Pergamon

PII: S0031-9422(97)00954-0

Phytochemistry, Vol. 48, No. 5, pp. 801–805, 1998

Printed in Great Britain
0031-9422/98 $19.00+0.00

HETEROCYST GLYCOLIPIDS FROM NITROGEN-FIXING CYANOBACTERIA OTHER THAN NOSTOCACEAE‡

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(*Received* 24 *July* 1997)

Key Word Index—Scytonemataceae; Rivulariaceae; Stigonematales; cyanobacteria; heterocyst glycolipids.

Abstract—Six cyanobacteria representative of families able to form heterocysts, other than Nostocaceae, have been examined for their heterocyst glycolipids (HGs) content. While the HGs found in Nostocaceae are dominated by the presence of triols and of the corresponding C-3 ketones as aglycones, those found in the other families reported here are mainly characterized by tetrols and by the corresponding ketones at the ω-3 position. The latter were found in four out of the six species examined, *Scytonema hofmanni*, *Calothrix desertica*, *Chlorogloeopsis fritschii* and *Fischerella muscicola*. Triols and the corresponding ketone glycosides were found only in a *Microchaete* sp. and in *Tolypothrix tenuis*, the latter showing the most complex array of HGs found so far. In *Calotrhix desertica* and *Chlorogloeopsis fritschii*, α-mannosides have been found for the first time. The relevance of these data as a chemotaxonomic tool is discussed.

INTRODUCTION

In some filamentous cyanobacteria, the fixation of N_2 takes place in specialized cells, called heterocysts, which possess a thick envelope. These thick-walled cells contain unique glycolipids organized in the so-called "laminated" layer [1], which seems to play a role in restricting air diffusion [2, 3], allowing sufficient N_2 penetration but restricting O_2, which is dangerous to the enzyme, nitrogenase.

Cyanobacteria are classified into five subgroups [4], only two of them (subgroups 4 and 5) include species in which differentiation of vegetative cells into heterocysts occurs. All the cyanobacteria investigated to date [5–8] for their heterocyst glycolipids (HGs) content belong to the Nostocaceae (subgroup 4). In particular, the structure [7] and biosynthesis [9] of HGs have been recently reinvestigated in *Anabaena cylindrica*, the "*E. coli* of cyanobacteria" [10].

The results of previous studies can be summarized as follows. The HG aglycones belong to four structural types consisting of long-chain triols (**1**–**4**) and tetrols (**5**–**7**), and the corresponding C-3 ketones (**11**–**13** and **14**–**16**), which in the examined species differ only in chain-length. The only different location of the carbonyl group in the aglycones so far encountered was found in *Cyanospira rippkae*, where it was located at the ω-1 position, instead of C-3 [6]. The triol glycosides and the corresponding ketones are ubiquitous, the former generally being the major HGs found in the species examined to date [8]. As far as the sugar moiety is concerned, the HGs of Nostocaceae occur as α-glucosides, with the notable exception of *Anabaena* sp. WSAF which contains only α-galactosides (**3**, **7**, **13**, **16**) and the β-glucoside **4** [8].

We report herein an extension of studies to cyanobacteria representative of the other families of subgroup 4 and 5 able to form heterocysts. The chemical structure of the HGs has been investigated in: (i) *Scytonema hofmanni* and *Tolypothrix tenuis* (Scytonemataceae, subgroup 4); (ii) *Calothrix desertica* and *Microchaete* sp. (Rivulariaceae, subgroup 4); (iii) *Chlorogloeopsis fritschii* and *Fischerella muscicola* (Stigonematales, subgroup 5).

RESULTS AND DISCUSSION

As expected, the cyanobacteria grown in media containing combined nitrogen did not differentiate heterocysts and, accordingly, did not yield HGs when extracted. When the cyanobacteria were grown in

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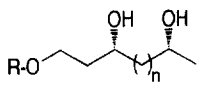
‡ Part 6 in the series "The Heterocyst Glycolipids of Cyanobacteria". For previous papers in the series see Refs [5–9].



