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Low molecular weight oligo-ß-hydroxybutyric acids and 3-hydroxy-N-phenethyl-butyramide – new products from microorganisms*

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Summary

A new group of low-molecular weight channel-forming oligo(hydroxybutyric acids) (cPHBs, **1** with n = 8-30; main component MW ≈ 1300 dalton) was isolated from microorganisms of different origin. Inclusion bodies were electron-microscopically visible in cells in the state of autolysis, not in cells in the exponential phase of growth. cPHB and high-molecular poly(ß-hydroxybutyric acid) (sPHB) is cleaved by phenylethylamine and forms the corresponding monomeric hydroxybutyramide and – under drastic conditions, the crotylamide. One of these compounds, the 3-hydroxy-N-phenethyl-butyramide (**5**), was isolated as a new natural product now.

Introduction

Beside the peptides, polysaccharides and the nucleic acids, the poly-(ß-hydroxyalkanoates) are forming another group of biopolymers of increasing importance. Among these, poly-(R)-ß-hydroxybutyric acid **1** is the most important one, and well-known as a biologically degradable and environmentally benign plastic material [1]. In many microorganisms, **1** serves as energy reservoir and forms there electron-microscopically visible so-called inclusion bodies [2]. This type of PHB is normally named as sPHB (s stands for "storage"). The molecular masses of the polymers depend strongly on the producing microorganisms and the condition of their cultivation.

* Marine Bakterien, XVII. XVI: R. P. Maskey, R. N. Asolkar, E. Helmke, and H. Laatsch, Chalcomycin B, a new antibiotic from a marine Streptomyces *sp.* B7064. J. Antibiot., submitted 2002

Values between 100.000 and 800.000 are typical, however, also higher masses were reported. The commercial BIOPOL™ product is, nowadays, a co-polymer of 3-hydroxybutyric acid and 3-hydroxypentanoic acid and forms a white powder with only low solubility in organic solvents.
Additionally to the known high-polymeric storage-PHB (sPHB), we isolated now a new group of readily soluble short-chain oligomers **1** (n = 8-30) from microorganisms of varying origin. Also the first example of a rare amide type, the 3-hydroxy-N-phenethyl-butyramide (**5**) was found.

Experimental

Strains and fermentation: Cytophaga marinoflava strain AM13.1 and *Alteromonas distincta* Hel69 were isolated from sea water at the isle of Helgoland and are kept in the culture collection of the Gesellschaft für Biotechnologische Forschung (Brunswig). The marine Streptomycetes B7700, B7835 and B8894 were obtained from the collection of the Alfred-Wegener-Institut für Polar- und Meeresforschung in Bremerhaven (Germany), the terrestrial Streptomycetes GW97/1541, GW62/2497 und GW2/577 were obtained from the strain collection of bioLeads, Heidelberg (Germany).
The marine Streptomycetes were cultured in M_2^+ medium [3], terrestrial Streptomycetes in M_2 medium [4], *Alteromonas distincta* Hel69 and *Cytophaga marinoflava* AM13.1 in LB medium [5] with 50 % artificial sea water [6] and 5 g/l glucose. Fermentation and work-up was done as previously described [6].

Oligo-(ß-hydroxybutyric acid (**1**, n = 10 - 22): The crude ethyl acetate extract from a 20 l fermentation of GW62/2497 was triturated with CH_2Cl_2/50 % MeOH and the white solid (1.2 g **1**-mixture, n = 11 – 22) was filtered off. In the same way, **1** (n = 8-30, 122 mg) was obtained from a 20 L-culture of strain GW 2/577. – IR (KBr): ν = 2978, 2935, 1724, 1458, 1382, 1280, 1226, 1187, 1132, 1100, 1055, 978 cm^{-1}. – ^1H NMR ($CDCl_3$, 200 MHz): δ = 5.26 (sext, J = 6.0 Hz, CH), 2.61 (dd, ^2J = 15.5 Hz, ^3J = 7.6 Hz, CH_2, H_A), 2.48 (dd, ^3J = 15.5 Hz, ^3J = 5.8 Hz, CH_2, H_B), 1.27 (d, J = 6.0 Hz, CH_3). – (+)-ESI-MS: *m/z (%)* = 987 ($[M^1 + Na]^+$, 54), 1073 ($[M^2 + Na]^+$, 82), 1159 ($[M^3 + Na]^+$, 100), 1245 ($[M^4 + Na]^+$, 68), 1331 ($[M^5 + Na]^+$, 48), 1417 ($[M^6 + Na]^+$, 37), 1503 ($[M^7 + Na]^+$, 26), 1589 ($[M^8 + Na]^+$, 14), 1675 ($[M^9 + Na]^+$, 6), 1761 ($[M^{10} + Na]^+$, 3), 1847 ($[M^{11} + Na]^+$, 1), 1933 ($[M^{12} + Na]^+$, 0.5). – (-)-ESI-MS: *m/z (%)* = 963 ($[M^1 - H]^-$, 42), 1049 ($[M^2 - H]^-$, 54), 1135 ($[M^3 - H]^-$, 99), 1221 ($[M^4 - H]^-$, 82), 1307 ($[M^5 - H]^-$, 100), 1393 ($[M^6 - H]^-$, 82), 1479 ($[M^7 - H]^-$, 84), 1565 ($[M^8 - H]^-$, 66), 1651 ($[M^9 - H]^-$, 48), 1737 ($[M^{10} - H]^-$, 42), 1823 ($[M^{11} - H]^-$, 22), 1909 ($[M^{12} - H]^-$, 5).

