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Structure of the "Heterocyst Glycolipids" of the Marine Cyanobacterium *Nodularia harveyana*

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Abstract: *The "heterocyst glycolipids" have been reported to take part in the protection of the specialized cyanobacterial cells capable of N_2 fixation against the penetration of O_2. For the first time such glycolipids have been isolated in a pure form from a cyanobacterium, Nodularia harveyana, and their structures have been established by spectroscopic and chemical means to be 1-(O-α-D-glucopyranosyl)-3R,25R-hexacosanediol, 1-(O-α-D-glucopyranosyl)-3S,25R-hexacosanediol and 1-(O-α-D-glucopyranosyl)-3-keto-25R-hexacosanol.*

Introduction

The majority of cyanobacteria capable of N_2 fixation has evolved specialized cells, the heterocysts, which allow for the spatial separation of the basically incompatible processes of O_2 production and N_2 fixation. It has been reported evidence[1] that the barrier to the penetration of oxygen into heterocysts depends upon two layers of the heterocyst envelope, the glycolipid layer and the polysaccharide layer. The glycolipid layer is essentially constituted of "heterocyst glycolipids", unique non-saponifiable glycolipids which have not been detected in any other class of algae[2,3], nor in other natural sources, to the best of our knowledge. Despite the relevant biological function of these lipids, their structural features have been partially elucidated only in *Anabaena cylindrica*[4,5] where they occurr as a complex mixture of mono-glucoside and -galactoside derivatives of long chain polyhydroxy alcohols, the most abundant having been formulated as 1-(O-α-D-glycopyranosyl)-3,25-hexacosanediol[5]. The presence of compounds reputed to be glucose and galactose esters of C_{26} and C_{28} hydroxy fatty acids was also noticed[5]. The above mentioned studies did not succeed in the isolation and characterization of pure compounds, nor in the elucidation of the stereochemistry of the aglycone moieties because of the complexity of the mixture and of the limited avalaibility of separation and spectroscopic techniques. A subsequent TLC-GLC study[6] on the heterocyst glycolipids of five species of *Anabaena* and *Nostoc* showed different patterns of lipids and the presence of a lipid with chromatographic properties comparable with those of the above mentioned 1-(O-α-D-glycopyranosyl)-3,25-hexacosanediol. We wish to report in this paper the isolation and the complete structure elucidation of the heterocyst glycolipids of the cyanobacterium *Nodularia harveyana*.

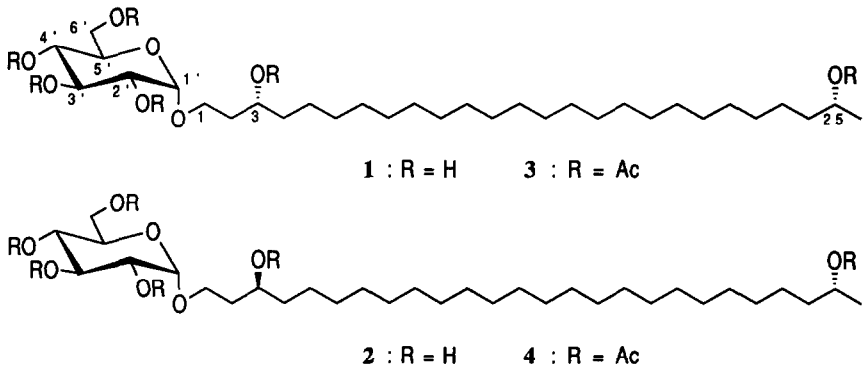
Results and Discussion

N. harveyana was grown in a 90 l fermentor under continuous illumination and aeration. Since it is well documented that the occurrence of this unique glycolipid class is restricted to the heterocysts[3,6,7], no attempt was made in the separation of the vegetative cells from heterocysts and therefore the intact cyanobacterium filaments were lyophilized and extracted. The examination of the $CHCl_3$/*i*-PrOH extract showed the presence of two bands in the R_f range characteristic[2,6] of heterocyst glycolipids, the major being more polar. The extract was sequentially chromatographed on Sephadex LH-20 and on RP-8 to give the mixture of the two glycolipid bands which were finally separated by preparative SiO_2 TLC. The major components (**1** and **2**) constituting the more polar band behaved like a single compound on TLC and showed in the FABMS spectrum a single quasi-molecular ion, m/z 577 $(M+H)^+$, which was consistent with the previously reported 1-(O-α-D-glycopyranosyl)-3,25-hexacosanediol structure. However, examination of ^1H- and ^{13}C-NMR spectra showed that the band was constituted of two closely related compounds in a ca. 2:1 ratio. In particular, in the ^1H-NMR spectrum two anomeric protons (2:1 ratio) at δ 5.47 (J=3.8 Hz) and 5.45 (J=3.6 Hz) were present, while in the ^{13}C-NMR spectrum the resonances attributed to the carbons surrounding the C-3 of the aglycone moieties were also doubled in a 2:1 approximate ratio. Acid hydrolysis of the mixture furnished only D-glucose as sugar component.

