# Sequencing Microbial Copolymers of 3-Hydroxybutyric and 3-Mercaptoalkanoic Acids by NMR, Electrospray Ionization Mass Spectrometry, and Size Exclusion Chromatography NMR

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Copolymers of 3-hydroxybutyrate (3HB) and 3-mercaptopropionate (3MP) or 3-mercaptobutyrate (3MB) units and minor amounts of 3-hydroxypropionate (3HP), 3-hydroxyvalerate (3HV), or 3-mercaptovalerate (3MV) were investigated regarding their microstructure by NMR, electrospray ionization mass spectrometry, and size exclusion chromatography NMR. These copolymers were produced by *Ralstonia eutropha* strain H16 when cells were cultivated in a mineral salts medium with gluconate as a carbon source for growth and 3MP or 3MB as precursor substrates for incorporation of 3-mercaptoalkanoates. Mass spectrometry analysis of partially methanolyzed or pyrolyzed samples proved the presence of true copolymers or terpolymers. $^{13}C$ NMR spectroscopy of intact polymer samples, with values of average block length and degree of randomness deviating from a random sequence model, suggested microblock structures; however, composition analysis by $^{1}H$ NMR of fractions obtained by size exclusion chromatography showed significant variations with molecular weight, revealing the presence of blends of poly(3HB-*co*-3MP-*co*-3HP) or poly(3HB-*co*-3MB) with poly(3HB). The experimental NMR carbonyl dyad signal intensities were satisfactorily matched by a random sequence model when the presence of poly(3HB) was taken into account.

## Introduction

Thermoplastic biopolymers represent an interesting group of environmentally friendly materials for various technical applications, considering in particular their origin from renewable resources.[1] Polyhydroxyalkanoates (PHAs) are cytoplasmatic water-insoluble storage compounds in prokaryotes and have attracted much interest over the past few decades, because they can be molded and processed into different devices such as compostable packaging materials or resorbable materials for medical applications.[2] The key enzymes of PHA biosynthesis, the PHA synthases, are well-known for their low substrate specificity, which is illustrated by the large number of different PHA constituents found in microbial PHAs.[3] Recently, the range of polymers produced by PHA synthases has been extended to a new dimension: 3-Mercaptoalkanoates (3MAs), which are first intracellularly converted to their activated coenzyme A derivatives, can also be polymerized by PHA synthases, as has been demonstrated for 3-mercaptopropionate (3MP), 3-mercaptobutyrate (3MB), and 3-mercaptovalerate (3MV).[4−7] Employing PHA-accumulating bacteria such as *Ralstonia eutropha*, which typically accumulates poly(3-hydroxybutyrate) (poly(3HB)) and PHAs consisting of other short carbon chain length hydroxyalkanoates (HAs), and distinct fermentation techniques, copolymers with various 3MA compositions can be obtained revealing interesting physical and material properties.[8] These were the first described biopolymers containing thioester linkages in the polymer backbone, and they were therefore referred to as polythioesters (PTEs) representing a separate new class of biopolymers. Most intriguing are the biodegradation properties of PTEs: Although traces of 3MP have been found as alleged poly(3HB-*co*-3MP) hydrolysis products of the PHA depolymerase of *Ralstonia pickettii* T1,[9] detailed studies on poly(3HB-*co*-3MP) biodegradation revealed that only the oxoester but not the thioester bonds were enzymatically hydrolyzed.[10] This was, for example, concluded from the abundant presence of 3MP oligomers comprising 7−16 units in the remaining water-insoluble fraction after biodegradation of the copolymers.[10] After PTE homopolymers became available,[11,12] the biodegradability of PTEs was further investigated with great effort employing various techniques and experimental continuance.[13] Astoundingly, poly(3MP) has been shown to be persistent in numerous environments, and the non-biodegradability of poly(3MP) represents a unique feature of a biopolymer.[13,14] Although the reason for the non-biodegradability of poly(3MP) remains to be elucidated, a relation to other unique physical properties of PTEs, such as thermal stability or extremely poor solubility in various solvents, seems obvious.[15] Therefore, information on the composition and microstructure of PTEs and in particular of copolymers consisting of 3HB and mercaptoalkanoic acids

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might be of high value for the understanding of the unique characteristics of PTEs such as environmental persistence.