sPHB (**1**, n > 50,000): A 25 l-fermentation of *Cytophaga marinoflava strain* AM13.1 afforded on extraction with ethyl acetate 9.0 g crude extract. On trituration with MeOH 76 mg PHB remained undissolved as a white solid. Crude extracts of 20 l-cultures of *Streptomyces* sp. B7700, 50 l *Streptomyces* sp. B8894 and 25 l *Alteromonas distincta* Hel69 yielded on dissolving in acetonitril/water (azeotrop) or CH_2Cl_2/50% MeOH 13 mg, 1.92 g and 156 mg PHB, respectively, as white solids. The crude extract of 20 l GW2/577 culture gave 122 mg sPHB. – IR (KBr): ν = 3442, 2924, 1736, 1458, 1282, 1057, 670 cm^{-1}. – ^1H and ^{13}C NMR data were identical with those of the cPHBs.

Methylation of cPHB (**1**, n = 10-22): To a solution of 10 mg cPHB in 5 ml CH_2Cl_2 an excess of etherial diazomethane solution was added and the mixture brought to dryness after 5 min. – ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 5.26 (sext, ^3J = 6.0 Hz, CH), 3.66 (s, OCH3), 2.60 (dd, 15.6 Hz, ^3J = 7.3 Hz, CH_2, H_A), 2.48 (dd, 15.6 Hz, ^3J = 5.9 Hz, CH_2, H_B), 1.27 (d, J = 6.3 Hz, CH_3).

3-Hydroxy-N-phenethyl-butyramide (**5**): The defatted crude extract (3.2 g) from a 25 l fermentation (M_2 medium, pH 6.50 ± 1.25) of a terrestrial Streptomycete strain GW 2/577 was chromatographed on Sephadex LH-20 (3 × 120 cm, MeOH) and separated under TLC control into four fractions. The second fraction (1.67 g) was further separated on Sephadex LH-20 (3 × 60 cm, MeOH) into four sub-fractions. The sub-fraction 3 (420 mg) on purification over RP18 HPLC (gradient: MeCN/10 % H_2O to 100 % MeCN in 25 min) gave 2.5 mg of **5** as colourless powder that gave a pink colouration with anisaldehyde/sulphuric acid. – ^{1}H NMR ([D_6]acetone, 300 MHz): δ = 7.24 (m, 5 H, Ph), 4.03 (AB*X*, 1 H, 3''-H), 3.42 (m, 2 H, 1'-H_2), 2.79 (t, ^3J = 7.5 Hz, 2 H, 2'-H_2), 2.24, 2.19 (*AB*X, J_{AB} = 14.7 Hz, J_{AX} = 4.2 Hz, J_{BX} = 8.0 Hz, 2 H, 2''-H_2), 1.09 (d, ^3J = 6.0 Hz, 3 H, 4''-H_3). – ^{13}C NMR ([D_6]acetone, 300 MHz): δ = 172.3 (C-1''), 138.8 (C-1), 128.7 (C-2, C-6), 128.6 (C-3, C-5), 126.4 (C-4), 64.8 (C-3''), 43.9 (C-1'), 40.4 (C-2'), 35.5 (C-2''), 22.8 (C-4''). – EI-MS (70 eV): *m/z* (%) = 207 (M^+, 83), 192 ($[M-CH_3]^+$, 7), 116 (43), 104 ($PhCH=CH_2^+$, 100), 91 (24), 69 (21), 59 (17), 43 (28). – HREI-MS (70 eV): calcd. for $C_{12}H_{17}NO_2$ 207.1259, found 207.1259.

