selectivity of their immune stimulatory activity, e.g. in inducing Interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), could be evaluated.
2. Results and Discussion

2.1. Synthesis of Trisaccharides

The target trisaccharide structure 2 has two α-glycosidic linkages, which can be assembled readily from known building blocks, donor 5 [22] and acceptor 6 [23]. The route to the 2,3-O-benzoyl-protected triose donor 5 was slightly modified compared to the literature method (Scheme 1) [22]. D-(−)-Arabinose was treated with HCl, freshly prepared by addition of acetyl chloride to anhydrous methanol at 0 ºC. Work up with pyridine rather than ammonium carbonate [24], gave methyl-α, β-D-Araf, 7 (R = H), with predominant formation of the α-anomer (α/β-D, 3:2) [25,26]. Rather than esterifying with benzoxane, the mixture was directly esterified to give triacetate 9b (R = Ac), 65%, (c) NaOCH3, pyridine, 0 ºC, RT, 3 h, 83%; (d) tBuOH, CH2Cl2, DMAP, CH2Cl2 at 0 ºC (81%); (e) TBAF, THF, 0 ºC/rt, 16 h, (b) THF, 0 ºC/rt, 8 h, (b) THF/CH2Cl2 at 0 ºC, 16 h, (f) NaOCH3, pyridine, 0 ºC/rt, 16 h, (i) NaOCH3, pyridine, 0 ºC, RT, 3 h, 83%; (d) TBAF, THF, 0 ºC/rt, 16 h, (b) THF, 0 ºC/rt, 8 h, (b) THF/CH2Cl2 at 0 ºC, 16 h, (f) NaOCH3, pyridine, 0 ºC/rt, 16 h, (i) NaOCH3, pyridine, 0 ºC, RT, 2 h, 83%. The next step was the coupling of trisaccharide 10b with the structurally defined synthetic mycolic acids, which had been prepared earlier [27-29]. Firstly, a model tri-arabino-di mycolate was prepared by coupling tosylate 10b with palmitic acid through the alkylative esterification strategy using cesium hydrogen carbonate in dry DMF; THF at 70 ºC for four days [14], to give compound 11a, which was then debenzylated to give compound 12a (Scheme 3). The synthesis of this glycolipid has been reported using a different method. Data obtained for 12a was identical to that reported [16].

A series of triarabino-dimycolates were then prepared according to the above procedure; the structures of the mycolic acid moieties, including α-, methoxy- and keto-classes are shown in Scheme 3. In each case, the products the proton NMR spectra included the expected three characteristic broad single-hydrogen singlets for the acetal hydrogens in the region δ 4.9 – 5.2.

The initial step in the synthetic route to acceptor 6 [23], the separation of the two anomers of 7 (R = H), was carried out using two methods (Supplementary data, Schemes 1 and 2).

Liu et al. [16] have reported the coupling of the monosaccharide building blocks to prepare the trisaccharide through the reaction of a 2-O-benzylated glycosyl acceptor and thioglycoside 5 using silver trifluoromethane sulfonate and N-methylimidazole in dry CH2Cl2. In the present work, the benzoylated glycosyl acceptor 6 and thioglycoside 5 were reacted under the reported conditions (Scheme 2). Trisaccharide 9a was obtained as a single stereoisomer in 87% yield, which is similar to that reported for the benzyl compound [16]. The product showed the expected three acetal signals in the proton NMR spectrum, two broad singlets at δ 5.10 and 5.33, for the newly formed α-glycosidic links, and a third singlet at 5.56; these correlated by HSQC with three signals in the carbon spectrum for the glycosidic links at δ 107.0, 106.0 and 105.3 respectively. Compound 9a was deprotected with sodium methoxide to give 9b as a thick oil in 81% yield. This was benzylated to protect the five secondary hydroxyl groups using benzyl bromide and sodium hydride in dry DMF to give 9c. Desilylation of the two primary hydroxyl groups gave 10a. Direct esterification of the primary hydroxyl group of Araf with a carbonyl group in natural mycolic acid mixtures has been achieved in a low yield (30%) [14], while activating the sugar as a tosylate raised the yield to 79%. Therefore, the two hydroxyl groups in compound 10a were tosylated using p-toluenesulfonyl chloride in dry pyridine in the presence of catalytic 4-dimethylaminopyridine to afford the tosylate 10b (Scheme 2).
2.2. IL-6 and TNF-α secretion assays with the synthesized compounds

