Pergamon 0031-9422(94)00648-2 Phytochemistry, Vol. 38, No. 3, pp. 641–645, 1995

0031-9422/95 $9.50 + 0.00

REINVESTIGATION OF HETEROCYST GLYCOLIPIDS FROM THE CYANOBACTERIUM, *ANABAENA CYLINDRICA**

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(*Received* 6 *June* 1994)

Key Word Index—*Anabaena cylindrica*; *A. torulosa*; cyanobacteria; heterocyst glycolipids; stereochemistry of 1,3-diols.

Abstract—The previously proposed structures for the heterocyst glycolipids of the nitrogen-fixing cyanobacterium *Anabaena cylindrica* have been revised. ^{13}C-enriched acetonides have been utilized for the determination of the relative stereochemistry of the 1,3-diol moiety in the aglycone of two glycolipids. The same glycolipids, which are thought to protect the nitrogenase from O_2, have also been detected in *A. torulosa*.

INTRODUCTION

In nitrogen-fixing cyanobacteria [1], the incompatible processes of photosynthetic O_2 production and N_2 reduction are spatially or temporally separated since the enzyme nitrogenase is greatly affected by oxygen. Temporal separation occurs in some genera of unicellular cyanobacteria, while some filamentous ones when deprived of fixed nitrogen elaborate differentiated cells, called heterocysts, whose primary function is the fixation of N_2. Differentiation of vegetative cells into heterocysts results, *inter alia*, in the formation of thick glycolipid layers in heterocyst walls which may restrict air diffusion [2], allowing sufficient N_2 penetration but restricting O_2 diffusion to a level which can be removed by respiratory scavenging. Constituents of the heterocyst glycolipid layers are some unusual long chain glycolipids, whose occurrence is restricted to the heterocystous cyanobacteria, which have attracted earlier [3–5] and recent [6, 7] research work.

Among the nitrogen-fixing cyanobacteria, the most widely studied is *Anabaena cylindrica* which has even been referred to as the '*E. coli*' of cyanobacteria [1]. Earlier investigations [4, 5] resulted in the proposal of the partial structures **I**–**IV** for the heterocyst glycolipids of *A. cylindrica* (Fig. 1); we report herein that structures **1**–**4** should be assigned to these compounds.

RESULTS AND DISCUSSION

The recently reported isolation procedure of heterocyst glycolipids [6, 7] has now been improved by using in the first step silica gel flash chromatography, instead of a LH-20 based procedure, and subsequently other chromatographic steps. Compounds **1**–**4** were eluted in order from the silica gel column chromatography and correspond to compounds **I**–**IV** (Fig. 1) previously reported [5]. The relative ratio between the compounds is *ca* 30:2.5:100:8. It should be noted that **1**–**4** are pure glucose derivatives; the minor galactose derivatives of the same aglycones, which were previously reported as companions of the glucosides [4, 5], have not been detected.

Compounds **1** and **3** have been previously isolated from *Nodularia harveyana* [6], where **3** co-occurs with its 3*S*-epimer, and were identified by their spectral and chemical properties. In particular, the stereochemistry at C-25 in both compounds, which cannot be deduced from spectral data or optical rotations, has been derived from the Mosher esters of the aglycones as previously described [6].

The structure of the ketone **1** deserves further comment. We anticipated [6] that the glucose ester structure **I** assigned to this compound by previous workers [5] should be revised. In the earlier work [5], the principal argument favouring the ester structure was that, besides acid-catalysed methanolysis, **1** was also cleaved by mild alkaline methanolysis giving a compound which was erroneously identified as the methyl ester of 25-hydroxyhexacosanoic acid. We have found that both acid- [6] and, more cleanly, base-catalysed methanolysis afford the same compound **5** which is isomeric with methyl 25-hydrohexanoate. The formation of **5** should be envisaged as a β-elimination process from **1** followed by both acid- and base-catalysed methanol addition to the unsaturated ketone intermediate **6**.

NMR and mass spectral data established that **2** and **4** differ from **1** and **3**, respectively, in having a common

*Part 3 in the series 'The Heterocyst Glycolipids of Cyanobacteria'. For part 2 see ref. [6].

