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POLY-3-HYDROXYALKANOATES FROM MARINE AND FRESHWATER CYANOBACTERIA

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Abstract—Polyesters of (*R*)-3-hydroxybutanoic and (*R*)-3-hydroxypentanoic acids have been isolated from *Aphanothece* species, a freshwater cyanobacteria The presence of similar polyesters from a number of marine cyanobacteria involved in blue-green algal mats and stromatolites is also documented

INTRODUCTION

Poly-β-hydroxybutyrate (PHB) is an important energy storage and carbon reserve product in a wide variety of bacteria [1]. Recently a polyester composed primarily of β-hydroxypentanoate and β-hydroxybutyrate units has been isolated from activated sludge [2]. We now report the presence of similar polyesters in a freshwater blue-green alga, *Aphanothece* sp., from Lake Joondalup, Western Australia, and in halophilic blue-green algae found in the algal mats and stromatolites of Shark Bay, Western Australia.

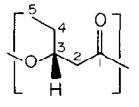
RESULTS

Polyesters in Aphanothece *sp*

In a study of the periphyton and metaphyton communities in Lake Joondalup, the largest permanent freshwater lake in the Swan coastal plain of Western Australia, Rose and McComb [personal communication] have observed the existence of a significant metaphyton mass [3]. The dominant algal species of the metaphyton, which ranged from 1 to 30 cm in depth, was found to be the colonial cyanophyte, *Aphanothece* sp. (family, Chroococcaceae; order, Chroococcales).

A sample of *Aphanothece* sp was freeze-dried and the material extracted with methylene chloride. The extract was washed with petrol and then ether to afford a resilient semi-translucent white film which was purified by elution with methanol through Sephadex LH-20. Combustion analysis yielded values consistent with the formula $(C_{14}H_{22}O_6)_n$. The polymeric nature of this material was suggested by its physical appearance, the wide mp range (100–205°), the formation of viscous solutions at moderate concentrations (10 mg/ml in chloroform) and the inability to elute this material through Si gel or alumina. Determination of the MW distribution by gel permeation chromatography (indirect calibration with polystyrene standards) gave the following approximate values: Z average MW, $\bar{M}_z$, 713 000; $\bar{M}_n$, 42 500; $\bar{M}_w$, 138 500; and dispersity, 3.26. The IR spectrum of the polymer showed an intense broad absorption band at 1740 cm^{-1} indicating the presence of carboxylic ester linkages. The ^1H NMR spectrum revealed resonances attributable to a 3-oxypentanoate (**1**) and a 3-oxybutanoate (**2**) system in a 2:1 ratio ^1H NMR measurements and a ^{13}C NMR spectrum provided evidence for these assignments (see Experimental).

Base hydrolysis of the polymer yielded an ether soluble fraction containing two compounds which from their spectral characteristics were identified as (*E*)-pent-2-en-1-oic acid and (*E*)-but-2-en-1-oic acid. Comparison of the GC and spectral characteristics of the derived methyl esters with those of authentic samples confirmed the assignment. Continuous extraction of the hydrolysate with ether afforded a mixture of two hydroxy acids which were separated as their methyl ester derivatives by prep. GC. Comparison with authentic samples showed them to be methyl-3-hydroxypentanoate and methyl-3-hydroxybutanoate The mixture of hydroxy acids showed



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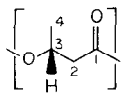

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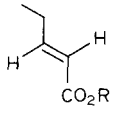

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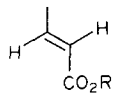

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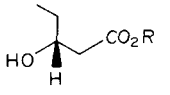

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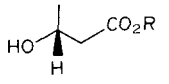