Due to the unfavourable solubility properties of **1** and **2**, soluble among the most common solvents only in Py or in $CHCl_3$-MeOH mixtures, a direct HPLC separation proved unfeasible and then the separation was achieved by normal phase HPLC on the peracetates **3** and **4** obtained by acetylation of **1** and **2**. Compounds **3** and **4** were isomeric since had the same molecular weight of 828 [FABMS m/z 851 $(M+Na)^+$]. The NMR data (Table), obtained by 1D and 2D spectra, readily accomodated for the 1-(O-α-D-glucopyranosyl)-3,25-hexacosanediol hexaacetate gross structure for both compounds. In particular, the presence of the α-glucose moiety was evident by comparison with the NMR data of α-methylglucoside peracetate, while the glucosidation site of the aglycone moieties was supported both by the ^{13}C chemical shift of C-1 (glycosidation shift[8]) and by the chemical shift of the C-1 protons. Finally the ^1H-^1H COSY spectra secured the position of the oxygenated functions on the aglycone moieties, while the unbranched nature of the methylene chain was ascertained by DEPT sequence.

The gross structure of the two compounds was confirmed by the spectral data of the two glucosides **1** and **2** obtained by methanolysis of **3** and **4**. At this point it was evident that the two compounds were diastereomeric and therefore that their aglycone moieties should be enantiomeric or, conceivably, epimeric at C-3 because of the small differences observed in the ^{13}C chemical shift values of the C-3 surrounding carbons. The $[\alpha]_D$ values, both positive, of the aglycones **5** and **6** obtained by acid hydrolysis of **1** and **2** confirmed the latter view. In addition, **5** and **6** showed practically superimposable ^1H- and ^{13}C-NMR spectra; however when a ^{13}C spectrum of a ca. 1:1 mixture of **5** and **6** was recorded, doubling of several signals was observed.

The stereochemistry at C-3 and C-25 in both **5** and **6** was elucidated as follows. The 1,3 diol moiety was suitable for the application of a recently developed CD exciton chirality method[9] and therefore **5** and **6** were transformed into their tris (*p*-bromobenzoate) derivatives **7** and **8** which exhibited opposite CD spectra (Fig. 1).



1 : R = H **3** : R = Ac

2 : R = H **4** : R = Ac

Table. ^{1}H and ^{13}C Spectral Data for **3** and **4**[a].

Position	^{13}C-NMR		^{1}H-NMR(multiplicity; J, Hz)	
	3	**4**	**3**	**4**
1'	96.1	95.7	4.99 (d; 3.7)[c]	5.05 (d; 3.8)
2'	70.7	70.8	4.86 (dd; 3.7, 9.8)[d]	4.86 (dd; 3.8, 9.8)[e]
3'	70.2	70.1	5.47 (t; 9.8)	5.47 (t; 9.8)
4'	68.8	68.5	5.04 (t; 9.8)	5.07 (t; 9.8)
5'	67.3	67.2	3.98 (ddd; 2.2, 4.5, 9.8)	4.06 (m)[f]
6'	62.0	61.8	*a* 4.25 (dd; 4.5, 12.3)	*a* 4.29 (dd; 4.4, 12.4)
			b 4.08 (dd; 2.2, 12.3)	*b* 4.07 (dd; 2.2, 12.4)[f]
1	65.1	65.3	*a* 3.71 (m)	*a* 3.72 (m)
			b 3.35 (m)	*b* 3.46 (m)
2	34.1	33.8		
3	71.1	71.5	5.00 (m)[c]	5.00 (m)
4	34.6	34.5		
5	25.3[b]	25.2[b]		
23	25.4[b]	25.4[b]		
24	36.0	35.9		
25	71.2	71.1	4.90 (m)[d]	4.90 (m)[e]
26	20.0	19.9	1.19 (d; 6.3)	1.20 (d; 6.3)
6-22	29.7	29.7	1.25	1.26
CH$_3$CO	21.4, 21.1, 20.7	21.1, 20.7	2.09, 2.08, 2.03	2.11, 2.09, 2.06
			2.023, 2.020, 2.00	2.04, 2.03, 2.02
CH$_3$*CO*	170.6, 170.4, 170.2, 169.8	170.6, 170.4, 169.8		