Synthesis of **5**: Phenylethylamine (120 mg; 1 mmol) and BuLi (0.4 ml solution in *n*-hexane, 1 mmol) was dissolved in 20 ml of dry ether and a solution of PHB (86 mg, ≈1 mmol of 3-HB) in 20 ml of dry ether was added dropwise. The reaction mixture was kept for 12 h under N_2 in an ice bath and washed three times with 50 ml of 0.2 N hydrochloric acid. PTLC (20 × 20 cm, CH_2Cl_2/5 % MeOH) gave 55 mg (26 %) colourless **5**, which was identical in all respects with the natural product **5**.

Crotonic acid β-phenylethyl amide (**6**): A mixture of 242 mg (2 mmol) of ß-phenylethyl amine and 220 mg (≈ 2.5 mmol of 3-HB) of PHB gave on boiling for 1 min a gel-like product which was dissolved in 50 ml of ethyl ether and the excess of amine was extracted three times with each 50 ml of 0.2 N hydrochloric acid. Column chromatography of the etherial residue on Sephadex LH-20 (methanol) and PTLC ($CHCl_3$/5% CH_3OH) afforded 60 mg (16 %) of **6** as a colourless powder, m.p. 77-79 °C – ^{1}H NMR ([D_6]acetone, 200 MHz): δ = 7.24 (m, 5 H, Ph), 6.76 (dq, ^3J = 15.4 Hz, ^3J = 6.8 Hz, 1 H, 3''-H), 5.96 (dq, ^3J = 15.4 Hz, ^4J = 1.7 Hz, 1 H, 2''-H), 3.45 (m, 2 H, 1'-H_2), 2.79 (t, ^3J = 7.5 Hz, 2 H, 2'-H_2), 1.78 (dd, ^3J = 6.8 Hz, ^4J = 1.7 Hz, 3 H, 4''-H_3). – ^{13}C NMR ([D_6]acetone, 300 MHz): δ = 165.9 (C-1''), 139.9 (C-3''), 138.9 (C-1), 128.7 (C-2,6), 128.6 (C-3,5), 126.4 (C-4), 124.9 (C-2''), 40.5 (CH_2-1'), 35.6 (CH_2-2'), 17.7 (CH_3-4''). – EI-MS (70 eV): *m/z* (%) = 189 (M^+, 63), 105 (18), 104 (76), 98 (35), 91 (17), 69 (100), 65 (5), 41 (6).

Results and discussion

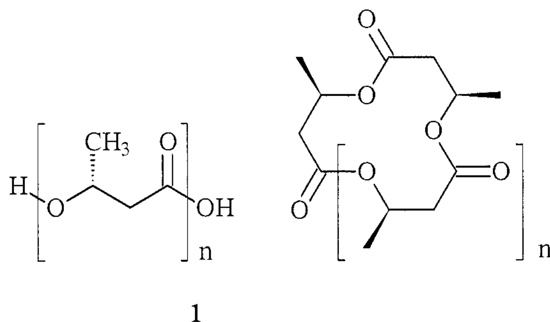
From the crude ethyl acetate extracts of several marine and terrestrial bacteria (Hel69 and *Streptomycetes* GW62/2497, GW2/577, and B8894) white solids were precipitated by treatment with methanol or acetonitril/water. They gave voluminous gels with chloroform or dichloromethane which went only slowly into solution and gave ductile foils on drying.

The ^{1}H NMR spectra of all these samples were nearly identical and showed sharp signals with the relative intensities of 1 at $\delta = 5.2$ (sext), 2.6 (dd), 2.5 (dd), and a doublet of the intensity 3 at $\delta = 1.3$ (for a figure see [7]). The ^{13}C NMR spectra showed three signals at $\delta = 67.6$ (CH-O), 40.8 (CH_2-sp^2-C) and 19.8 (CH_3), and a carbonyl signal at $\delta = 169.1$, corresponding to the partial structure $-(O-CHMe-CH_2-CO)_n-$ (fragment mass = 86 Da).