Since the physicochemical and biodegradability properties of PTE copolymers depend both on comonomer composition and sequence distribution of repetitive units, it is important to determine this latter parameter also. Previous papers reported that PTEs possess a somewhat blocky structure, based on NMR,[4] differential scanning calorimetry (DSC),[15] and matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS)[10] evidence. This was unexpected, because in the case of PHA copolymers it was shown by $^{13}C$ NMR dyad and triad analysis[16−20] that some of the bacterially synthesized PHAs are random copolymers, whereas others are mixtures of random copolymers with different compositions.[18,21] Additional information can be obtained by applying the method of Yamadera and Murano[22] to the intensities of carbonyl dyads and triads in $^{13}C$ NMR spectroscopy. However, NMR can hardly discern beyond the triad level, and the distinction between a single copolymer and a mixture of copolymers is sometimes too difficult.[18] Mass spectrometry is able to look at the masses of individual molecules in a mixture and is therefore an interesting alternative to NMR spectroscopy. Soft ionization techniques in mass spectrometry such as fast atom bombardment (FAB), electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), which enable the production of gas-phase molecular ions from neutral natural or synthetic macromolecules, can be adopted.[23]

We have previously described how microbial poly(3HB-*co*-3HV)[24,25] and PHAs consisting of medium chain length 3-hydroxyalkanoates, which are referred to as poly(3HA$_{mcl}$),[26−28] can be analyzed for the determination of the sequence distribution of repetitive units by FAB-MS,[24−26] MALDI-MS,[27] or ESI-MS[28] of oligomers obtained by partial methanolysis or partial pyrolysis of copolymers. In this paper we present an integrated study for the structural characterization and sequencing of some microbial PTEs by using NMR spectroscopy for non-degraded samples, ESI-MS of the oligomers generated by partial degradation, and $^1H$ NMR analysis of size exclusion chromatography fractions (SEC-NMR).

## Materials and Methods

**Production of Microbial Polymers.** Different poly(3HB-*co*-3MP) and poly(3HB-*co*-3MB) samples were obtained from *R. eutropha* H16 (DSM 428). The cells were grown in a mineral salts medium with sodium gluconate or fructose as a carbon source for growth and with 3-mercaptopropionate or 3-mercaptobutyrate as a second carbon source and precursor substrate for the incorporation of mercaptoalkanoates. The cells were cultivated exactly as described previously.[8] Polymers were extracted from lyophilized cells with chloroform, filtered, precipitated in 10 volumes of ethanol, and dried under a constant air stream. The precipitation procedure was repeated three times to obtain highly purified polymers.

**Composition Analysis by Gas Chromatography and Gas Chromatography Coupled to Mass Spectrometry.** For gas chromatography (GC) analysis approximately about 3−5 mg of the isolated polymers was subjected to methanolysis in presence of methanol and sulfuric acid, and the resulting methyl esters were analyzed. This analysis included also coupled GC/MS analysis using an HP6890 gas chromatograph equipped with a model 5973 mass-selective detector (Hewlett-Packard). The mass spectra obtained were compared with the NIST '98 Mass Spectral Library with the Windows search program version 1.6, National Institute of Standards and Technology (U. S. Department of Commerce). These analyses were exactly done as described previously.[4,11]

**Partial Methanolysis.** A 1 N solution of HCl in dry methanol was prepared by bubbling gaseous HCl through redistilled anhydrous methanol. The amount of dissolved HCl was determined gravimetrically, and the concentration was adjusted by adding an appropriate volume of methanol. Approximately 5 mg of each sample was dissolved in 4 mL of $CHCl_3$, and 0.3 mL of a freshly prepared 1 N solution of HCl in methanol was added. The mixture was allowed to react at room temperature for 25, 50, 80, and 100 h, after which the solvent was evaporated.

**Partial Pyrolysis.** Partial pyrolyses of microbial PTEs were performed with a Perkin-Elmer TGS/2 thermogravimetric (TG) apparatus under a nitrogen atmosphere (60 mL/min) at a heating rate of 10 °C/min. The temperature was increased until the weight loss was 20%. After being cooled rapidly (100 °C/min) to room temperature, the residue was recovered and dissolved in chloroform.

**Electrospray Ionization Mass Spectrometry.** ESI-MS experiments were carried out using a Mariner mass spectrometer (Perseptive Biosystems) equipped with an atmospheric pressure ionization (API) source. Partial methanolysis or partial pyrolysis products were dissolved in a 1% acetic acid solution of $CH_3Cl/CH_3OH$ 60:40 at a concentration of 0.1 mg/mL. The samples were introduced into the ESI source with a syringe pump at a flow rate of 7 $\mu$L/min. The spray tip potential was 4.0 kV, with 6 s of acquisition per spectrum; the nozzle potential was 85 V. The quadrupole interface and nozzle temperature were set at 140 °C. Mass spectra were scanned over the range $m/z$ 100−2000 in positive-ion mode. The final spectrum for each sample was an average of at least 20 scans.