[a] $CDCl_3$; 500 MHz for proton and 125 MHz for carbon spectra; assignments were made by ^{1}H-^{1}H COSY, ^{1}H-^{13}C HETCOR, DEPT and by comparison with proton and carbon spectra of α-methylglucopyranoside.
[b] Assignments may be interchanged. [c-f] Signals with identical superscripts were partially or totally overlapped in the proton spectra

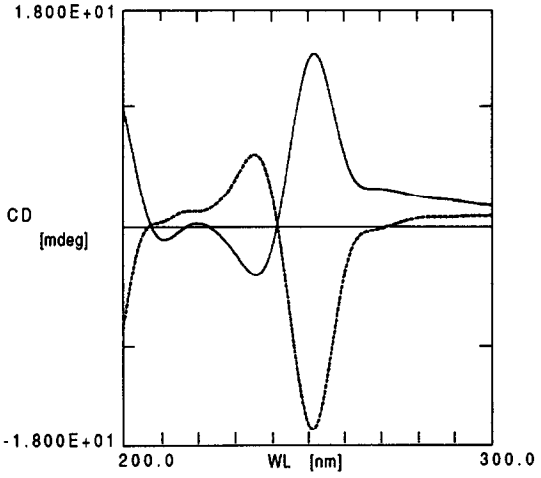
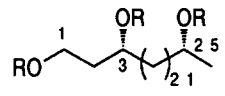
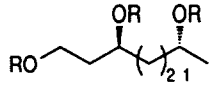


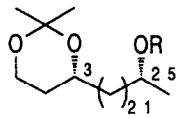
Fig. 1 CD spectra of 1,3*R*,25*R*-hexacosanetriyl tris (p-bromobenzoate) (**7**; dotted line) and 1,3*S*,25*R*-hexacosanetriyl tris (p-bromobenzoate) (**8**; continuous line). [11]



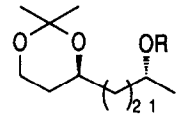
5 : R = H
7 : R = *p*-Br-C_6H_4CO



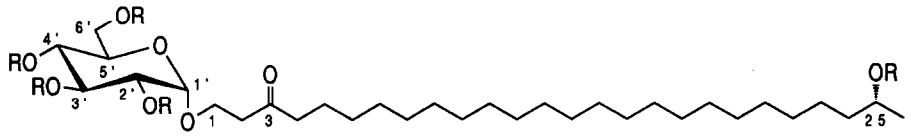
6 : R = H
8 : R = *p*-Br-C_6H_4CO



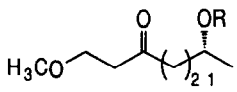
9 : R = H
9a : R = *R*-MTPA
9b : R = *S*-MTPA



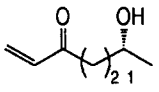
10 : R = H
10a : R = *R*-MTPA
10b : R = *S*-MTPA



11 : R = H
12 : R = Ac



13 : R = H
13a : R = *S*-MTPA



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Compound **7** shows first the negative and then secondly the positive Cotton effects and hence the absolute configuration at C-3 is *R*[9], while **8** has the reverse figure and, accordingly, its absolute configuration at C-3 is *S*. The absolute configuration at C-25 was elucidated by Mosher's method[10]. The triols **5** and **6** were converted into the acetonides **9** and **10** from which the (*R*)- and (*S*)-MTPA esters were prepared. The C-25 methyl group in the MTPA esters of compound **9** exhibited a negative $\Delta\delta$ (-0.08, $\delta_{(R)MTPA}$-$\delta_{(S)MTPA}$) in the ^{1}H-NMR spectra and thus the absolute configuration in **9**, and hence in **5**, is *R*. Similarly, the $\Delta\delta$ of the C-25 methyl group of the MTPA esters of compound **10** showed a -0.08 value disclosing also in this case the *R* configuration at C-25. Thus the glucoside **1** is 1-(O-α-D-glucopyranosyl)-3*R*,25*R*-hexacosanediol, while **2** is 1-(O-α-D-glucopyranosyl)-3*S*,25*R*-hexacosanediol.