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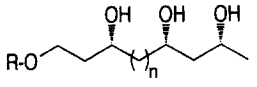

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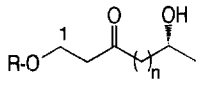
8 R = α-Man, n = 21
9 R = α-Glu, n = 25
10 R = α-Man, n = 25



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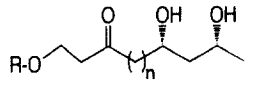

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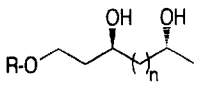

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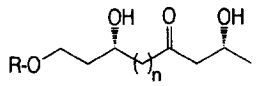

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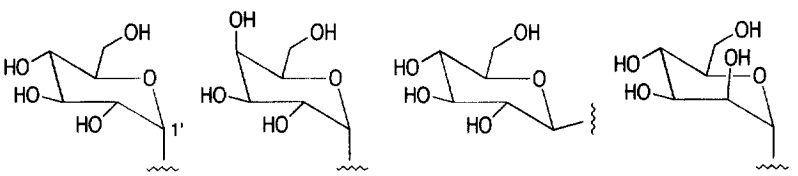
23 R = α-Man, n = 25



α-Glu α-Gal β-Glu α-Man

media without combined nitrogen they formed heterocysts, the frequency of which depended on the microorganism. In *Calothrix desertica*, basal heterocysts appeared in the late phase of growth, when the thrichomes were mature. In *Tolypothrix tenuis*, single thricomes and false branches associated with a single terminal heterocyst appeared. In *Chlorogloeopsis fritschii*, at the end of the growth, several thricomes with terminal heterocysts were observed, although several stages of development were simultaneously found. *Fischerella muscicola* showed, at the end of the growth, pseudoakinetes, in addition to heterocysts. In *Microchaete*, sp. the heterocysts were observed in terminal position and not intercalary as reported in Bergey's manual [4], while they were intercalary in *Scytonema*. Results obtained are summarized in Table 1. Isolation of HGs and their structural determination were carried out as previously reported [5–8]; as far as the stereochemistry is concerned, in the previous work [5–8] the absolute configuration of the stereogenic centres of the aglycone moieties was determined by Mosher method on the aglycones obtained by acid hydrolysis of the parent glycosides. However, hydrolysis of the new ketone glycosides **19–23** did not afford the expected aglycones since the β-hydroxy ketone moiety present in these molecules was sensitive to the hydrolytic conditions. We thus attempted the preparation of the Mosher esters directly on the glycosides, with excellent results in the case of the triol and tetrol glycosides, but with poor results in the case of ketone glycosides. For triol and tetrol glycosides, the Mosher esters were obtained in good yields and the stereochemistry at the centres far from the carbohydrate moiety was safely derived from the $\Delta\delta$ values since the chemical shift values cannot be affected by the esters residues present on the carbohydrate moiety. In all the HGs reported herein, the absolute configuration at the ω-1 and ω-3

Table 1. Occurrence of heterocyst glycolipids (HGs)

Cyanobacterium	HGs (approximate relative ratio)
Scytonemataceae	
Scytonema hofmanni	**5** (1.5), **21** (1)
Tolypothrix tenuis	**1** (4), **4** (1.5), **5** (2), **11** (0.5), **14** (0.5), **18** (1.5)
Rivulariaceae	
Calothrix desertica	**6** (1), **8** (0.8), **19** (2), **20** (1.2)
Microchaete sp.	**1** (1), **11** (3)
Stigonematales	
Fischerella muscicola	**9** (1.5), **22** (1)
Chlorogloeopsis fritschii	**9** (4.5), **10** (traces), **22** (4.5), **23** (0.6)

carbons was found to be the same as in the previously isolated glycosides [5–8]. On the other hand, the stereochemistry at C-3 can be derived from the ^1H and ^{13}C NMR data, as previously reported [5]. By contrast, when compounds **19–23** were subjected to the usual procedure of Mosher ester preparation [5], retroaldol cleavage occurred, affording methyl ketones lacking the last two carbon atoms of the alkyl chain of the parent glycosides. Therefore, the stereochemistry at the ω-1 carbon in **19–23** was assumed to be the same as in the co-occurring tetrol glycosides.

Scytonema hofmanni contains the tetrol α-glucoside (**5**) previously found in *A. sphaerica* [8], together with the α-glucoside (**21**) carrying an aglycone moiety not found before. The ^1H NMR data (COSY) showed that the carbonyl group was located at C-27, since the diastereotopic C-28 protons resonated as two mutually coupled double doublets at δ 2.70 (J = 15.3 and 4.6 Hz) and 2.92 (J = 15.3 and 8.2 Hz), both coupled with the C-29 proton at δ 4.70; the latter was coupled with the methyl doublet at δ 1.45. The absolute configuration at C-3 was evident from the ^1H and ^{13}C NMR data [5], also in comparison with those of the tetrol **5**, while the absolute configuration at C-29 was derived from the $\Delta\delta$ values of the terminal methyl group in the Mosher esters.