8 R = H
10 R = Me

$[\alpha]_D - 28°$ which is expected for a 2.1 mixture of (*R*)-3-hydroxypentanoic acid ($[\alpha]_D - 35°$) [4] and (*R*)-3-hydroxybutanoic acid ($[\alpha]_D - 25°$) [5]. The optical rotation of the corresponding methyl esters ($[\alpha]_D - 29°$, lit [6] $-18.6°$) and ($[\alpha]_D - 21°$, lit values for the enantiomer $[\alpha]_D + 23.8°$ [6]; $+33.7°$ [7]) confirmed the assignment of configuration

At this point it seemed likely that the polymer was in fact a heterpolymer consisting of (*R*)-3-hydroxypentanoic acid and (*R*)-3-hydroxybutanoic acid units in a ratio of 2 1. However, as described below, by further fractionation of this polymer the ratio of the two units could be increased to 3 1 indicating that the sample contained some poly-β-hydroxybutyrate

Polyesters from marine cyanobacteria

Blue-green algal mats and stromatolites are the dominant autotrophs of the hypersaline waters of Shark Bay, Western Australia [8] Algal mat extends from shallow subtidal depths (*ca* 4 m) to an elevation of *ca* 2 m above low water level and is differentiated into four basic types and three intergradational types that can be distinguished on the basis of surface texture and colour. Each mat type is colonized by a dominant cyanophyte [8] In connection with another project we had the opportunity of examining three of the seven types of algal mats (Table 1) for the presence of polyalkanoates

Samples of algal mats were frozen on collection and freeze-dried prior to extraction The freeze-dried powders were extracted with 10% methanol in methylene chloride and the material obtained was washed with petrol and ether to give white translucent films of polymers. The ^{1}H NMR spectra of the isolated polymers showed them to contain 3-oxybutanoate (methyl doublet at δ 1.28) and 3-oxypentanoate units (methyl triplet at δ 0.89) by comparison with the ^{1}H NMR spectrum of the polymer obtained from an *Aphanothece* sp as described above

The ratio of pentanoate to butanoate was different for each sample (Table 1) and for sample 2 pure PHB could be isolated by precipitation from chloroform with methanol The remaining polymer from sample 2, and those of samples 3 and 4, could be fractionated in a similar way to a final ratio of pentanoate to butanoate of 3 1 suggesting that a certain amount of PHB is present

DISCUSSION

The results presented above establish the presence of polyesters in cyanobacteria growing in vastly different environments, viz a permanent freshwater lake and a variable hypersaline intertidal zone It also seems clear that one of the polyesters is PHB, a chiral polymer which normally occurs as hydrophobic granules in the cells of a wide variety of bacteria [1] Only a few reports of its presence in cyanobacteria have appeared The occurrence and identification of PHB in the blue-green alga, *Chlorogloea fritschii*, after growth in the presence of acetate has been reported [9] No PHB could be detected after growth of *C fritschii* on an autotrophic medium in the absence of acetate, or growth of *Anabaena variabilis* in the presence or absence of acetate [9] PHB has been detected also in an *Oscillatoria* sp [10]

Whether the polyester containing the 3-hydroxypentanoate unit is a homopolymer of C_5 units or a heteropolymer made up of C_5 and C_4 units remains an open question One report [2] describes the isolation of a heteropolymer composed primarily of C_5 and C_4 3-hydroxy acids (probably in a ratio of *ca* 5 1), along with lesser amounts of higher MW components, from activated sludge, but there are indications for the presence of blocks of PHB in the heteropolymer [11]

In our work repeated attempts to remove PHB from the samples by selective precipitation did not improve the ratio of C_5 C_4 beyond 3 1 suggesting the presence of a heteropolymer On the other hand, the presence of PHB along with a poly-3-hydroxypentanoate polymer of different molecular sizes cannot be excluded.