The less polar band (**11**) was judged to be homogeneous by chromatographic and spectral data. The FABMS spectrum showed a $(M+H)^+$ peak at m/z 575, that is 2 mass units less than the glucosides **1** and **2**. The ^{13}C spectrum indicated the presence of a ketone carbonyl group (δ 209.0) which was flanked by two methylenes since two deshielded triplets at δ 2.88 and 2.49 were found in the ^{1}H-NMR spectrum. The ketone function was located at C-3 because the triplet at δ 2.88 (C-2 protons) was found coupled with the C-1 protons by ^{1}H-^{1}H COSY. Compound **11** by acetylation gave a pentaacetate (**12**) and by $NaBH_4$ reduction yielded a ca. 1:1 mixture of two compounds which after separation as their peracetates by HPLC showed ^{1}H-NMR spectra identical to those of **3** and **4**. These data allow the assignment of the gross structure **11** to the less polar glycolipid. In order to establish the stereochemistry at C-25, **11** was hydrolyzed in the same conditions as **1** and **2** (2N H_2SO_4; 9:1 CH_3OH-H_2O). Notably, the acid catalyzed methanolysis yielded the methyl ether **13**, whose structure was evident by MS and NMR data, which should arise by acid catalyzed conjugate addition of CH_3OH to the expected unsaturated ketone **14**. Since **13** was obtained in a very limited amount, insufficient to prepare both the *R*- and *S*-MTPA derivatives, only the *S*-MTPA derivative **13a** was prepared. The chemical shift value of the C-25 methyl group (δ 1.33) was the same as in the *S*-MTPA derivatives **9b** and **10b** and therefore the absolute configuration at C-25 in **11** should be *R* as in **1** and **2**.

It should be noted that compound **11** has a chromatographic behaviour very similar to that reported for two compounds of *Anabaena cylindrica* for which the structure of hydroxy fatty acid esters of glucose and galactose has been proposed[5]. The proposed structures need reinvestigation since they rest mainly on mass spectral data which in the case could give ambiguous indications. In fact, one of the supposed esters is isobaric with **11** and, in addition, it was reported that by acid catalyzed methanolysis it gives rise to a methyl ester, characterized only by mass spectra, once again isobaric with the methyl ether **13**.

Experimental

Optical rotations were measured on a JASCO DIP 370 polarimeter. CD spectra were measured on a JASCO 710 spectropolarimeter. NMR spectra were recorded on a Bruker AM 250 (250.13 MHz for ^{1}H and 62.89 MHz for ^{13}C) and Bruker AMX 500 (500.13 MHz for ^{1}H and 125.75 MHz for ^{13}C) spectrometers. Chemical shifts are given in ppm (δ) scale ; for the spectra in $CDCl_3$ the $CHCl_3$ signal was used as internal standard (δ 7.26 ^{1}H; δ 77.0 ^{13}C), while for the spectra in C_5D_5N-D_2O the downfield signal of pyridine was used as internal standard (δ 8.80 ^{1}H; δ 150.0 ^{13}C); J values are given in Hz. Mass spectra were taken on a Kratos MS-50 instrument equipped with a Kratos fast atom bombardment (FAB) source, by dissolving the

samples in a glycerol matrix. Gas-chromatography was performed on a Carlo Erba Fractovap 2900 gas-chromatograph using an OV 101 capillary column (25 m). Column chromatographic separations were carried out using Sephadex LH-20 (Pharmacia), Lobar RP-8 (Merck) or Silica gel 60 (Merck). Preparative TLC was performed on precoated Silica gel 60 plates (0.5 mm; Merck). HPLC was performed on LDC Analytical apparatus equipped with an UV detector, using a μ-Porasil (Waters) preparative column (30x0.78 cm).

Organism and growth conditions.

A culture of *Nodularia harveyana* (Thwaites) Thuret 44.85 was obtained from Sammlung von Algenkulturen Gottingen. It was grown in large scale at 25° into a 90 l fermentor with low mechanical agitation and an aeration flux of 30 ml/min using 70 l of seawater medium[12] at pH 8.0; continuous illumination was provided by four 18 W cool white fluorescent tubes arranged parallel to the fermentor. The fermentor culture was started with a 2.5% inoculum obtained in a 1 l fermentor. The biomass concentration was monitored by determining the cell dry weight on 200 ml aliquots of the cultures withdrawn daily. After 16 days the cells were harvested by continuous flow on an Alfa-Laval model LAB 102B-20 centrifuge. The pellet was washed twice with an iso-osmotic saline solution, collected by centrifugation at 5.000 g for 30 min and then lyophilized. The yields of different preparations ranged from 14 to 21 g of lyophilized cells.

Isolation of the glycolipids.