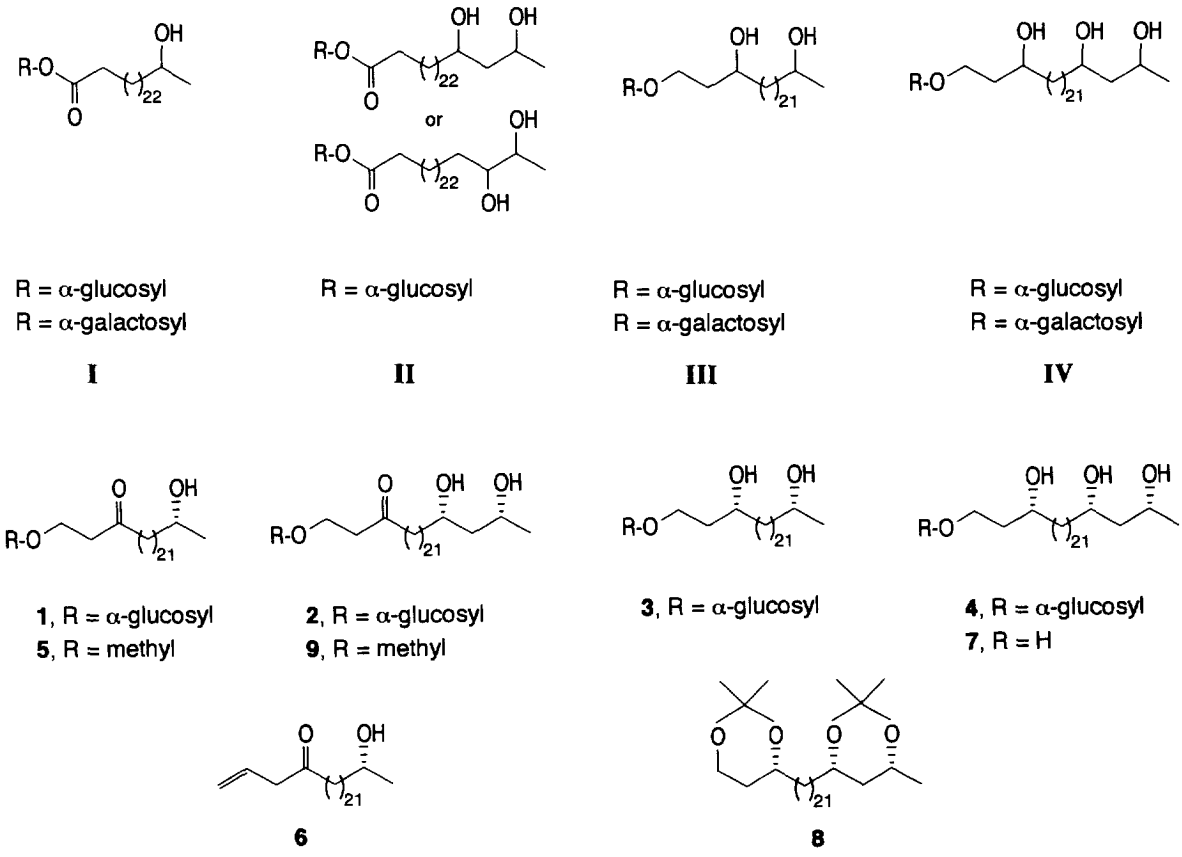

Fig. 1. Proposed structures for heterocyst glycolipids of *Anabaena cylindrica* (adapted from ref. [5]).

additional C_2H_4O unit. The proton spectrum of both compounds contains a well resolved spin system for the C-26 methylene protons at δ2.01 (*dt*, J = 13.8, 9.2 Hz) and δ1.82 (*dt*, J = 13.8, 3.5 Hz) which was assigned by a COSY spectrum. Consequently, the COSY spectrum allowed the assignment of the chemical shift values to the carbinol protons at C-25 (δ4.22) and C-27 (δ4.44), since both protons were coupled to CH_2-26 while only the proton at δ4.44 was also coupled with the methyl protons. The remaining parts of the ^{1}H- and ^{13}C NMR spectra of both **2** and **4** were identical, where relevant, with those **1** and **3**.