Ishiwata et al. reported the synthesis of a series of mono-, di- and tetra-arabinomycolates found in the terminal position of the cell-wall skeleton of bacillus calmette Guerin from M. bovis, by using natural mycolic acid mixtures extracted from the cell wall and showed they differentially induce TNF-α secretion in a murine macrophage cell line, some at levels similar to trehalose dinimycolate (TDM) [14]. Arabinomycolates obtained by acid hydrolysis from the originally prepared CWS (SMP-105) of M. bovis BCG Tokyo 172, consisting mainly of mono-arabinobise mono-mycolate, penta-arabinobise tetra-mycolate and hexa-arabinobise tetra-mycolate fractions, significantly induced TNF-α production with an intensity comparable to that of cell-wall skeleton, a potent adjuvant, and enhanced delayed type per sensitivity reactions against inactivated tumour cells. The induced TNF-α production was completely dependent on TLR2 and MyD88 pathways [6]. To provide an initial evaluation of the activities of synthesised tri-arabinobis di-mycolates reported here (12c and 12f) as immune potentiators, secretion inducing assays for IL6 and TNF-α, cytokines involved in inflammation, were conducted, in comparison with TDM and synthetic monoarabinobiso mono-mycolates [30]. Surprisingly, neither compound was strongly stimulatory in either assay when compared to synthetic TDMs, trehalose dinimycolates or arabinobis mycolates [31, 32]. Further work to evaluate the biological effects of these molecules is under way.

3. Experimental Section

For general experimental detail, see Supplementary Information. Unless otherwise stated, all products were single components by TLC and by proton and carbon NMR.

3.1. Methyl 2,3-di-O-benzyl-5-O-p-toluensulfonyl-arabinofuranosyl-(1→3)-(2,3-di-O-benzyl-5-O-p-toluencesulfonyl-arabinofuranosyl-(1→5))-2-O-benzyl-a-D-Araf

(a) Molecular sieves 4 Å (6.6 g) were added to a stirred solution of the acceptor 6 (0.9 g, 3.3 mmol) and the donor 5 (6.0 g, 8.5 mmol) in dry CH2Cl2 (30 mL) at r.t. under a nitrogen atmosphere. The reaction mixture was stirred for 30 min, then cooled to -60 °C and N-iodosuccinimide (2.09 g, 9.29 mmol) was added followed by the addition of AgOTf (0.36 g, 1.40 mmol). The mixture was stirred at the same temperature until the color became red/dark brown and TLC showed no starting material. It was then quenched by the addition of triethylamine (1 mL) until it turned yellow. The mixture was diluted with CH2Cl2 (100 mL) and filtered through celite and the solvent was evaporated under reduced pressure. Column chromatography on silica eluting with hexane/ethyl acetate (5:1) gave compound 9a as a thick oil (4.2 g, 87%) [Found (MALDI (M+Na)+): 1447.6, C54H56NaO12S12], requires: 1447.7], [M]+ + 16 (c 0.1, CHCl3) which showed δH (400 MHz, CDCl3): 8.07 (2H, d, J 7.9 Hz), 8.02 – 7.91 (8H, m), 7.70 – 7.62 (9H, m), 7.59 – 7.41 (8H, m), 7.40 – 7.28 (18H, m), 5.64 (1H, d, J 4.5 Hz), 5.61 (1H, d, J 4.2 Hz), 5.56 (1H, s), 5.57 (1H, s), 5.53 (1H, br. s), 5.41 (1H, s), 5.32 (1H, br. s), 5.10 (1H, br. s), 4.49 – 4.35 (4H, m), 4.08 (1H, dd, J 11.3, 5.1 Hz), 3.97 (5H, m), 3.44 (3H, s), 1.01 (9H, s), 0.98 (9H, s); δC (100 MHz, CDCl3): 165.5, 165.4, 165.2.

165.1, 135.63, 135.6, 135.5, 133.3, 133.2, 133.15, 133.1, 133.0, 129.96, 129.9, 129.8, 129.78, 129.7, 129.6, 129.4, 129.3, 129.2, 129.1, 128.4, 128.31, 128.3, 128.2, 127.6, 127.0, 106.0, 105.3, 84.0, 83.4, 82.5, 82.0, 81.8, 81.6, 80.6, 77.2, 77.1, 66.1, 63.5, 63.3, 54.7, 26.7, 26.6, 19.3, 19.2; νmax: 3069, 3010, 2929, 2859, 1724, 1652, 1645, 1602, 1451, 1070, 708 cm⁻¹.