**NMR Spectroscopy.** $^1H$ NMR spectra of polymer samples and of SEC fractions were recorded at 500 MHz on a Varian Unity INOVA spectrometer in $CDCl_3$ at 27 °C. Spectra were acquired with a spectral width of 5000 Hz in 32 000 data points, an excitation pulse of 60°, an acquisition time of 3.3 s, processed using WINNMR (Bruker), and referenced with internal tetramethylsilane (TMS). $^{13}C$ spectra were acquired either at 50 MHz on a Bruker AC200 spectrometer or at 125 MHz on the Varian instrument described above.

**Size Exclusion Chromatography.** SEC fractionation was performed by using a Waters 515 high-performance liquid chromatography (HPLC) pump with 4 Styragel HR columns connected in a series (in the order HR4, HR3, HR2, and HR1) and a model 401 refractive index detector. Chloroform was used as the eluent at a flow rate of 1.0 mL/min; 200 $\mu$L of a 5 mg/mL solution was injected for each sample. Fractionation of samples for subsequent 500 MHz $^1H$ NMR analysis was performed by collecting the polymer peak into 5−7 fractions of equal volume, evaporating the SEC solvent, and dissolving the residue in the NMR solvent.

## Results and Discussion

**Microbial Production, Isolation, and Composition Analysis of HA/MA Copolymers.** Cells of *R. eutropha* strain H16 were cultivated in a mineral salts medium containing sodium gluconate and 3-mercaptopropionate or 3-mercaptobutyrate. Whereas the latter thiochemicals served as precursor substrates for incorporation of the 3-mercaptoalkanoates, gluconate served as a carbon and energy source for growth. After harvest of the cells, the polymers were extracted from the cells with chloroform and precipitated with ethanol as described in the Materials and Methods section. The comonomer compositions of five HA/MA copolymers, as obtained by $^1H$ NMR spectroscopy or GC, are reported in Table 1. Both analysis methods revealed very similar compositions: The GC analysis was less sensitive only for detection of 3HP.

**Analysis of Comonomer Distribution in Partial Methanolysis Products.** Sample 1 has a molar composition 3HB/3HV/3MV of 94:2:4 mol % (NMR) or 93:3:4 mol % (GC). Although by $^{13}C$ NMR we assigned the different carbons, it was not possible to obtain sequence information because signals of carbons of 3HV and 3MV units were of very low intensity.

**Table 1.** Content of Monomer Units, Average Block Length, and Degree of Randomness of PTE Copolymers[a]

| sample | 3HB/3MP/3HP[b] (mol %) | 3HB/3MB[b] (mol %) | 3HB/3HV/3MV[b] (mol %) | $L_{3HB}$[c] | $L_{3MP}$[c] | $L_{3MB}$[c] | DR[d] |
|---|---|---|---|---|---|---|---|
| 1 | | | 94/2/4[e]<br>93/3/4[f] | | | | |
| 2 | 62/35/3[e]<br>65/35/traces[f] | | | 10.1 | 4.0 | | 0.35 |
| 3 | 42/55/3 [e]<br>46/54/traces[f] | | | 4.1 | 4.3 | | 0.48 |
| 4 | | 28/72[e]<br>28/72[f] | | 1.8 | | 4.4 | 0.79 |
| 5 | | 32/68[e]<br>38/62[f] | | 2.3 | | 4.7 | 0.64 |

[a] Polymers were obtained as described in the Materials and Methods section. [b] 3HB, 3-hydroxybutyrate; 3MP, 3-mercaptopropionate; 3HP, 3-hydroxypropionate; 3HV, 3-hydroxyvalerate; 3MV, 3-mercaptovalerate. [c] Average block length calculated from $^{13}$C NMR carbonyl resonances according to ref 22. [d] Degree of randomness calculated from $^{13}$C NMR carbonyl resonances according to ref 22. [e] Composition as measured by $^{1}$H NMR spectroscopy. [f] Composition as measured by GC.