The ESI and MALDI-TOF mass spectra of the product from a terrestrial *Streptomycete* GW62/2497 displayed the typical signal pattern as for a mixture of oligomers with molecular masses of $(n \times 86) + H_2O + Na^+$ (fig. 1a). Correspondingly, the product was a mixture of oligo-(ß-hydroxybutyric acid) (**1**, cPHB) with n = 10-22 (GW62/2497) or n = 8-30 (GW2/577). Accordingly, treatment with diazomethane gave cPHB methyl ester which exhibited a methoxy signal at $\delta = 3.66$ in the ^{1}H NMR spectrum. The intensity of the methoxy signal indicated an average chain length of cPHB (**1**) to be $n \approx 15$ units (GW62/2497). From the North Sea bacterium *Cytophaga marinoflava* strain AM13.1 and several other strains not mentioned here, we isolated a sPHB mixture (**1**) with n > 2.500. This polymer resembled the sPHB from *Alcaligenes eutrophus*, yet did not contain 3-hydroxypentanoic acid. Also in most other bacterial samples, either high *or* low molecular weight PHBs were found. According to the intensity of the methoxy signal and the mass spectra, the sample from another Streptomycete GW2/577 was, however, a mixture of cPHB (n = 8-30) and high-molecular weight polymers (MW > 50.000, MALDI-TOF). Beside from special feeding experiments, only isotactic polyhydroxybutyric acids with *all*-(R)-configuration have been reported [2]. We assume therefore also an (R)-configuration in our oligomers.

Low molecular weight PHBs were often found as constituents of prokaryotic and eukaryotic cell membranes, however, had sizes of 100-200 units [7]. The function of these oligomers is still in debate, concerning e.g. the formation of ion channels or the stabilization of proteins. This group of polymers is therefore named as cPHB (c for "channel-forming"). To the best of our knowledge, such short ß-hydroxybutyric acid oligomers as described now have not been isolated from natural sources before. They have just a chain length corresponding to the thickness of a cell membrane, and their pore or channel forming capabilities might be of interest.

It should be mentioned that two related butyrolides are known from nature, the cyclodimeric DG-1 [8] **2** (n = 0) from the Ascomycete *Diplogelasinospora grovesii*, and pinnatifolide [9] **2** (n = 1) from the red alga *Laurencia pinnatifida* Lamour. The latter is also obtained in good yield by acid treatment of PHB [2]. Higher oligolides or open-chain oligomers in the range up to 100 units have been obtained by total synthesis or by acid-catalyzed transesterification of **1** [2].



The mass spectrometric analysis showed typical differences between these two groups of high polymeric (sPHB) and very low oligomeric hydroxybutyric acids (cPHB): The oligomers (**1**, n = 10-30) gave clear ESI, FAB, and MALDI-TOF mass spectra with the characteristic pattern of a polymer distribution (Fig. 1a). The polymeric PHB, however, did not show any molecular signal below 200.000 Da in the ESI or MALDI-TOF mass spectra, due to molecular weights out of the measurable range. On CI or EI ionization, both groups gave signals of decreasing intensities with $\Delta m = 86$ (Fig. 1b). Obviously, a fragmentation of the chains by loss of butyrolides **2** with increasing ring size or by formation of open-chain compounds with a terminal crotyl residue occurs.

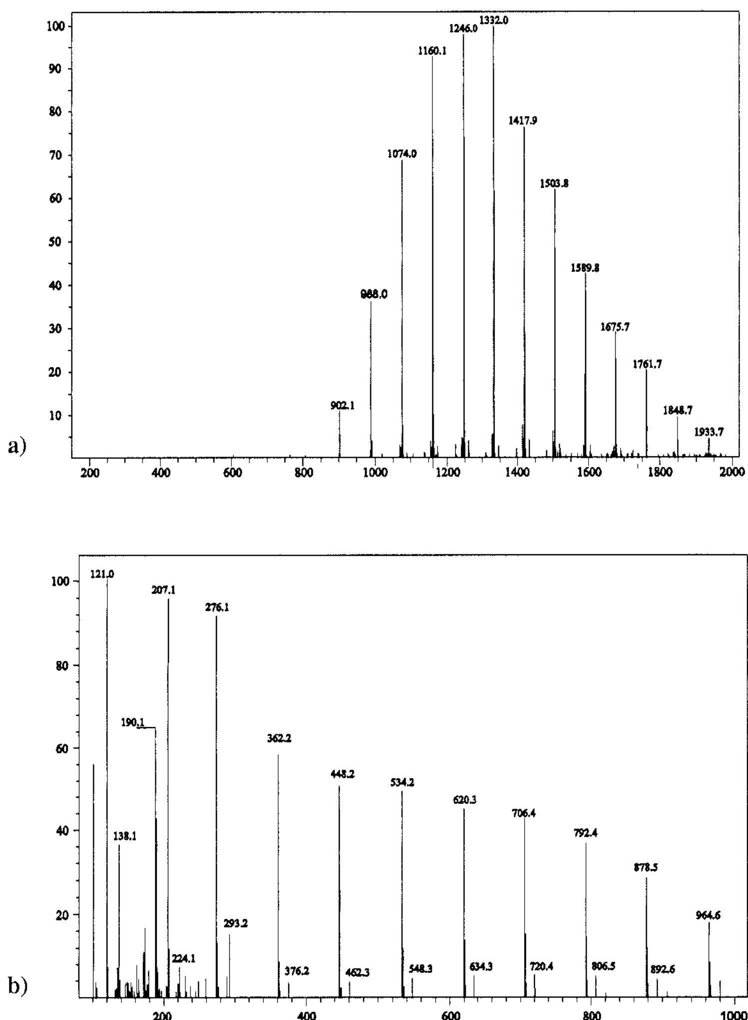
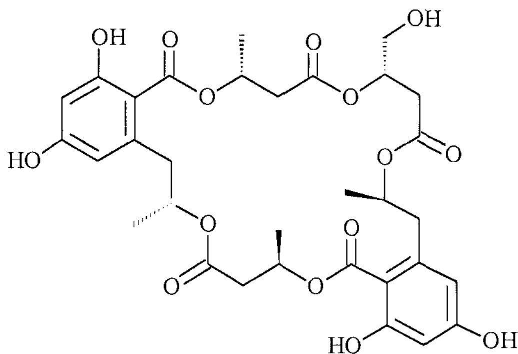