(b) A solution of sodium methoxide (2 mL, 1 M, in methanol) was added to a stirred solution of 9a (0.20 g, 0.14 mmol) in dry MeOH: CH2Cl2 (1:1, 5 mL) at r.t. until a pH of 11 was obtained. The mixture was stirred at r.t. for 2 h then the solvent was evaporated under reduced pressure to give an oil. The residue was purified by column chromatography on silica eluting with CH2Cl2:MeOH (5:2) to give 9b compound as a thick oil (0.1 g, 83%) [Found (MALDI (M+Na)+): 927.1, C54H56NaO12S12, requires: 927.3], [M+H]+ + 50 (c 0.1, CHCl3) which showed δH (400 MHz, CDCl3: C, 4.2 g, 96%) [Found (MALDI (M+Na)+): 1377.0, C54H56NaO12S12, requires: 1377.6], [M]+ + 40 (c 0.1, CHCl3) which showed δH (400 MHz, CDCl3): 7.66 – 7.51 (7H, m), 7.42 – 7.17 (38H, m), 5.19 (1H, s), 5.17 (1H, s), 4.94 (1H, s), 4.59 – 4.37 (10H, m), 4.28 (1H, dd, J 6.3, 2.3 Hz), 4.22 – 4.14 (2H, m), 4.14 – 4.03 (5H, m), 4.03 – 3.95 (2H, m), 3.86 – 3.74 (5H, m), 3.37 (3H, s), 1.03 (18H, s); δC (100 MHz, CDCl3): 135.7, 135.65, 135.62, 135.6, 129.6, 129.54, 129.51, 128.5, 128.4, 128.35, 128.34, 128.3, 128.22, 128.2, 127.9, 127.8, 127.79, 127.75, 127.73, 127.66, 127.63, 127.6, 127.56, 127.53, 127.4, 127.35, 127.33, 127.3, 127.28, 107.2, 105.3, 84.0, 83.4, 82.5, 82.0, 81.8, 81.6, 80.6, 77.2, 77.1, 66.1, 63.5, 63.3, 54.7, 26.7, 26.6, 19.3, 19.2; νmax: 3069, 3010, 2929, 2859, 1724, 1602, 1451, 1070, 708 cm⁻¹.

(Pd(OH)2-C, 12 mg, 0.3 fold by weight) was added to a stirred
(d) Tetrabutylammonium fluoride (0.3 mL, 0.3 mmol, 1 M) was added dropwise to a stirred solution of 9e (0.2 g, 0.147 mmol) in dry THF (10 mL) at 0 °C under nitrogen atmosphere. The mixture was allowed to reach r.t. and stirred for 16 h so the solvent was evaporated under reduced pressure to give an oil which was purified by column chromatography on silica eluting with hexane/ethyl acetate (1:1) to give compound 10a as a colourless thick oil (0.1 g, 77%) [Found (MALDI) (M+Na)+: 901.3, C39H54NaO3,S, requires: 901.3, [M]+: 970.9 g/mol].