The glycolipids were isolated several times from different biomass preparations and showed always the same composition. In a typical isolation procedure, 14 g of lyophilized cells were extracted for five days with 0.9 l (0.3x3) of 1:1 $CHCl_3$/*i*-PrOH. The extract (2.4 g) dissolved in 2:1 $CHCl_3$-MeOH was chromatographed in two portions on a Sephadex LH-20 (70x3 cm) column and the fractions were eluted with 9:1 MeOH-$CHCl_3$ and monitored by TLC (8:2 $CHCl_3$-MeOH). The fractions having a R_f intermediate between monogalactosyldiacylglycerols and digalactosyldiacylglycerols (usual cyanobacterial lipids) were pooled (878 mg). This material was dissolved in 2:1 $CHCl_3$-MeOH and chromatographed in two portions on a RP-8 column (30x2.8 cm) eluted with 9:1 MeOH-$CHCl_3$. The appropriated fractions (193 mg) were dissolved in 2:1 $CHCl_3$-MeOH and subjected to preparative TLC on eight SiO_2 plates which were eluted with 8:2 $CHCl_3$-MeOH. The two glycolpid bands were evidenced by exposure to I_2 vapours and eluted from the silica with 2:1 $CHCl_3$-MeOH, obtaining 4.0 mg of the less polar band (**11**) and 19.1 mg of the mixture of **1** and **2**.

1-(O-α-D-glucopyranosyl)-3-keto-25R-hexacosanol (**11**): amorphous powder. $[\alpha]_D$ +10.7 (c, 0.4; 2:1 $CHCl_3$-MeOH). FABMS, m/z 575$(M+H)^+$, 413 (cleavage of the glucosidic bond). NMR data (C_5D_5N-D_2O; assignments made by ^1H-^1H COSY, ^1H-^{13}C HETCOR and DEPT): δ ^1H 5.47 (d, J 3.8; H-1'), 4.65 (t, J 9.1; H-3'), 4.61 (bd, J 9.9; H-6'a), 4.48 (m; H-6'b), 4.43 (m; H-5'), 4.41 (m; H-1a), 4.31 (t, J 9.1; H-4'), 4.23 (dd, J 3.8, 9.1; H-2'), 4.11 (sextet, J 5.8; H-25), 4.04 (m; H-1b), 2.88 (bt, J 6.3; H-2), 2.49 (t, J 7.3; H-4), 1.44 (d, J 5.9; 25-Me), 1.37, 1.36, 1.28 (methylene chain). δ^{13}C 209.0 (C-3), 100.9 (C-1'), 75.5 (C-3') 74.5 (C-5'), 73.9 (C-2'), 72.3 (C-4'), 67.2 (C-25), 63.8 (C-1), 63.0 (C-6'), 43.4 (C-4), 43.0 (C-2), 40.4 (C-24), 26.6 (C-23), 24.5 (C-26), 24.0 (C-5); 30.2, 30.1, 29.9, 29.6 (methylene chain).

Mixture of **1** and **2**: amorphous powder. FABMS, m/z 577 $(M+H)^+$, 415 (cleavage of the glucosidic bond) . NMR data (C_5D_5N-D_2O), δ ^1H 5.47 and 5.45 (ca. 2:1; doublets J 3.8 and 3.6 respectively; anomeric

protons); δ ^{13}C 100.7, 75.6, 74.5, 74.0, 72.3, 69.3 and 68.7 (ca. 1:2), 67.2, 66.7 and 66.3 (ca. 1:2), 63.1, 40.4, 39.0 and 38.8 (ca. 1:2), 38.2 and 38.1 (ca. 2:1), 30.2, 26.7, 26.5, 24.5. The sugar moiety was identified to be D-glucose as follows. To 1.3 mg of **1+2** mixture, placed in a capped vial, dry 2N HCl-MeOH (0.5 ml) was added and the solution was heated at 80° for 16 h. After cooling, the solution was neutralized with an excess of Ag_2CO_3 and the surnatant was recovered by centrifugation and taken to dryness with a N_2 stream. The mixture of the obtained methylgycosides was carefully dried under vacuum for 1 h, Trisil-Z (Pierce; 0.05 ml) was added and after few min the mixture was analyzed by GLC in comparison with trimethylsilylated methyl-glucosides and -galactosides prepared similarly. The GLC trace of the sample prepared from the **1+2** mixture showed the presence of only trimethylsilylated methylglucosides. 2 mg of **1+2** mixture were hydrolyzed with 2N H_2SO_4 (0.5 ml; 80°; 16 h). After cooling the solution was diluted with H_2O (1.5 ml) and extracted with $CHCl_3$. The optical rotation measured on the remaining water was positive (α= +0.23), thus establishing the D configuration of glucose.

Acetylation of the 1 and 2 mixture and separation of the hexaacetates 3 and 4.

The mixture of **1** and **2** was dissolved in Py (0.5 ml) and Ac_2O (0.05 ml) was added. After 28 h at r.t. the solvents were removed with a N_2 stream and the crude reaction mixture (29 mg), dissolved in $CHCl_3$, was subjected to preparative HPLC (99:1 *n*-hexane/*i*-PrOH) to obtain, in order of increasing polarity, 15 mg of **3** and 6 mg of **4**.