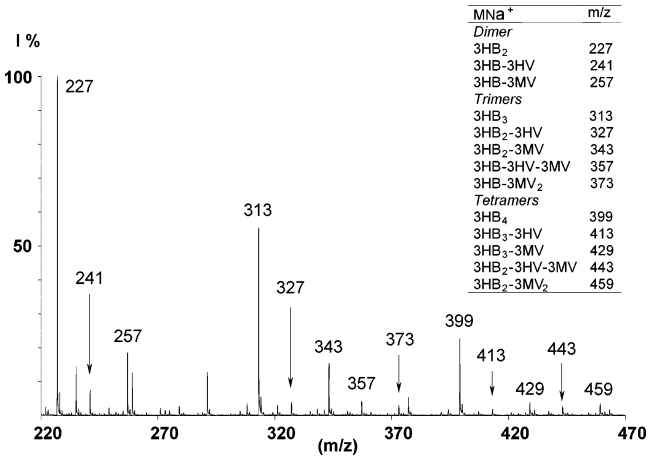



**Figure 1.** ESI-MS analysis of partially methanolyzed poly(3HB-*co*-3HV-*co*-3MV) (sample 1). The ESI-MS spectrum is shown on the left, whereas the assignments are shown on the right.

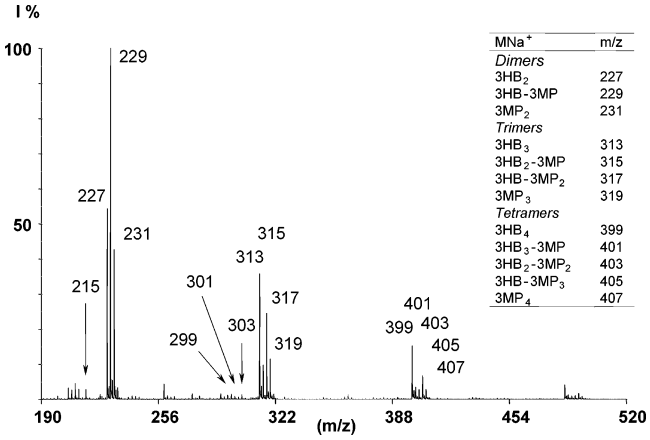



**Figure 2.** ESI-MS analysis of partially methanolyzed poly(3HB-*co*-3MP-*co*-3HP) (sample 3). The ESI-MS spectrum is shown on the left, whereas the assignments are shown on the right.

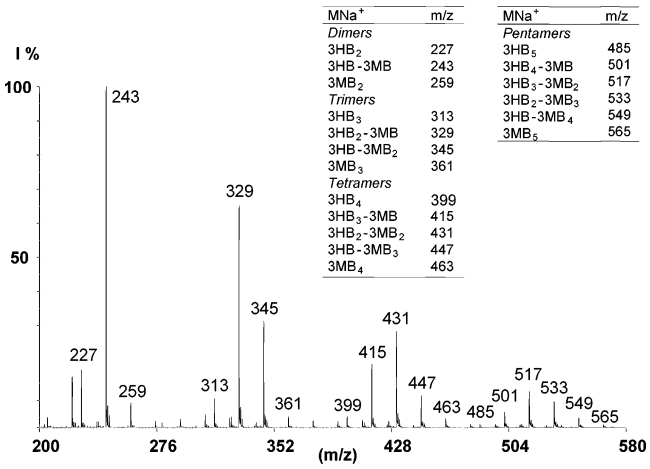



**Figure 3.** ESI-MS analysis of partially methanolyzed poly(3HB-*co*-3MB) (sample 5). The ESI-MS spectrum is shown on the left, whereas the assignments are shown on the right.

Therefore, this copolymer was partially methanolyzed (50 h at room temperature), and the oligomers obtained were analyzed by ESI-MS. The positive ESI-MS spectrum essentially consisted of sodiated ions, and their assignments are shown in Figure 1. Of interest, among the others, are the peaks at *m*/*z* 357, corresponding to the trimer 3HB-3HV-3MV, and at *m*/*z* 443, corresponding to the tetramer $3HB_2$-3HV-3MV, which unequivocally demonstrates the presence of a true terpolymer. Other minor peaks not labeled in Figure 1 correspond to protonated pseudo-molecular ions. Application of statistical analysis showed a poor correlation between the experimental ion intensities and those calculated by assuming a random sequence of the copolymer, leading to the hypothesis of some degree of blockiness. However, this interpretation of the data relies on the assumption that the degradation reaction used to generate the oligomers, i.e., methanolysis, occurs with the same rate constant at the oxoester and thioester bonds, which was not proved. Alternatively, the polymer could also consist of a mixture of copolymers of different compositions. To overcome this difficulty, the intact polymer sample was subjected to fractionation by size exclusion chromatography, and five fractions were collected and analyzed by $^{1}$H NMR with regard to their compositions (SEC-NMR). The first two fractions represented pure poly(3HB) homopolymer, whereas fractions 3, 4, and 5 gave compositions of 93:3.5:3.5, 93:3:4, and 81:8:11 mol % (3HB/3HV/3MV). These results show that sample 1 is a mixture of poly(3HB) and poly(3HB-*co*-3HV-*co*-3MV). The relative amounts of homopolymer and terpolymer and the composition of the terpolymer could not be determined.