The stereochemistry of **4** was established as follows. The ^{13}C chemical shift of C-3 establishes the *R* configuration at the centre, since the proximity of the glucose moiety makes different the C-3 chemical shift of *R* and *S* epimers [6, 7]. Acid methanolysis of **4** furnished the tetrol **7**, which was transformed into the *bis*-acetonide **8** in order to deduce the relative stereochemistry at C-25 and C-27 by inspection of the ^{13}C chemical shift of the acetonide methyls according to Rychnovsky [8, 9]. In fact, in 1,3-*syn* diols the acetonide methyls resonate at two distinct chemical shift values at *ca* δ20 and 30, while in 1,3-*anti* diols they resonate very close in the δ25 zone. In order to facilitate the assignment of the acetonide methyls and also because of the small amount of **7** in our hands, we decided to use 1,3-^{13}C-enriched 2,2-dimethoxypropane to generate ^{13}C-enriched **8**. 1,3-^{13}C-enriched 2,2-dimethoxypropane was simply prepared by reacting 2,2-dimethoxypropane with commercially available [1,3-$^{13}C_2$] acetone, in the presence of catalytic *p*-TsOH. The assignment of the ^{13}C chemical shift of the methyls in acetonides enriched in such a way is straightforward because (i) the enriched signals dominate the spectrum and (ii) each methyl signal appears as a doublet because of the $^2J_{CC}$ (*ca* 4.5 Hz) between the two acetonide methyls. In the acetonide **8**, two close pairs of doublets were observed, a pair at δ19.3 and δ30.0, which was assigned to the acetonide methyls of the C-1, C-3 diol moiety [8], also in comparison with the acetonide prepared from the aglycone of **3**, and a pair at δ19.8 and δ30.4 which was due to the methyls of the acetonide of the C-25, C-27 diol moiety. From this evidence, the C-25, C-27 diol should have a *syn* relative stereochemistry. ^{13}C-enriched acetonides have been previously used for the determination of the stereochemistry of the macrolactins [10]. However, to generate the acetonides the authors used the Noyori's procedure [11], which involves firstly silylation of the diol and then reaction with [1,3-$^{13}C_2$] acetone, in the presence of TMSOTf and in strictly aprotic conditions. In our hands, this method gave very poor yields when small amounts of material (1–3 mg) were available, as also previously experienced [10]; in contrast, the use of ^{13}C-enriched 2,2-dimethoxypropane gives excellent yields of acetonides.

The absolute configuration was established by the Mosher method [12]. Because of the presence of the 1,3-diol moiety which prevents the use of the $\Delta\delta$ value of the central carbon protons (C-26), influenced from both sides by the flanking MTPA esters, and since the C-24 methylene resonance is obscured by other signals, the absolute configuration was established to be *R* at C-27 by consideration of the negative $\Delta\delta$ value [12] of the terminal methyl group. Accordingly, the absolute configuration at C-25 is *S*. The same absolute configuration at C-25 and C-27 was established in **2** by inspection of the ^{1}H NMR spectra of the MTPA esters of the alkaline hydrolysis product **9**.

We have detected glycolipids **1**–**4**, in the same approximate ratio, in another *Annabaena* species, *A. torulosa*. From *A. torulosa* all four compounds were isloated, while the absolute configuration was confirmed to be the same as in *A. cylindrica* only for the major compound **3**, which was isolated in sufficient amounts.

EXPERIMENTAL

General. For procedures see ref. [7].

Anabaena *species and growth conditions. A. cylindrica* 10 C was obtained from the culture collection of Centro di Studio dei Microrganism Autotrofi (Firenze); *A. torulosa* B26.79 was obtained from Sammlung von Algenkulturen, Gottingen. *A. cylindrica* was grown in BG-11$_0$ medium (BG-11 with omission of $NaNO_3$) [13], while *A. torulosa* was grown in the medium Z of Zender, with omission of combined N[14]. The 2 species were grown on a larger scale at 25° in a 90 l fermentor with an aeration flux of 30 ml min^{-1} using 70 l medium at pH 7; continuous illumination was provided by 4 18W cool white fluorescent tubes arranged parallel to the fermentor. The fermentor culture was started with 2.5% inoculum obtained in a 1 l fermentor. The biomass concn was monitored and the cells were collected as previously reported [6]. The yields of different prepns of the two species ranged from 9 to 10 g of lyophilized cells per 70 l fermentor.