Table 1 Polyesters from cyanobacteria

Sample	Locality	Form	Cyanophyte	Polymer yield (pentanoate–butanoate)
1	Lake Joondalup Perth	metaphyton	*Aphanothece* sp	0.2%* (2 1)
2	Hutchison Bay Shark Bay	pustular mat	*Microcoleus* sp	0.02%† (n d)
3	Hamelin Pool Shark Bay	smooth mat	*Schizothrix calcicola*,‡ *Symploca laete-viridis*, *Scytonema* sp	0.03%† (1 1)
4	Hamelin Pool Shark Bay	tufted mat	*Lyngbya aestuarii* *Microcoleus chthonoplastes*	0.03%† (1 4)
5	Hamelin Pool Shark Bay	pustular mat	*Entophysalis deusta*	0

*Yield based on wet wt

†Yield based on freeze dried wt of algal mat

‡Dominant cyanophyte

A search of the literature reveals that 3-hydroxypentanoic acid is an uncommon natural product but, in the polyesters from the cyanobacteria described in this report, it is more abundant than the ubiquitous 3-hydroxybutanoic acid. In passing, it is worth noting that the pentanoic acid forms part, as a 4-hydroxy-3-oxypentanoate moiety, of the aplysiatoxins and oscillatoxins present in some cyanobacteria [10].

Finally, given recent interest in the production and use of natural polymers [11–13], the occurrence of polyesters containing the (*R*)-3-hydroxypentanoate unit from a range of cyanobacteria is interesting and formally these polymers can be regarded as a source of chiral monomers [13].

EXPERIMENTAL

General exptal details are described in ref. [14].

Extraction of Aphanothece *sp.* A suspension of the blue-green algae in *ca* 20 l H_2O, collected from Lake Joondalup, 32 km N of Perth, Western Australia in 1979, was filtered through several layers of cheesecloth to yield a thick green-black biomass (200 g). Extraction of the freeze-dried biomass with CH_2Cl_2 yielded a dark green oil (2 g) which was partitioned into petrol soluble (1 4 g) and Et_2O soluble (0.1 g) fractions The residue (0 5 g) was dissolved in MeOH and eluted through a column of Sephadex LH-20 to afford fractions of the polymer (0.2% wet wt) The polymer was soluble in $CHCl_3$, but gave viscous solns at concns of 10 mg/ml or higher, and appeared as a resilient, semi-translucent white film which could readily be cut with scissors The mp range was from 100° to 205° [Found. C, 58 57, H, 7 76. $(C_{14}H_{22}O_6)_n$ requires C, 58 77, H, 7 75%] IR $\nu_{max}^{CHCl_3}$ cm^{-1}. 1740, ^1H NMR ($CDCl_3$; 80 MHz) δ 0 89 (*t*, J = 7 4 Hz, H_3-5), 1.63 (*dq*, $J_{3,4}$ = 6 3 Hz, $J_{4,5}$ = 7 4 Hz, H_2-4), 2 55 (AA'X 2H, *m*, H_2-2), 5 16 (*m*, H-3) for the 3-oxypentanoate unit, **1**, 1.28 (*d*, J = 6 3 Hz, H_3-4), 2 55 (2H, AA'X *m*, H_2-2), 5 23 (3H, *m*) for the 3-oxybutanoate unit, **2** ^{13}C NMR ($CDCl_3$, 20 1 MHz) δ 9 34 (*q*, C-5), 26 87 (*t*, C-4), 38 77 (*t*, C-2), 72.08 (*d*, C-3), 169.5 (*s*, C-1), for the 3-oxypentanoate unit, **1**, 19 81 (*q*, C-4), 40 94 (*t*, C-2), 67 75 (*d*, C-3), 169.97 (*s*, C-1) for the 3-oxybutanoate unit, **2**. Optical rotation $[\alpha]_{589}^{25}$ + 8 6°, $[\alpha]_{578}^{25}$ + 9 2°, $[\alpha]_{546}^{25}$ + 10 8°, $[\alpha]_{436}^{25}$ + 19 2°, $[\alpha]_{365}^{25}$ + 30 3° ($CHCl_3$, *c* 0 4).