Samples 2, 3, 4, and 5 were also subjected to partial methanolysis, and subsequent ESI-MS analysis gave good interpretable spectra, allowing the assignments of the various monomers and oligomers obtained. For example, Figures 2 and 3 show parts of the spectra of samples 3 and 5, respectively. In the first spectrum, besides intense signals due to oligomers containing the more abundant constituents 3HB and 3MP, we

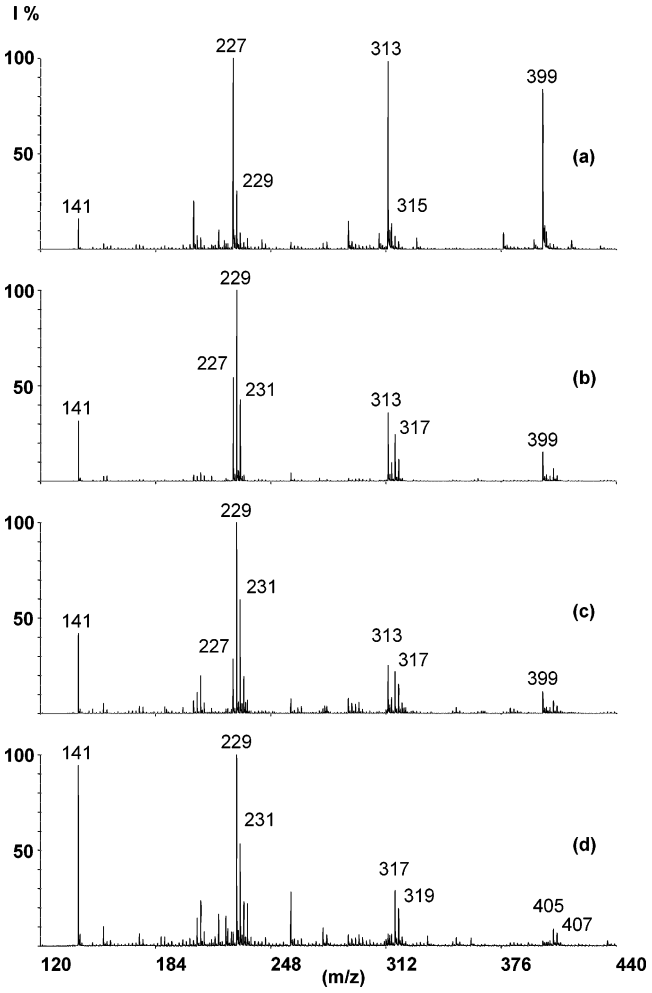



**Figure 4.** ESI-MS analysis of oligomers obtained from poly(3HB-*co*-3MP-*co*-3HP) (sample 3) after exposure to methanolysis for various time periods: (a) after 25 h; (b) after 50 h; (c) after 80 h; (d) after 100 h.

can assign the low-intensity peak at *m*/*z* 215 to the dimer 3HP-3MP and those at *m*/*z* 299, 301, and 303 to the trimers $3HB_2$-3HP, 3HB-3MP-3HP, and $3MP_2$-3HP. These data confirm the presence of poly(3HB-*co*-3MP-*co*-3HP) with low abundance of the comonomer 3HP. In the second spectrum the assignments shown in Figure 3 demonstrate the presence of a 3HB/3MB copolymer.