Isolation of glycolipids. Lyophilized cells of *A. cylindrica* (*ca* 60 g), originating from 6 fermentors, were extracted as previously described [6] to afford 6.1 g of extract which was dissolved in $CHCl_3$–MeOH (2:1) and flash-chromatographed in 1 g portions on a silica gel column (50 × 3 cm) eluted with $CHCl_3$–MeOH (4:1). Frs containing **1** and **2** (412 mg) were flash chromatographed on a silica gel column (115 × 3 cm) eluted with $CHCl_3$–MeOH (9:1) to afford crude **1** (70 mg) and crude **2** (11 mg). Crude **1** (30 mg) was dissolved in $CHCl_3$–MeOH (2:1) and chromatographed on a LH-20 column (87 × 3 cm) eluted with MeOH–$CHCl_3$ (9:1) giving 18 mg of pure **1**, $[\alpha]_D$ + 38.8 [($CHCl_3$–MeOH, 2:1; *c* 1.1) lit. [6]. $[\alpha]_D$ + 10.7 ($CHCl_3$–MeOH, 2:1; *c* 0.4) discrepancies in the $[\alpha]_D$ values with previously reported data are due to a previous misworking of the polarimeter which caused aberrant results, especially with dilute samples], identified by MS and NMR data. Crude **2** (11 mg), dissolved in $CHCl_3$–MeOH (2:1), was subjected to prep. TLC on 5 silica gel plates (20 × 20 cm; 0.5 mm thick) which were eluted with $CHCl_3$–MeOH (17:3). The band due to **2** was visualized by exposure to I_2 vapours and eluted from the silica with $CHCl_3$–MeOH (2:1) to give 2.9 mg of **2**.

1-((O-α-D-*glucopyranosyl*)-3-*keto*-25S, 27R-*octacosanediol* (**2**). $[\alpha]_D$ + 14.4 ($CHCl_3$–MeOH, 2:1; *c* 0.29). FABMS, *m/z* 619 $[M + H]^+$, 457 (cleavage of glucosidic bond). IR (liquid film), ν_{max} cm^{-1} 1710. NMR data (C_5D_5N; assignments made by ^{1}H–^{1}H COSY, ^{1}H–^{13}C HETCOR, DEPT and comparison with **1** and **4**): aglycone moiety, δ^1H 4.44 (*m*, overlapped with other signals; H-27), 4.40 (*dt*, *J* = 10.1, 6.4; H-1a), 4.22 (*m*, overlapped with other signals; H-25), 4.04 (*dt*, *J* = 10.1, 6.4; H-1b) 2.88 (*dt*, *J* = 6.4, 1.3, H-2), 2.50 (*t*, *J* = 7.4; H-4), 2.01 (*dt*, *J* = 13.8, 9.2; H-26a), 1.82 (*dt*, *J* = 13.8, 3.5; H-26b), 1.48 (*d*, *J* = 6.1; H-28), 1.38, 1.37, 1.30 (methylene chain); δ^{13}C 208.9 (C-3), 71.6 (C-25), 67.8 (C-27), 63.9 (C-1), 46.7 (C-26), 43.4 (C-4), 43.0 (C-2), 39.1 (C-24), 30.3, 30.1, 29.9, 29.6 (methylene chain), 26.2 (C-23), 24.8 (C-28), 24.0 (C-5). Glucose moiety, δ^1H 5.41 (*d*, *J* = 3.7; H-1), 4.63 (*t*, *J* = 9.3; H-3), 4.60 (*dd*, *J* = 11.1, 2.0; H-6a), 4.47 (*m*, H-5 + H-6b), 4.29 (*t*, *J* = 9.3; H-4), 4.21 (*dd*, *J* = 9.6, 3.8; H-2); δ^{13}C 100.9 (C-1), 75.5 (C-3), 74.6 (C-5), 73.9 (C-2), 72.3 (C-4), 63.0 (C-6).