Hydrolysis of polymer from Aphanothece *sp* A suspension of polymer (100 mg) in EtOH was treated with 2 N NaOH and the mixture heated under reflux for 2 hr in a N_2 atmosphere Extraction of the acidified mixture with Et_2O afforded a 2 1 mixture (40 mg, 40%) of (*E*)-pent-2-en-1-oic acid (**3**) and (*E*)-but-2-en-1-oic acid (**4**) ^1H NMR ($CDCl_3$, 80 MHz) δ 1 09 (*t*, = 8 Hz, H_3-5), 2 29 (*ddq*, $J_{4,5}$ = 8 0 Hz, $J_{3,4}$ = 6 0 Hz, $J_{2,4}$ = 2.0 Hz, H_2-4), 5 86 (*dt*, $J_{2,3}$ = 16 3 Hz, $J_{2,4}$ = 2 0 Hz, H-2), 7 23 (*dt*, $J_{2,3}$ = 16 3 Hz, $J_{3,4}$ = 6 0 Hz, H-3) for the (*E*)-pent-2-en-1-oic acid part, and δ 1 93 (*dd*, $J_{3,4}$ = 7 5 Hz, $J_{2,4}$ = 2 0 Hz, H_3-4), 5 86 (*dt*, $J_{2,3}$ = 16 3 Hz, $J_{2,4}$ = 2 0 Hz, H-2), 7 23 (*dt*, $J_{2,3}$ = 16 3 Hz, $J_{3,4}$ = 7 5 Hz, H-3) for the (*E*)-but-2-en-1-oic acid part ^{13}C NMR ($CDCl_3$, 20 1 MHz) δ 12 07 (*q*, C-5), 25 51 (*t*, C-4), 120 78 (*d*, C-2), 153 73 (*d*, C-3), 172 06 (*s*, C-1) due to **3** and 18 05 (*q*, C-4), 122 47 (*d*, C-2), 147 52 (*d*, C-3), 172 34 (*s*, C-1) due to **4** These values are in good agreement with lit. values [16, 17]

A portion of the mixture of **3** and **4** was treated with CH_2N_2 to yield the corresponding Me esters **5** and **6**, IR ν_{max}^{film} cm^{-1} 1720, UV λ_{max}^{EtOH} nm 203 (ε 1400), 231 (ε 800). GC analysis (10% Carbowax 20M, 2 0 mm × 3 0 m glass column, 100° isothermal) and GC/MS analysis (Carbowax 20M, 0 3 mm × 25 m glass capillary column, 80° at 20°/min) showed the esters to be identical with Me but-2-enoate (**6**), R_t 4.3 min, MS(CI_{CH_4}) *m/z* 101 [M + 1]$^+$ (2%), 87 (100), 69 (15) and Me pent-2-enoate (**5**), R_t 5.0 min, MS (CI_{CH_4}) *m/z* 115 [M + 1]$^+$ (1%), 101 (100), 83 (18) A sample of **6** was obtained by CH_2N_2 treatment of (*E*)-but-2-en-1-oic acid and a sample of **5** was obtained by similar treatment of (*E*)-pent-2-en-1-oic acid prepared as described in the lit [15].

Continuous extraction of the aq layer with Et_2O over 24 hr yielded a 2.1 mixture (45 mg, 45%) of (*R*)-3-hydroxypentanoic acid (**7**) and (*R*)-3-hydroxybutanoic acid (**8**), $[\alpha]_D$ − 28° ($CHCl_3$, *c* 4 0) ^1H NMR ($CDCl_3$, 80 MHz) δ 0 95 (*t*, J = 7 0 Hz, H_3-5), 1.50 (*m*, H_2-4), 2 49 (*m*, H_2-2), 3 98 (*m*, H-3), 7.09 (*br s*, D_2O exchangeable) for **7** and 1 23 (*d*, J = 8 0 Hz, H_3-4), 2 49 (*m*, H_2-2), 4 20 (*m*, H-3), 7.09 (*br s*, D_2O exchangeable) for **8** ^{13}C NMR ($CDCl_3$, 20 1 MHz) δ 9 74 (*q*, C-5) 29 49 (*t*, C-4) 40 82 (*t*, C-2), 69.63 (*d*, C-3), 177 3 (*s*, C-1) for **7**, 22 49 (*q*, C-4), 42.70 (*t*, C-2), 64.51 (*d*, C-3), 177.5 (*s*, C-1) for **8**.