Hexaacetate **3**: oil. $[\alpha]_D$ +59.9 (c, 1.5; $CHCl_3$). FABMS, m/z 851 $(M+Na)^+$, 829 $(M+H)^+$. NMR data, Table.

Hexaacetate **4**: oil. $[\alpha]_D$ +64.7 (c, 0.6; $CHCl_3$). FABMS, m/z 851 $(M+Na)^+$, 829 $(M+H)^+$; NMR data, Table.

Methanolysis of hexaacetates 3 and 4: glucosides 1 and 2.

10 mg of **3** were dissolved in MeOH (3 ml) and 10 mg of solid Na_2CO_3 were added. The mixture was stirred at r.t. for 16 h, then 6 ml of $CHCl_3$ were added and the suspension was centrifuged. The surnatant was filtered through a short silica gel column (2:1 $CHCl_3$-MeOH) to give 5 mg of **1**.

1-(O-α-D-glucopyranosyl)-3R,25R-hexacosanediol (**1**): amorphous powder. $[\alpha]_D$ +40.8 (c, 0.36; 2:1 $CHCl_3$-MeOH). FABMS, m/z 577 $(M+H)^+$, 415 (cleavage of the glucoside bond). NMR data (C_5D_5N-D_2O; assignments made by ^1H-^1H COSY, ^1H-^{13}C HETCOR and DEPT): δ ^1H 5.48 (d, J 3.8; H-1'), 4.72 (t, J 9.1; H-3'), 4.60 (bd, J 11.2; H-6'a), 4.49 and 4.46 (m; H-5' + H-6'b), 4.40 (m; H-1a), 4.30 (t, J 9.1; H-4'), 4.25 (dd, J 9.6, 3.6; H-2'), 4.20 (m; H-3), 4.11 (m; H-1b + H-25), 2.09 (m; H-2); 1.47 (d, J 6.2; 25-Me), 1.40, 1.37, 1.35 (methylene chain). δ ^{13}C 100.7 (C-1'), 75.5 (C-3'), 74.5 (C-5'), 73.9 (C-2'), 72.3 (C-4'), 68.7 (C-3), 67.4 (C-25), 66.3 (C-1), 63.0 (C-6'), 40.4 (C-24), 38.8 (C-4), 38.2 (C-2), 30.4 (methylene chain), 26.7 and 26.6 (C-5 and C-23), 24.4 (C-26).

6 mg of **4** were subjected to methanolysis as above to yield 3 mg of **2**.

1-(O-α-D-glucopyranosyl)-3S,25R-hexacosanediol (**2**): amorphous powder. $[\alpha]_D$ +40.0 (c,0.3; 2:1 $CHCl_3$-MeOH). FABMS, m/z 577 $(M+H)^+$, 415. NMR data (C_5D_5N-D_2O; assignments made by ^1H-^1H COSY, ^1H-^{13}C HETCOR and DEPT): δ ^1H 5.48 (d, J 3.6; H-1'), 4.75 (t, J 9.2; H-3'), 4.62 (bd, J 11.5; H-6'a), 4.54 (m; H-1a + H-5'), 4.44 (dd, J 11.5, 5.6; H-6'b), 4.30 (t, J 9.2; H-4'), 4.28 (dd, J 9.2, 3.6; H-2'),

4.17 (m; H-3 + H-25), 4.03 (m; H-1b), 2.13 (m; H-2), 1.49 (d, J 6.2; 25-Me), 1.41, 1.37, 1.36 (methylene chain). δ ^{13}C 100.6 (C-1'), 75.6 (C-3'), 74.5 (C-5'), 73.9 (C-2'), 72.4 (C-4'), 69.3 (C-3), 67.5 (C-25), 66.6 (C-1), 63.1 (C-6'), 40.4 (C-24), 39.1 (C-4), 38.1 (C-2), 30.3 (methylene chain), 26.8 and 26.7 (C-5 and C-23), 24.5 (C-26).

Acid hydrolysis of 1 and 2: triols 5 and 6.

3.6 mg of **1** were dissolved in 2N H_2SO_4 (9:1 MeOH-H_2O; 2ml) and refluxed for 22 h. After cooling, 2 ml of H_2O were added, the MeOH was removed under vacuum and the resulting suspension was extracted with $CHCl_3$ (3x3 ml). After removal of the solvent the product was purified by preparative TLC (9:1 $CHCl_3$-MeOH) and recovered from the silica with 2:1 $CHCl_3$-MeOH to obtain 1.3 mg of **5**.