Compounds **3** and **4** were recovered as a mixt. with other compounds in 3 frs weighing 100, 430 and 92 mg. The fr. of 430 mg was dissolved in $CHCl_3$–MeOH (2:1) and flash-chromatographed in 2 portions on a silica gel column (50 × 3 cm) eluted with $CHCl_3$–MeOH (17:3) to afford 106 mg of crude **3** and 35 mg of *ca* 1:1 mixt. of **3** and **4**. Crude **3** (50 mg) was dissolved in $CHCl_3$–MeOH (2:1) and chromatographed on a LH-20 column (87 × 3 cm) eluted with MeOH–$CHCl_3$ (9:1) to give 25 mg of pure **3**, $[\alpha]_D$ + 47.8 ($CHCl_3$–MeOH, 2:1; *c* 1.2) lit. [6]. $[\alpha]_D$ + 40.8 ($CHCl_3$–MeOH, 2: 1; *c* 0.36), identified by MS and NMR data. The mixt. of **3** and **4** (35 mg), dissolved in $CHCl_3$–MeOH (2:1) was subjected to prep. TLC on 18 silica gel plates eluted with $CHCl_3$–MeOH (17:3). The bands due to **3** and **4** were visualised by exposure to I_2 vapour and eluted from the silica with $CHCl_3$–MeOH (2:1) to give **3** (18 mg) and **4** (12 mg).

1-(O-α-D-*glucopyranosyl*)-3R,25S,27R-*octacosanetriol* (**4**). $[\alpha]_D$ − 48.9 ($CHCl_3$–MeOH, 2: 1, *c* 1.0). FABMS, *m/z* 621 $[M + H]^+$, 459 (glucosidic bond cleavage). NMR data (C_5D_5N; assignments made by ^{1}H–^{1}H COSY, ^{1}H–^{13}C HETCOR, DEPT and comparison with **1** and **3**); aglycone moiety, δ^1H 4.44 (*m*, partially overlapped with other signals; H-27), 4.38 (*dt*, *J* = 9.9, 6.2; H-1a), 4.22 (*m*, overlapped with other signals; H-25), 4.20 (*m*, partially overlapped with H-25; H-3), 4.10 (*dt*, *J* = 9.9, 6.8; H-1b), 2.07 (*m*, H-2), 2.01 (*dt*, *J* = 13.8, 9.2; H-26a), 1.82 (*dt*, *J* = 13.8, 3.5; H-26b), 1.48 (*d*, *J* = 6.1; H-28), 1.38, 1.37, 1.36 (methylene chain); δ^{13}C 71.6 (C-25), 68.6 (C-3), 67.8 (C-27), 66.2 (C-1), 46.7 (C-26), 39.1 (C-24), 38.8 (C-4), 38.2 (C-2), 30.4, 30.3, 30.1, 29.7 (methylene chain), 26.4 (C-5), 26.2, (C-23), 24.8 (C-28). Glucose moiety δ^1H 5.47 (*d*, *J* = 3.7; H-1), 4.69 (*t*, *J* = 9.3; H-3), 4.58 (*dd*, *J* = 13.9, 5.0; H-6a), 4.47 (*dd*, *J* = 14.0, 5.1; H-5 + H-6b), 4.30 (*t*, *J*

= 8.9; H-4), 4.23 (*dd*, J = 9.6, 3.7; H-2); δ^{13}C 100.7 (C-1), 75.7 (C-3), 74.5 (C-5), 74.0 (C-2), 72.4 (C-4), 63.0 (C-6).

Lyophilized cells of *A. torulosa* (9.9 g), originating from one fermentor, afforded 1.6 g of an extract which was processed as above to give 1.4 mg of **1**, trace amounts of **2**, 5.2 mg of **3** and 1.1 mg of impure **4**.