3.2: MethylS-(1-hydroxy-17S,18S)-17-methoxy-18-methylhexatriacontyl)cyclopropyl]octadecyl[hexacosanoic acid (28) [0.092 g, 0.073 mmol] in dry DMF: THF (1:5, 3 mL) at r.t. under a nitrogen atmosphere. The mixture was stirred at 70 °C for 4 days, then worked up and purified as before to afford 11b as a colourless thick oil (0.051 g, 45%) [Found (MALDI) (M+Na)+: 3731.9, C72H138NaO3,S, requires: 3731.9, [M]+: 3731.0 g/mol] which showed δ0 (400 MHz, CDCl3): 7.37 – 7.23 (25H, m), 5.18 (1H, br. s), 5.13 (1H, br. s), 4.91 (1H, br. s), 4.49 – 4.37 (9H, m), 4.34 (1H, d, J = 7.6 Hz), 4.32 – 4.24 (6H, m), 4.18 (1H, ddd, J = 9.7, 6.3, 3.0 Hz), 4.11 (1H, dd, J = 4.4, 2.7 Hz), 4.08 (1H, br. d, J = 2.9 Hz), 4.00 (1H, dd, J = 2.9, 0.6 Hz), 3.96 (1H, br. d, J = 2.3 Hz), 3.93 (1H, ddd, J = 7.8, 3.9 Hz), 3.88 – 3.78 (2H, m), 3.76 (1H, dd, J = 11.4, 1.8 Hz), 3.67 – 3.57 (2H, m), 3.57 (3H, s), 3.35 (6H, s), 2.99 – 2.93 (2H, m), 2.67 (1H, d, J = 6.1 Hz), 2.65 (1H, d, J = 6.5 Hz), 2.41 (2H, dt, J = 9.0, 5.8 Hz), 1.68 – 1.03 (29H, m), 0.89 (12H, t, J = 6.8 Hz), 0.86 (6H, d, J = 6.9 Hz), 0.71 – 0.61 (4H, m), 0.57 (2H, dt, J = 7.6, 4.0 Hz), -0.33 (2H, br. q, J = 5.1 Hz), δc (101 MHz, CDCl3): 175.1, 175.0, 173.7, 173.6, 173.5, 173.4, 128.5, 128.4, 128.4, 128.3, 128.2, 128.1, 127.9, 127.8, 127.8, 127.8, 127.1, 106.0, 105.3, 85.8, 82.0, 81.9, 80.9, 79.7, 73.2, 72.2, 72.0, 71.9, 71.8, 64.9, 62.8, 62.7, 54.9; νmax: 3467, 3050, 3017, 2926, 2861, 1609, 1494, 1050, 824 cm⁻¹.

(e) 4-Toluene sulfonyl chloride (2.82 g, 14.7 mmol) was added to a stirred solution of 10a (1.30 g, 1.47 mmol), pyridine (1.17 g, 19 mL, 174 mmol) and DMAP (catalytic amount) in dry CH2Cl2 (10 mL) at 0 °C under nitrogen. The mixture was allowed to reach r.t. and stirred for 16 h, then diluted with ethyl acetate (100 mL) and water (50 mL). The organic layer was separated and the aqueous layer was re-extracted with ethyl acetate (3x50 mL). The combined organic layers were washed with water (50 mL) and brine (50 mL), dried. The solvent was evaporated under reduced pressure to give an oil; column chromatography on silica eluting with hexane/ethyl acetate (1:1) afforded the title compound as a colourless thick oil 10b (1.3 g, 74%) [Found (MALDI) (M+Na)+: 1209.2, C89H140NaO5,S, requires: 1209.3, δ0 (400 MHz, CDCl3): 7.7 (2H, d, J = 8.3 Hz), 7.8 (2H, d, J = 8.4 Hz), 7.37 – 7.23 (29H, m), 5.09 (1H, br. s), 5.08 (1H, br. s), 4.93 (1H, s), 4.61 – 4.42 (9H, m), 4.35 (1H, d, J = 12.0 Hz), 4.30 – 4.24 (1H, m), 4.22 – 4.10 (6H, m), 4.10 – 4.05 (2H, m), 4.0 (1H, ddd, J = 3.3, 1.2 Hz), 3.96 (1H, ddd, J = 3.1, 1.0 Hz), 3.87 (2H, dd, J = 6.1, 3.1 Hz), 3.84 (1H, ddd, J = 12.0, 4.0 Hz), 3.69 (1H, dd, J = 12.0, 2.5 Hz), 3.38 (3H, s), 2.38 (3H, s), 2.4 (3H, s); δc (101 MHz, CDCl3): 144.8, 144.7, 137.5, 137.4, 137.3, 137.2, 137.1, 132.7, 132.6, 129.8, 129.7, 128.4, 128.3, 128.35, 128.33, 128.3, 128.0, 127.93, 127.9, 127.85, 127.82, 127.8, 127.7, 127.69, 127.67, 106.9, 106.4, 105.5, 88.0, 87.6, 87.4, 82.8, 82.7, 80.5, 80.4, 79.0, 78.7, 72.2, 72.0, 71.9, 71.8, 81.1, 71.7, 68.7, 68.6, 65.7, 54.8, 21.5; νmax: 3088, 3064, 3031, 2924, 2826, 1598, 1454, 1177, 738 cm⁻¹.
3.3: Methyl 5-O-(2-[(R)-1-hydroxy-18-[(1R,2S)-2-[(17S,18S)-17-methoxy-18-
 methylhexatriacontyl]cyclopropyl]octadecyl]tetracosanoate)-
 a-D-arabinofuranosyl-[(1→3)]5-O-[(2R)-1-hydroxy-18-[(1R,2S)-2-[(17S,18S)-17-methoxy-18-
methylhexatriacontyl]cyclopropyl]octadecyl]tetracosanoate)-a-D-
arabinofuranosyl-[(1→5)]-a-D-Araf