Fig. 1: a) ESI mass spectrum of cPHB (**1**, n = 10-22) from a chloroform solution and b) DCI mass spectrum (reactand gas NH_3) of sPHB (**1**, n > 50,000).

Biosynthetically, sPHB is formed on the surface of the inclusion bodies by chain

elongation. It is unlikely therefore that our cPHBs are intermediates of the sPHB formation. Also an origin during work-up by partial hydrolysis of the sPHBs of the bacteria is less plausible, as the products were detectable in freshly harvested cultures and did not have any unphysiological acid contact. They differ also clearly in their molecular weight distribution from partial hydrolysates obtained by acid-treatment of sPHB.

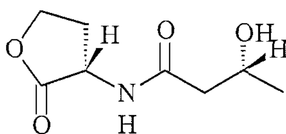
In the *Streptomycete* B7835 inclusion bodies were electronmicroscopically visible, and correspondingly sPHB was found. The cPHB producing Strain GW62/2497, however, did not show inclusion bodies in the exponential phase of growth. Only in deteriorating cells, particles resembling inclusion bodies could be detected. They might be products of phase separation, containing cPHB. This is the first example that bacteria are found to produce cPHBs and not to contain inclusion bodies and it is again a hint that low-molecular weight cPHBs may have another function than as storage material. The small oligomers may play a role in the complexation of calcium or be involved in the formation of channels [2]. The real physiological importance of these compounds, however, remains still open.



3

ß-Hydroxybutyric acid amides

Bacteria may contain up to 80 % PHB of their dry weight [10]. Although they are obviously abundant molecules, nature makes little use of ß-hydroxybutyric acid for the synthesis of other metabolites. Less than 70 out of more than 25.000 compounds from microorganisms contain HBA derivatives: 30 are esters with one or more HB units, e.g. the antifungal antibiotic BK-223A (**3**), also of interest as potentiator of nerve growth factor [11]. Most of the other compounds are siderophores, where ß-hydroxybutyric acid is bound *via* an amide bond to a peptide unit. Only one compound is a simple amide, the homoserine lactone II (**4**) [12]. We have now isolated another amide **5** with ß-phenylethyl amine from the terrestrial Streptomycete GW2/577.



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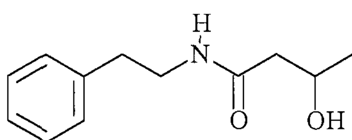

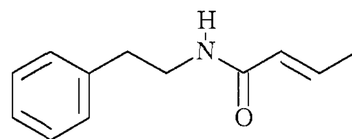

5: **6**

The structure of the amide was determined by means of spectroscopic methods. Synthetically it should be easily accessible by treatment of PHB with ß-phenylethyl amine. At moderate temperature (120 °C, toluene), however, no reaction occurred, and in boiling amine the crotyl-phenylethylamide (**6**) was formed. We obtained **5** finally by cleavage of sPHB with the corresponding lithium amide. We have not found any biological activity of **5** against bacteria, fungi, or algae.

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3. M_2^+ medium: 10 g malt extract, 4 g yeast extract and 4 g glucose in 0.5 l artificial sea water and 0.5 l tap water. The solution was adjusted to pH 7.8 with 2 N NaOH before autoclaving
4. M_2 medium: A solution of 10 g malt extract, 4 g yeast extrakt and 4 g glucose in 1 l tap water was adjusted with 2 N NaOH to pH 7.8 and autoclaved
5. LB medium: A solution of 10 g trypton, 5 g yeast extrakt and 10 g NaCl in 1 l tap water
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