Base-catalysed methanolysis of ketones **1** *and* **2**. Compound **1** (8 mg) was dissolved into 2 ml of 0.5 N KOH in MeOH, the soln stirred for 3 hr at 0° and then filtered through a short silica gel column to obtain **5** (5 mg), $[\alpha]_D$ − 4.8 ($CHCl_3$–MeOH 2:1; *c* 0.5); MS and ^{1}H NMR identical to those of the same product previously obtained by acid-catalysed methanolysis [6]. ^{13}C NMR δ($CDCl_3$) 209.2 (weak), 68.2, 67.6, 58.8, 43.5, 42.7, 39.4, 29.7, 29.5, 29.4, 29.2 25.8, 23.6, 23.5. *R*- and *S*-MTPA esters of **5** were prepd as previously described [6]. *R*-MTPA ester: ^{1}H NMR δ($CDCl_3$) 7.53 (*m*, 2H), 7.40 (*m*, 3H), 5.13 (*sextet*, J = 6.3; H-25), 3.63 (*t*, J = 6.2; H-1), 3.60 (*s*,-OMe), 3.33 (*s*, -OMe), 2.64 (*t*, J = 6.3; H-2), 2.43 (*t*, J = 7.4; H-4), 1.25 (methylene chain + 25-Me). *S*-MTPA ester, ^{1}H NMR identical to that previously described [6]. 25-Me, $\Delta\delta$ = − 0.08 ($\delta_{R-MTPA} - \delta_{S-MTPA}$).

Compound **2** (2.8 mg) was methanolized as described above to afford 1.4 mg of **9**, $[\alpha]_D$ − 21.1 ($CHCl_3$–MeOH, 2:1; *c* 0.14). FABMS, *m/z* 471 $[M + H]^+$. ^{1}H NMR δ(C_5D_5N) 4.45 (*m*, 1H), 4.22 (*m*, 1H), 3.76 (*t*, J = 6.2; H-1), 3.68 (*s*, -OMe), 2.77 (*t*, J = 6.1; H-2), 2.51 (*t*, J = 7.3; H-4), 2.01 (*dt*, J = 13.8, 9.2; H-26a), 1.82 (*dt*, J = 13.8, 3.5; H-26b), 1.47 (*d*, J = 6.1; H-28), 1.45, 1.38, 1.25 (methylene chain). ^{13}C NMR δ(C_5D_5N) 209.1 (weak), 71.6, 68.1, 67.8, 58.8, 46.7, 43.3, 43.1, 39.1, 30.3, 30.1, 29.9, 26.2, 24.8, 24.0. Compound **9** was divided in two portions from which the MTPA diesters were prepd [6, 7]. *R*-MTPA diester: ^{1}H NMR δ($CDCl_3$) 7.53 (4H), 7.40 (6H), 5.11 (*quintet*, J = 6.6; H-25), 5.03 (*sextet*, J = 6.4; H-27), 3.64 (*t*, J = 6.2; H-1), 2.64 (*t*, J = 6.3; H-2), 2.43 (*t*, J = 7.5; H-4), 2.04 (*dt*, J = 14.3, 6.9; H-26a), 1.76 (*dt*, J = 14.3, 6.7; H-26b), 1.25 (methylene chain), 1.22 (*d*, J = 6.3; 27-Me). *S*-MTPA diester: ^{1}H NMR δ($CDCl_3$) 7.53 (4H), 7.40 (6H), 5.16 (*sextet*, J = 6.4; H-27), 5.03 (*pentet*, J = 6.2; H-25), 3.64 (*d*, J = 6.3; H-1), 2.65 (*t*, J = 6.3; H-2), 2.43 (*t*, J = 7.4; H-4), 2.07 (*dt*, J = 14.2, 7.1; H-26a), 1.77 (*dt*, J = 14.2, 6.0; H-26b), 1.37 (*d*, J = 6.3; 27-Me), 1.26 (methylene chain). 27-Me $\Delta\delta$ = − 0.15.

Acid hydrolysis of **4**. Compound **4** (11.0 mg) was dissolved in 2 ml of 2N H_2SO_4 in MeOH–H_2O (9:1) and refluxed for 24 hr. After cooling, 2 ml of H_2O were added, the MeOH removed with a N_2 stream and the resulting suspension extracted with $CHCl_3$ and then with *n*-BuOH. The BuOH extract, after removal of solvent, was chromatographed on a short silica gel column eluted with $CHCl_3$–MeOH (9:1) to afford 3.5 mg of **7**. $[\alpha]_D$ − 166.9 ($CHCl_3$–MeOH, 2:1; *c* 0.35). FABMS, *m/z* 459 $[M + H]^+$. ^{1}H NMR δ(C_5D_5N) 6.25, 6.11, 6.07, 5.87 (*br*, OH protons) 4.45 (*m*, 1H), 4.28 (*m*, 2H), 4.22 (*m*, 1H), 2.12 (*m*, H-2), 2.01 (*dt*, J = 13.8, 9.2; H-26a), 1.82 (*dt*, J = 13.8, 3.5; H-26b), 1.47 (*d*, J = 6.1; H-28), 1.39, 1.37 (methylene chain). ^{13}C NMR δ (C_5D_5N) 71.6 (C-25), 69.8 (C-3), 67.8 (C-27), 60.5 (C-1), 46.8 (C-26), 41.3 (C-2), 39.1 (C-24), 38.9 (C-4),30.3, 30.1 (methylene chain), 26.4 (C-5), 26.2 (C-23), 24.8 (C-28).