12c

(a) Cesium hydrogen carbonate (0.081 g, 0.417 mmol) was added to a stirred solution of 10b (0.050 g, 0.042 mmol) and (2R)-2-[(1R)-1-hydroxy-18-[(2S)-2-[(17S,18S)-17-methoxy-18-
 ethylhexatriacontyl]cyclopropyl]octadecyl]tetracosanoic acid [3](0.113 g, 0.092 mmol) in dry DMF : THF (1:5, 6 mL) at r.t. under nitrogen. The mixture was stirred at 70 ºC for 4 days, then work up and purification as before gave the title compound

11c as a colourless oil (56 mg, 18%) [Found (M+Na)+: 3139.7057, C_{20}H_{38}NaO_{17}, requires: 3139.7041].

[b] Palladium (II) hydroxide on activated charcoal (20% Pd(OH)$_2$-C, 6.6 mg, 0.30 fold by weight) was added to a stirred solution of compound 11d (0.030 g, 0.009 mmol) in dry CH$_2$Cl$_2$ : MeOH (1:1, 2 mL) at r.t. under hydrogen. After 16 h, work up and purification as before gave the title compound 12d as a white oil (21 mg, 65%) [Found (M+Na)+: 2689.4694, C$_{27}$H$_{38}$NaO$_{17}$, requires: 2689.4694, 2685.6640].

[b] Palladium (II) hydroxide on activated charcoal (20% Pd(OH)$_2$-C, 6.6 mg, 0.30 fold by weight) was added to a stirred solution of compound 11d (0.030 g, 0.009 mmol) in dry CH$_2$Cl$_2$ : MeOH (1:1, 2 mL) at r.t. under hydrogen. After 16 h, work up and purification as before gave the title compound 12d as a white oil (21 mg, 65%) [Found (M+Na)+: 2689.4694, C$_{27}$H$_{38}$NaO$_{17}$, requires: 2689.4694, 2685.6640].
3.5: Methyl-5-O-(2R)-1-hydroxy-12-[(1S,2R)-2-[(14-(1S,2R)-2-
ecosylcyclopropyl)cyclopropyldodecan]hexacosanoyl]-
(1→5)-β-D-Araf 12e
(a) Cesium hydrogencarbonate (0.081 g, 0.417 mmol) was added to a stirred solution of 10b (0.050 g, 0.042 mmol) and 2R-1
hydroxy-12-[(1S,2R)-2-[(14-(1S,2R)-2-
ecosylcyclopropyl)cyclopropyldodecan]hexacosanoyl]-
(1→5)-β-D-Araf 12e

(b) Palladium (II) hydride on activated charcoal (20%
Pd(OH)2-C, 13.8 mg, 0.30 fold by weight) was added to a stirred solution of compound 11f (0.046 g, 0.013 mmol) in dry CH2Cl2:
MeOH (1:1, 2 mL) at r.t. under hydrogen. After 16 h, work up and purification as before gave the title compound 12f as a colourless oil (26 mg, 65%) [Found (M+Na)+: 2889.6404, C185H132NaO19s, requires: 2889.6470].

3.6: Methyl-5-O-(2R)-1-hydroxy-16-[(1R,2S)-2-[(20-methyl-19-oxo-16-octadecatriacetyl)cyclopropyl]hexacosanoyl]-
(1→5)-β-D-Araf 12f
(a) Cesium hydrogencarbonate (0.081 g, 0.417 mmol) was added to a stirred solution of 10b (0.050 g, 0.042 mmol) and 2R-1-
hydroxy-16-[(1R,2S)-2-[(20-methyl-19-oxo-16-octadecatriacetyl)cyclopropyl]hexacosanoyl]-
(1→5)-β-D-Araf 12f

(b) Palladium (II) hydride on activated charcoal (20%
Pd(OH)2-C, 13.8 mg, 0.30 fold by weight) was added to a stirred solution of compound 11f (0.046 g, 0.013 mmol) in dry CH2Cl2:
MeOH (1:1, 2 mL) at r.t. under hydrogen. After 16 h, work up and chromatography as before gave the title compound 12f as a colourless oil (26 mg, 65%) [Found (M+Na)+: 2889.6404, C185H132NaO19s, requires: 2889.6470].
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References and notes