1,3R,25R-hexacosanetriol (**5**): amorphous powder $[\alpha]_D$ +7.7 (c, 0.13; 2:1 $CHCl_3$-MeOH). FABMS, m/z 415 $(M+H)^+$. NMR data (C_5D_5N-D_2O; assignments made by proton decoupling experiments and DEPT): δ ^{1}H 6.17 (m; 1-OH), 5.97 (d, J 4.5; 3-OH), 5.85 (d, J 4.5; 25-OH), 4.32 (m; H-1 + H-3), 4.11 (m; H-25), 2.14 (m; H-2), 1.45 (d, J 6.1; 25-Me), 1.38 (methylene chain); δ ^{13}C 69.8 (C-3), 67.2 (C-25), 60.5 (C-1), 41.3 (C-2), 40.4 (C-24), 38.9 (C-4), 30.3, 30.2, 30.1 (methylene chain), 26.6 and 26.5 (C-5 and C-23), 24.5 (C-26).

3 mg of **2** were hydrolyzed as above to give 1.0 mg of **6**.

1,3S,25R-hexacosanetriol (**6**): amorphous powder. $[\alpha]_D$ +12.0 (c, 0.1; 2:1 $CHCl_3$-MeOH). FABMS, m/z 415 $(M+H)^+$. NMR data identical to those of **5**.

Tris (*p*-bromobenzoates) 7 and 8.

To 1.2 mg of the triol **5** dissolved in dry Py (0.1 ml), 4-dimethylaminopyridine (1 mg) and p-bromobenzoylchloride (3 mg) were added and the mixture was heated in a capped vial at 50° for 16 h. After cooling, MeOH (0.1 ml) was added and after additional 30 min the solvents were removed with a N_2 stream. The product was dissolved in $CHCl_3$ and chromatographed on a preparative SiO_2 TLC plate (9:1 *n*-hexane-EtOAc) and recovered from the silica with $CHCl_3$ to afford the *tris (p-bromobenzoate)* **7**, FABMS, no molecular ion; UV, λ_{max} (*n*-hexane) 244 nm[11]. ^{1}H-NMR δ ($CDCl_3$) 7.86 (6H), 7.57 (6H), 5.32 (m; 1H), 5.12 (m; 1H), 4.41 (m; 2H), 2.15 (q, J 6.3; 2H), 1.32 (d, J 6.5; 3H), 1.25 (methylene chain). CD spectrum, Fig 1.

1.1 mg of the triol **6** were *p*-bromobenzoylated as above to afford the *tris (p-bromobenzoate)* **8**, FABMS, UV and ^{1}H-NMR identical to those of **7**. CD spectrum, Fig. 1.

MTPA esters of the acetonides 9 and 10.

2.5 mg of triol **5** were dissolved in 2,2-dimethoxypropane (1ml) and a crystal of *p*-TsOH was added. After 24 h at r.t. the solution was chromatographed on a silica gel column ($CHCl_3$) to afford 2.5 mg of **9**; FABMS m/z 455 $(M+H)^+$. ^{1}H-NMR δ (C_5D_5N) 4.11 (sextet, J 5.9; H-25), 4.01 (dt, J 2.6, 12.1; H-1ax), 3.90 (m; H-1eq + H-3), 1.55 (3H, s), 1.50 (3H, s), 1.44 (d, J 6.1; 25-Me), 1.37 (methylene chain). The above acetonide (**9**; 2.5 mg) was divided in two portions and to each of them, dissolved in dry Py (0.1 ml), 4-dimethylaminopyridine (1 mg) was added. In one of the two portions an excess of *S*-(+)-MTPA-Cl (Fluka) was added, while in the other an excess of *R*-(-)-MTPA-Cl was added. After 24 h at r.t. spermine was added in

order to destroy the excess of the unreacted acyl chloride, the Py was removed with a N_2 stream, and the residue was dissolved in $CHCl_3$ and chromatographed on a silica gel column ($CHCl_3$) to afford the *R*-MTPA ester **9a** and the *S*-MTPA ester **9b** respectively.

R-MTPA ester **9a**: FABMS, no molecular ion. ^{1}H-NMR δ ($CDCl_3$) 7.52 (m; 2H), 7.40 (m; 3H), 5.14 (m; H-25), 3.95 (dt, J 2.4, 11.0; H-1ax), 3.82 (m; H-1eq + H-3), 3.55 (s; OCH_3), 1.45 (s; 3H), 1.38, (s; 3H), 1.25 (methylene chain + 25-Me)[13].