Tolypothrix tenuis exhibits a very complex array of HGs. For this reason only compounds **1**, **5**, **11** and **14** were isolated in a pure form and identified as those isolated previously from *A. sphaerica* [8]. Compounds **4** and **18** were isolated as a mixture also containing **1** and giving a single $[M]^+$ in the FAB mass spectrum at m/z 605. The ^{13}C NMR spectrum of the mixture showed three sets of signals in a ratio of ca 2:1:1, **1** being the major component. Comparison with data of previously isolated compounds [5, 8] allowed the identification of the three HGs. In particular, besides the signals of the major compound **1**, the representative signals of the β-glucoside **4** [8] resonated at δ 105.0, 78.7, 78.6, 75.3, 71.9, 68.1 and 38.9, while signals indicative for the presence of **18**, the C-3 epimer of **1**, were found at δ 69.2, 66.6, 39.1 and 38.1 [5]. It should be noted that a HG having a 3*S*-absolute configuration was previously isolated, as the lower homologue **17**, only from *Nodularia harveyana* [5], where it was found occurring with its major epimer **2** in a ratio of ca 1:2.

The four HGs isolated from *Calothrix desertica* consist of two pairs of α-glucosides and α-mannosides of the same aglycones. The tetrol α-glucoside (**6**) has been previously isolated from *Anabaena cylindrica* [7], while the ketone (**19**) is a lower homologue of **21** isolated from *S. hofmanni* (*vide supra*). Compounds **6** and **19** were accompanied by two HGs (**8** and **20**) slightly more polar on silica gel chromatography, which were isolated by repeated chromatographic purifications. The FAB mass spectra of **8** and **20** were identical to those of **6** and **19**, while comparison of the ^1H and ^{13}C NMR data readily showed that **8** and **20** had the same aglycones as **6** and **19**, respectively, but with a different carbohydrate moiety. The latter was identified as α-mannose by comparison of the ^1H and ^{13}C NMR data with those of authentic α-methyl mannoside. Since several signals of the α-mannose moiety were superimposed in the ^1H NMR spectrum of both **8** and **20**, assignments of the chemical shift values were made after acetylation and comparison with the data of α-methyl mannoside acetate. This is the first time that mannosides have been found within HGs, besides the widespread α-glucosides, only α-galactosides and β-glucosides have been isolated [8].

The *Microchaete* sp. examined contains HGs **1** and **11** previously isolated from *A. sphaerica* [8] and from *T. tenuis* (*vide supra*).

From *Fischerella musicicola*, compounds **9** and **22** were isolated, which are higher homologues of **5** and **21** isolated from *S. hofmanni* (*vide supra*). It is noteworthy that the aglycones of **9** and **22** are the longest (C_{32}) so far found, the C_{26} aglycones being the most common [5, 7, 8].

From *Chlorogloeopsis fritschii* two pairs of α-glucosides (**9** and **22**) and α-mannosides (**10** and **23**) have been isolated, as from *C. desertica* (*vide supra*). However, the aglycones are C_{32}, as in *F. muscicola*.

While the HGs found in Nostocaceae are dominated by the presence of triol aglycones (**1** and **2**) and of the corresponding C-3 ketones, those found in the other families reported here are mainly characterized by tetrols (**5–7** and **9**) and by the corresponding ketones at the ω-3 position, which were found in four

out of the six species examined. Triol glycosides and the corresponding ketones were found only in the *Microchaete* sp. and in *Tolypothrix tenuis*, the latter showing the most complex array of HGs so far found.

It is noteworthy that in two species, *Calotrhix desertica* and *Chlorogloeopsis fritschii* for the first time α-mannosides have been found, while until now variants to the usual α-glucosides were constituted only by α-galactosides and β-glucosides. In this respect, it is surprising that cyanobacteria showing a very close pattern of HGs are classified into two different subgroups. As previously noted [4], classification of cyanobacteria is in transition and may be subjected to a great modification. Criteria for classification include morphology, mode of reproduction, ultrastructure, physiology, chemistry and, sometimes, genetics. Looking only at the carbohydrate moieties and at the aglycone functionalization of the HGs, it should be noted the close similarity of the composition of the HGs of *Calothrix desertica* and *Chlorogloeopsis fritschii*, which could suggest the classification of the two cyanobacteria at least in the same subgroup. On the other hand, the HGs of *Chlorogloeopsis fritschii* and *Fischerella muscicola*, classified in the same family, have in common the unusually long aglycone chain (C_{32}). Finally, it should be also noted that the HGs of *Microchaete* sp. and *Tolypothrix tenuis* show a close similarity to those found in the Nostocaceae.

EXPERIMENTAL

General

For procedures see Ref. [6].