^{13}C-*Enriched* bis-*acetonide* **8**. A stock soln was prepd by dissolving [1,3-$^{13}C_2$]acetone (250 mg; 98 atom % ^{13}C; Aldrich Chem. Co.) in dry toluene (1 ml). To 0.3 ml of the stock soln. 0.3 ml of 2,2-dimethoxypropane were added together with a crystal of *p*-TsOH and the soln was left to stand at room temp. for 7 hr. After this period, a control by ^{13}C NMR showed that the ratio between the *C*-methyl groups (δ23.6) and *O*-methyl groups (δ47.8) in the resulting enriched 2,2-dimethoxypropane was > 40:1. The above soln was added to 3.5 mg of **7** and the mixt. left to stand overnight at room temp. The reaction mixt. was filtered through a short basic Al_2O_3 column eluted with Et_2O to give 2.9 mg of **8**. ^{1}H NMR δ ($CDCl_3$) 3.95 (*m*, 2H), 3.80 (*m*, 3H), 1.45, 144, 1.40, 1.38 (*s*, acetonide methyls). ^{13}C NMR δ($CDCl_3$) 98.3, 98.2, 69.0, 68.9, 65.2, 60.1, 38.8, 36.5, 36.4, 31.3, 30.9, 30.4 (*d*, J = 4.4), 30.0 (*d*, J = 4.4), 29.7, 29.6, 25.0, 24.9, 22.3, 19.8 (*d*, J = 4.4), 19.3 (*d*, J = 4.4).

MTPA esters of **7**. The acetonide **8** (2.9 mg) was dissolved in MeOH, a crystal of *p*-TsOH added and the soln was left to stand overnight at room temp. The solvent was removed under red. pres. and the product filtered through silica gel eluting with $CHCl_3$–MeOH (9:1) to afford 1.8 mg of tetrol **7**, which was divided into 2 portions and reacted [6, 7] to prepare the MTPA esters. *R*-MTPA tetraester: ^{1}H NMR δ ($CDCl_3$) 7.51 (*m*, 8H), 7.40 (*m*, 12H), 5.11 (*m*, H-3 + H-25), 5.03 (*sextet*, J = 6.3; H-27), 4.36 (*dt*, J = 11.1, 5.6; H-1a), 4.25 (*dt*, J = 11.1, 7.0; H-1b), 3.54 (*s*, 2 × OMe), 3.52 (*s*, 2 × OMe), 1.26, 1.24 (methylene chain), 1.22 (*d*, J = 6.3; 27-Me). *S*-MTPA tetraester: ^{1}H NMR δ($CDCl_3$) 7.51 (*m*, 8H), 7.40 (*m*, 12H), 5.15 (*sextet*, J = 6.4; H-27), 5.09 (*m*, H-3), 5.04 (*quintet*, J = 6.3; H-25), 4.21 (*m*, H-1a), 4.13 (*m*, H-1b), 3.55 (*s*, 3 × OMe), 3.53 (*s*, OMe), 1.37 (*d*, J = 6.3; 27-Me), 1.26 (methylene chain). 27-Me, $\Delta\delta$ = − 0.15.

Acknowledgements—This work was supported in part by MURST and CNR grants. We thank R. Di Maso for his help in growing *A. torulosa*. Thanks are also due to the ICMIB-NMR Service for the 500 MHz ^{1}H NMR and to the Servizio di Spettrometria di Massa del CNR e dell'Università di Napoli for MS.

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