In the case of PHAs, we usually compare the peak intensities for each set of oligomers (dimers, trimers, etc.) with those theoretically calculated for a random copolymer of a given composition (obtained by NMR or GC). In this case, however, we cannot use the same procedure because the oxoester and thioester bonds have different methanolysis rates. This was proved experimentally when samples subjected to different methanolysis times gave oligomer mixtures of varying ratios, as shown in Figure 4. When sample 3 was methanolyzed for 25, 50, 80, or 100 h, the amounts of the different oligomers (dimers, trimers, etc.) and their ratios in each set changed as a function of methanolysis time. For example, after methanolysis for 25 h (Figure 4a) oligomers rich in 3HB (e.g., the dimers $3HB_2$ and 3HB-3MP at *m*/*z* 227 and 229) were prevalently observed. After 50 h oligomers $3HB_n$ and $3HB_x$-$3MP_y$ and also $3MP_n$ (see dimers $3HB_2$, 3HB-3MP, and $3MP_2$ at *m*/*z* 227, 229, and 231) were preferentially observed (Figure 4b). After 80 h, the homo-$3HB_n$ oligomers decreased, whereas $3HB_x$-$3MP_y$ heterooligomers and $3MP_n$ homooligomers increased, and $3MP_2$ became more abundant than $3HB_2$ (Figure 4c). After methanolysis for 100 h, 3HB monomers at *m*/*z* 141 prevailed, and among the remaining species $3HB_n$ homooligomers were no longer found, while $3HB_x$-$3MP_y$ heterooligomers (for example, 3HB-3MP at *m*/*z* 229) and $3MP_n$ homooligomers (for example, $3MP_2$, $3MP_3$, and $3MP_4$ at *m*/*z* 231, 319, and 407) were still present (Figure 4d). These results suggest that methanolysis of the oxoester bonds occurred at higher rates than methanolysis of the thioester bonds. This is in contradiction with our expectation, because it is known that hydrolysis of thioester bonds occurs at a higher rate than that of the oxoester.[29] However, it was previously shown that poly(3MP) is thermally more stable than PHAs[15] and that PTEs, in contrast to PHAs, are also resistant to hydrolysis by PHA depolymerases and to cleavage by other enzymes.[10,13]

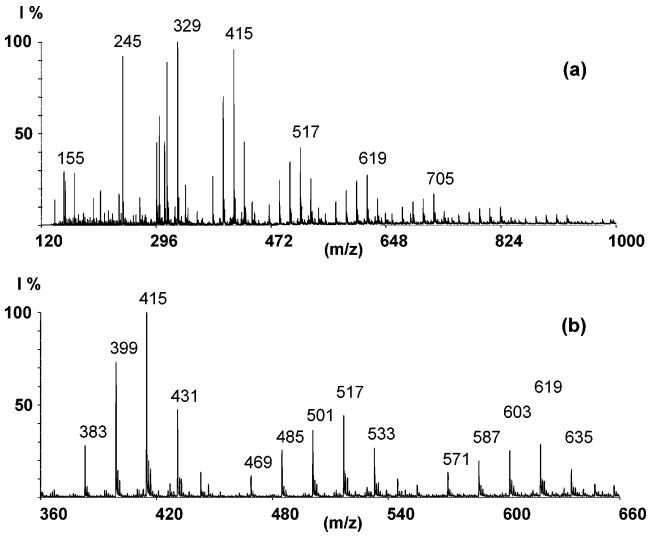
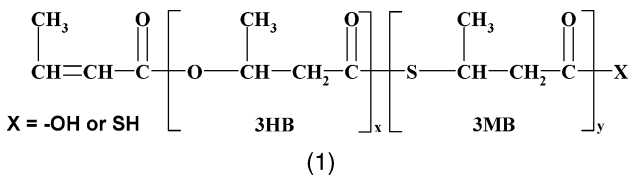



**Figure 5.** ESI-MS analysis of partial pyrolysis products of a poly-(3HB-*co*-3MB) copolymer (sample 4). The upper part (a) shows the entire spectrum, whereas in the bottom part (b) the range of the spectrum showing the tetramers, pentamers, and hexamers was expanded.

**Analysis of Comonomer Distribution in Partial Pyrolysis Products by ESI-MS.** We subjected the copolymers to partial pyrolysis and analyzed the oligomers obtained by ESI-MS using the same procedure employed for PHAs.[28] Among the PTEs studied, only samples 4 and 5, both representing poly(3HB-*co*-3MB) copolymers, gave interpretable spectra. Figure 5a shows the mass spectrum of sample 4, with signals up to about 1000 *m*/*z*. Figure 5b shows the range from tetramers to hexamers in more detail. Each spectrum essentially consisted of sodiated pseudo-molecular ions corresponding to oligomers of the general formula shown in structure **1**




(1)

These results indicate that the thermal decomposition of poly-(3HB-*co*-3MB) is similar to that of poly(3HB)[25] or poly-($3HA_{mcl}$)[26] and that it proceeds by a $\beta$-H transfer process, yielding oligomers with olefinic and oxocarboxylic or thiocarboxylic terminal groups. Kawada et al.[15] performed the thermogravimetric analysis of poly(3MB), finding that the temperature at 5% weight loss was practically coincident with that of poly(3HB) (256 vs 260 °C) and that their thermograms are