Characterization of hydroxy acids **7** *and* **8** A mixture of the two hydroxy acids obtained by hydrolysis of the polymer was treated with CH_2N_2 to yield the corresponding Me esters. These were separated by prep GC (15% Carbowax, 5 0 mm × 1.0 m glass column, 95° isothermal) to yield (a) Me (*R*)-3-hydroxybutanoate (**10**), R_t 6 0 min, $[\alpha]_D$ − 21° ($CHCl_3$, *c* 0 1) (lit for *S*-enantiomer $[\alpha]_D$ + 23.8° [6], $[\alpha]_D$ + 33 7° [7]). (Found. [M − Me]$^+$ 103 040. $C_4H_7O_3$ requires 103 040.) ^1H NMR ($CDCl_3$; 80 MHz) δ 1.23 (*d*, J = 6 0 Hz, H_3-4), 2 50 (*m*, H_2-2), 2 90 (*br s*, D_2O exchangeable, OH), 3 71 (*s*, OMe), 4 22 (*m*, H-3). MS (EI) *m/z* 103 [M − Me]$^+$ (31%), 100 (4), 87 (28), 74 (100), 71 (58), 69 (6), 59 (17), 45 (46), 43 (78). MS(CI_{CH_4}) *m/z* 119 [M + 1]$^+$ (68%), 101(100), 87(70). (b) Me (*R*)-3-hydroxypentanoate (**9**), R_t 9 0 min, $[\alpha]_D$ − 29° ($CHCl_3$, *c* 0 3) (lit [6] $[\alpha]_D$ − 18 6°) (Found [M − C_2H_5]$^+$ 103 040 $C_4H_7O_3$ requires 103 040) ^1H NMR ($CDCl_3$, 80 MHz) δ 0 97 (*t*, J = 7 0 Hz, H_3-5), 1 49 (*m*, H_2-4), 2 51 (*m*, H_2-2), 2.84 (*br s*, D_2O exchangeable, OH), 3 72 (*s*, OMe), 3 95 (*m*, H-3). MS(EI) *m/z* 114 [M − H_2O]$^+$ (4%), 103 (85), 101 (13), 83 (11), 74 (79), 71 (100), 59 (53), 43 (68); MS(CI_{CH_4}) *m/z* 133 [M + 1]$^+$ (18%), 115 (100), 101 (20), 83 (11) The GC (10% Carbowax 20 M, 2 0 mm × 3 0 m glass column, 130° isothermal) and spectral characteristics of the two esters were identical with those of authentic samples of racemic mixtures

Me 3-hydroxypentanoate was obtained by treatment of pent-2-en-1-oic acid with NaOH followed by methylation with CH_2N_2. Similar treatment of but-2-en-1-oic acid yielded Me 3-hydroxybutanoate

Determination of MW distribution of polymer. The equipment used was a Waters Associates ALC/GPC Model 244 consisting of a Model U6K injector, Model 6000A solvent delivery system and utilizing the Model 401 differential refractometer and Model 440 absorbance detector A Microbondagel E Linear Column (separation range 2000–2 000 000) was used with THF as mobile phase The polymer sample was prepared as a 0 25% (w/v) soln in $CHCl_3$, filtered through a 0 45 μm Millipore filter and then 20 μl of the soln was injected into the column fitted with an in-line pre-column filter (2 μm). Data recording and integration was performed on a Model 730 Data Module fitted with a GPC option The *Q*-factor method for indirect calibration was used, *Q* was determined for polystyrene samples

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