S-MTPA ester **9b**: FABMS, no molecular ion. ^{1}H-NMR δ ($CDCl_3$) 7.52 (m; 2H), 7.40 (m; 3H), 5.15 (m; H-25), 3.95 (dt, J 2.4, 11.0; H-1ax), 3.83 (m; H-1eq + H-3), 3.57 (s; -OCH_3), 1.44 (s; 3H), 1.38 (s; 3H), 1.33 (d, J 6.1; 25-Me), 1.25 (methylene chain).

1.0 mg of the triol **6** was treated as above to afford the acetonide **10**, FABMS and ^{1}H-NMR spectra identical to those of **9**. The acetonide **10** was divided in two portions and treated as above to afford the *R*-MTPA ester **10a** and the *S*-MTPA ester **10b** whose FABMS and ^{1}H-NMR spectra were identical to those of **9a** and **9b** respectively.

Acetylation of 11: pentaacetate 12.

1.5 mg of **11** were acetylated as above and the crude reaction product was purified by HPLC (99:1 *n*-hexane/*i*-PrOH) to obtain ca. 1 mg of **12**.

Pentaacetate **12**: FABMS, m/z 785 $(M+H)^+$. NMR data ($CDCl_3$; assignments made by ^{1}H-^{1}H COSY): δ ^{1}H 5.40 (t, J 9.8; H-3'); 5.06 (d, J 3.8; H-1'), 5.05 (t, J 9.8; H-4'), 4.87 (m; H-25), 4.85 (dd, J 3.7, 10.3; H-2'), 4.28 (dd, J 4.2, 12.4; H-6'a), 4.12 (dd, J 2.3, 12.4; H-6'b), 4.03 (m; H-5'), 3.99 (m; H-1a), 3.62 (m; H-1b), 2.70 (bt, J 5.9; H-2), 2.45 (t, J 7.5; H-4), 2.08, 2.05, 2.03 (2x), 2.00 (acetyl methyls), 1.24 (methylene chain), 1.20 (d, J 6.3; H-26). δ ^{13}C 208.1, 170.1, 169.5, 96.0, 71.1, 70.7, 68.6, 67.3, 63.3, 61.9, 43.6, 41.8, 35.9, 29.7, 25.4, 23.7, 21.4, 20.6, 19.9.

Acid hydrolysis of 11.

2.0 mg of **11** were dissolved in 2N H_2SO_4 (9:1 MeOH-H_2O; 2ml) and refluxed for 22 h. After cooling, 2 ml of H_2O were added, the MeOH was removed under vacuum and the resulting suspension was extracted with $CHCl_3$ (3x3 ml). After removal of the solvent the product was purified by preparative TLC (9.5:0.5 $CHCl_3$-MeOH) and recovered from the silica with 8:2 $CHCl_3$-MeOH to obtain the methyl ether **13**, FABMS, m/z 427 $(M+H)^+$, ^{1}H-NMR δ ($CDCl_3$) 3.79 (sextet, J 5.9; H-25), 3.64 (t, J 6.2; H-1), 3.33 (s; -OCH_3) 2.65 (t, J 6.2; H-2), 2.43 (t, J 7.4; H-4) 1.25 (methylene chain), 1.18 (d, J 6.3; 25-Me). The methyl ether **13** was treated with an excess of *R*-(-) -MTPA-Cl as above to afford the *S*-MTPA ester **13a**, FABMS, m/z 643 $(M+H)^+$, 409 $(MH^+$-MTPA). ^{1}H-NMR δ ($CDCl_3$) 7.52 (m; 2H), 7.40 (m; 3H), 5.15 (m; H-25), 3.64 (t, J 6.2 H-1), 3.56 (s; -OCH_3), 3.33 (s; -OCH_3) 2.64 (t, J 5.9; H-2), 2.42 (t, J 7.5; H-4), 1.33 (d, J 6.1; 25-Me), 1.25 (methylene chain).

$NaBH_4$ reduction of 11.

To 2 mg of **11**, dissolved in MeOH (0.5 ml), $NaBH_4$ (1 mg) was added and the mixture was stirred at r.t. for 15 min. AcOH (5 µl) was added and the mixture was applied to a preparative TLC plate (8:2 $CHCl_3$-MeOH) from which the product was recovered with 2:1 $CHCl_3$-MeOH. The product was acetylated with Ac_2O

(0.05 ml) in Py (0.5 ml) at r.t. for 24 h and, after removal of the solvents with a N_2 stream, subjected to preparative HPLC as above. Two peaks were collected (ca. 1:1) which were identified as **3** and **4** from the HPLC R_t and ^{1}H-NMR.

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