**Table 2.** Experimental and Calculated Relative Amounts of the Partial Pyrolysis Products from Poly(3HB-*co*-3MB) Samples 4 and 5

| | | sample 4 | | sample 5 | |
|---|---|---|---|---|---|
| | | | calculated[b] 3HB/3MB | | calculated[b] 3HB/3MB |
| oligomers | *m/z* ($MNa^+$) | ESI-MS[a] | (28/72) | ESI-MS[a] | (32/68) |
| | | Trimers | | | |
| $3HB_3$ | 281 | 2 | 2 | 1 | 3 |
| $3HB_2$-3MB | 297 | 20 | 17 | 13 | 21 |
| 3HB-$3MB_2$ | 313 | 39 | 43 | 61 | 44 |
| $3MB_3$ | 329 | 39 | 38 | 25 | 31 |
| | | Tetramers | | | |
| $3HB_4$ | 367 | 1 | 1 | 2 | 1 |
| $3HB_3$-3MB | 383 | 11 | 6 | 16 | 9 |
| $3HB_2$-$3MB_2$ | 399 | 30 | 24 | 22 | 28 |
| 3HB-3MB3 | 415 | 40 | 42 | 54 | 40 |
| $3MB_4$ | 431 | 18 | 27 | 6 | 21 |
| | | Pentamers | | | |
| $3HB_5$ | 453 | 1 | 0 | 3 | 0 |
| $3HB_4$-3MB | 469 | 8 | 2 | 14 | 4 |
| $3HB_3$-$3MB_2$ | 485 | 15 | 11 | 18 | 15 |
| $3HB_2$-$3MB_3$ | 501 | 25 | 29 | 15 | 32 |
| 3HB-$3MB_4$ | 517 | 32 | 38 | 44 | 34 |
| $3MB_5$ | 533 | 18 | 20 | 6 | 14 |
| | | Hexamers | | | |
| $3HB_6$ | 539 | 4 | 0 | 4 | 0 |
| $3HB_5$-3MB | 555 | 4 | 1 | 12 | 1 |
| $3HB_4$-$3MB_2$ | 571 | 11 | 5 | 15 | 7 |
| $3HB_3$-$3MB_3$ | 587 | 19 | 16 | 13 | 21 |
| $3HB_2$-$3MB_4$ | 603 | 23 | 31 | 17 | 33 |
| 3HB-$3MB_5$ | 619 | 27 | 33 | 35 | 28 |
| $3MB_6$ | 635 | 13 | 14 | 4 | 10 |

[a] Normalized experimental ion intensity. [b] Normalized ion intensity calculated assuming a random sequence. The composition reported is that measured by $^1$H NMR.

similar. Furthermore, direct pyrolysis into a mass spectrometer (DPMS)[23] showed that at the onset of thermal degradation there was evolution of products deriving from both 3HB and 3MB (our unpublished results). Pyrolysis of poly(3HB-*co*-3MB), therefore, may be assumed to occur randomly along the polymer chain.

Table 2 summarizes the peak assignments of Figure 5b (sample 4) and of the peaks obtained by identical analysis for the other poly(3HB-*co*-3MB) (sample 5), together with their experimental and calculated intensities. The calculation was carried out under the assumption that the comonomers follow a random sequence with a 3HB/3MB ratio of 28:72 mol % in sample 4 or of 32:68 mol % in sample 5. A rough fitting of the experimental data was obtained for sample 4 using the composition measured by $^1$H NMR spectroscopy.

We then recorded the $^{13}$C NMR spectrum of sample 4 and used the carbonyl dyad intensities for sequence analysis, applying the definition of Yamadera and Murano[22] to calculate the 3HB and 3MB average block lengths ($L_{3HB}$ and $L_{3MB}$) and the degree of randomness (DR), which are reported in Table 1. A DR of 0.79 suggests a moderate deviation from a random sequence. In fact, the theoretical value for a perfectly random copolymer is 1. In conclusion, MS and NMR analysis gave converging results for this copolymer, showing only a slight deviation from random statistics.