Organisms and culture conditions. Scytonema hofmanni (PCC 7110), *Tolypothrix tenuis* (PCC 7101), *Calothrix desertica* (PCC 7102), *Microchaete* sp. (PCC 7126) and *Chlorogloeopsis fritschii* (PCC 6912) were obtained from the Pasteur Culture Collection, while *Fischerella muscicola* (UTEX 1829) was kindly provided by Prof. C. P. Wolk, Michigan University. All cyanobacteria were enriched in BG11 medium plus 5 mM $NaHCO_3$ at pH 7.8 with the sole exception of *Fischerella* which was grown without $NaHCO_3$. They were grown large-scale at 25° into a 90 l fermentor with low mechanical agitation and an aeration flux of 30 ml min^{-1} using 70 l of $BG11_0$ (BG11 without combined nitrogen). The pH was monitored every 5 days and adjusted by fluxing CO_2 until the pH value was 7.5. Fermentor cultures were started with 1 l inoculum grown in media containing combined nitrogen, as described above. Illumination was provided continuously by four 18 W cool-white fluorescent tubes arranged parallel to the fermentor. Heterocyst formation and frequency were evaluated under a phase microscope (Zeiss) at 40× magnification and cultures were harvested at the maximum of heterocyst frequency by continuous flow on an Alfa-Laval model LAB 102B-20 centrifuge. The pellet was washed twice with an iso-osmotic saline soln, collected by centrifugation at 5000 *g* for 30 min and then lyophilized. The growth period varied between 15 days for *Scytonema* to 45 days for *Chlorogloeopsis* and *Calothrix*. Cell yields ranged between 4 g of dry wt for *Scytonema* to 17 g of dry wt for *Fischerella*.

Isolation of HGs. Lyophilized cells were extracted as previously reported [5] and glycolipids isolated using a combination of chromatographic techniques [5–7] leading to pure compounds which were characterized as previously described [5–8]. α-Mannosides were slightly more polar on silica gel chromatography than the corresponding α-glucosides.

Selected data for compounds of novel structural type

1-(*O*-α-D-glucopyranosyl)-29-keto-(3R,31R)-dotriacontanediol (**22**; isolated from *Chlorogloeopsis fritschii*). $[\alpha]_D$ +24.0 ($CHCl_3$–MeOH, 2:1; *c* 1.3). FABMS, *m/z* 675 $[M+H]^+$, 513 (cleavage of the glycosidic bond). NMR data (C_5D_5N). Aglycone moiety: δ ^1H 4.70 (*m*; H-31; overlapped with H-3 of glucose), 4.38 (*dt*, *J* = 9.9, 6.3; H-1a), 4.19 (*m*; H-3), 4.11 (*dt*, *J* = 9.7, 6.6; H-1b), 2.92 (*dd*, *J* = 15.3, 8.2; H-30a), 2.70 (*dd*, *J* = 15.3, 4.6; H-30b), 2.62 (*t*, *J* = 7.2; H-28), 1.45 (*d*, *J* = 6.2; H-32), 1.40 and 1.38 (methylene chain); δ ^{13}C 210.2 (C-29), 68.7 (C-3), 66.2 (C-1), 64.2 (C-31), 52.8 (C-30), 43.9 (C-28), 38.8 (C-4), 38.2 (C-2), 30.4, 30.3, 30.1, 29.7 (methylene chain), 26.4 (C-5), 24.4 (C-32), 24.0 (C-27). 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).

1-(*O*-α-D-mannopyranosyl)-29-keto-(3R,31R)-dotriacontanediol (**23**). FABMS, *m/z* 675 $[M+H]^+$, 513 (cleavage of the glycosidic bond). NMR data (C_5D_5N). Aglycone moiety, δ ^1H 4.41 (*dt*, *J* = 9.6, 6.3; H-1a), 4.15 (*m*; H-3), 4.03 (*dt*, *J* = 9.6, 4.0; H-1b), 2.92 (*dd*, *J* = 15.3, 8.2; H-30a), 2.70 (*dd*, *J* = 15.3, 4.6; H-30b), 2.62 (*t*, *J* = 7.2; H-28), 1.45 (*d*, *J* = 6.2; H-32), 1.40 and 1.38 (methylene chain); δ ^{13}C 210.2 (C-29), 68.4 (C-3), 65.4 (C-1), 64.2 (C-31), 52.8 (C-30), 43.9 (C-28), 38.8 (C-4), 38.5 (C-2), 30.4, 30.1, 29.9, 29.6 (methylene chain), 26.5 (C-5), 24.4 (C-27), 24.0 (C-32). Mannose moiety: δ ^1H 5.51 (*s*), 4.75 (*t*, *J* = 8.9), 4.6–4.7 (overlapping signals), 4.52–4.45 (overlapping signals); δ ^{13}C 102.1, 75.5, 73.3, 72.4, 69.4, 63.3. α-D-methylmannopyranoside: δ ^{13}C 102.8, 75.4, 73.2, 72.2, 69.2, 63.3, 54.7.

Hexaacetate of **23**. FABMS, *m/z* 927 $[M+H]^+$. NMR data ($CDCl_3$): Aglycone moiety, δ ^1H 5.26 (overlapped with H-2 of mannose; H-31), 4.96 (*m*; C-3), 3.73 (*dt*, *J* = 9.5, 6.1; C-1a), 3.47 (*dt*, *J* = 9.5, 6.5; C-1b), 2.77 (*dd*, *J* = 16.3, 7.3; H-30a), 2.52 (*dd*, *J* = 16.3, 5.9 H-30b), 2.40 (*t*, *J* = 7.3; H-28), 2.04, 2.0 (acetyl methyls), 1.26 (methylene chain). Mannose moiety: δ ^1H 5.32 (*dd*, *J* = 10.0, 3.2; H-3), 5.27 (*t*, *J* = 9.4; H-4), 5.23 (*bdd*; H-2), 4.79 (*bd*; H-1), 4.28 (*dd*, *J* = 12.2, 5.3; H-6a), 4.10 (*dd*, *J* = 12.2, 2.4; H-6b),

2.15, 2.09, 2.05, 1.99 (acetyl methyls). δ ^{13}C (whole molecule) 170.6, 169.9, 169.7, 97.8, 71.6, 69.5, 69.0, 68.5,67.1, 66.2, 65.2, 62.5, 48.5, 43.4, 34.4, 33.9, 29.7, 29.5, 29.1, 25.2, 23.6, 21.2, 20.9, 20.7, 20.0. α-D-methylmannopyranoside tetraacetate: δ ^{1}H 5.30 (*dd*, $J = 10.0$, 3.3; H-3), 5.26 (*t*, $J = 9.6$; H-4), 5.22 (*dd*, $J = 3.2$, 1.8; H-2), 4.72 (*d*, $J = 1.6$; H-1), 4.28 (*dd*, $J = 12.2$, 5.3; H-6a), 4.11, (*dd*, $J = 12.2$, 2.4; H-6b), 3.97 (*m*; H-5), 3.42 (*s*; CH_3), 2.15, 2.10, 2.05, 1.99 (acetyl methyls); δ ^{13}C 170.2, 169.6, 169.5, 169.4, 98.2, 69.1, 68.7, 68.0, 65.8, 62.1, 54.9, 20.4, 20.3, 20.2.

1-(*O*-α-D-mannopyranosyl)-(3R,25S,27R)-octacosanetriol (**8**). $[\alpha]_D$ +25 ($CHCl_3$–MeOH, 2:1; *c* 0.2). FABMS, *m/z* 621 $[M+H]^+$, 459 (cleavage of the glycosidic bond). NMR data (C_5D_5N). Aglycone moiety, δ ^{1}H 4.44 (*m*, H-25; overlapped with mannose signals), 4.40 (*dt*, $J = 9.8$, 6.3; H-1a), 4.23 (*m*; H-27), 4.16 (*m*; H-3), 4.03 (*dt*, $J = 9.6$, 7.1; H-1b), 2.0 (*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), 1.38 and 1.37 (methylene chain); δ ^{13}C 71.6 (C-25), 68.4 (C-3), 67.8 (C-27), 65.4 (C-1), 46.7 (C-26), 39.1 (C-24), 38.8 (C-4), 38.5 (C-2), 30.3, 30.1 (methylene chain), 26.4 (C-5), 26.2 (C-23), 24.8 (C-28). Mannose moiety: δ ^{1}H 5.50 (*d*, $J = 1.5$; H-1), 4.75 (*t*, $J = 9.0$; H-4), 4.7–4.6 (overlapping signals), 4.5–4.4 (overlapping signals). δ ^{13}C 102.0, 75.4, 73.3, 72.4, 69.4, 63.3.

Acknowledgements—We thank Prof. C. P. Wolk, University of Michigan, for a specimen of *Fischerella muscicola*. The 500 MHz NMR and MS were obtained from the ICMIB-NMR Service (Mr S. Zambardino) and from the Servizio di Spettrometria di Massa del CNR e dell'Università di Napoli respectively, the staff of both are gratefully acknowledged. This work has been supported by CNR and MURST. The Istituto per la Chimica M.I.B. is associated with the Istituto Nazionale per la Chimica dei Sistemi Biologici.

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