The same MS analysis for sample 5 is reported in Table 2. The calculated intensities were obtained for a copolymer

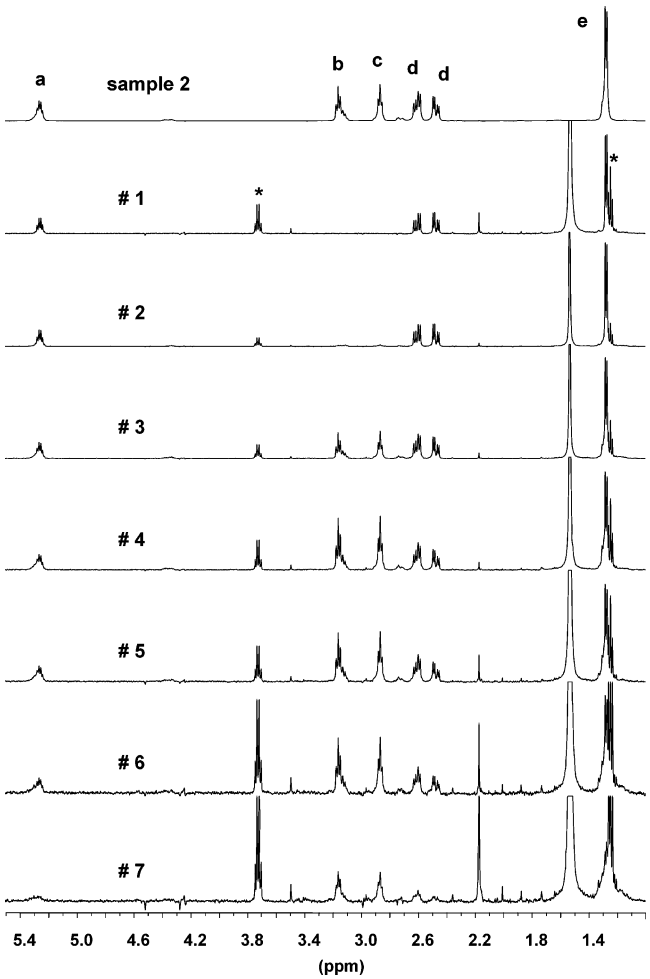
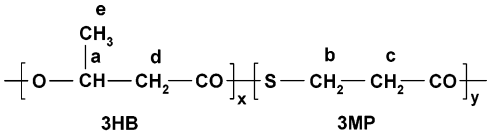



**Figure 6.** $^1$H NMR spectra of sample 2 and of its SEC fractions. Assignments are given in structure **2**. The peaks indicated with a star belong to ethanol contamination.

composition 3HB/3MB of 32:68 mol %. For this sample there was a greater discrepancy between the experimental and the theoretical values. The values of $L_{3HB}$, $L_{3MB}$, and DR are reported in Table 1. The DR is 0.64, thereby suggesting that the copolymer has a microblock structure.

**Fractionation of Polymer Samples by SEC and Analysis of Comonomer Composition.** The ESI-MS spectra of partial pyrolysis products obtained from samples 2 and 3, which are 3HB/3MP/3HP copolymers, were of limited quality. This may be due to the fact that the two most abundant repetitive units (3HB and 3MP) have different thermal degradation mechanisms and different thermal stabilities, as was demonstrated by a previous study,[15] showing that poly(3MP) is degraded at a higher temperature than poly(3HB). To investigate these polymers further, we recorded the $^1$H NMR spectra of fractions collected after SEC separation and measured the comonomer contents of the various fractions (Table 3). Figure 6 shows the $^1$H NMR spectra of sample 2 and of its SEC fractions with peak assignments according to structure **2**




(2)

A very strong variation of composition with molecular weight was observed. The first SEC fraction was even pure poly(3HB),

**Table 3.** Compositions of SEC Fractions of Poly(3HB-*co*-3MP-*co*-3HP) Samples 2 and 3 as Measured by $^1$H NMR Spectroscopy

| 3HB/3MP/3HP[a] (mol %) | fraction1 | fraction2 | fraction 3 | fraction 4 | fraction5 | fraction 6 | fraction 7 |
|---|---|---|---|---|---|---|---|
| | | | Sample 2 | | | | |
| 62/35/3 | 100/0/0 | 94/4/2 | 64/32/4 | 51/46/3 | 52/45/3 | 51/46/3 | 44/52/4 |
| area %[b] | 2.8 | 12.5 | 30.8 | 28.7 | 15.5 | 7.3 | 2.4 |
| | | | Sample 3 | | | | |
| 42/55/3 | 75/17/8 | 70/27/3 | 42/53/5 | 33/63/4 | 31/64/5 | 31/65/4 | 35/62/3 |
| Area %[b] | 3.7 | 6.8 | 22.9 | 28.5 | 21.5 | 10.7 | 5.9 |

[a] 3HB, 3-hydroxybutyrate; 3MP, 3-mercaptopropionate; 3HP, 3-hydroxypropionate. [b] Area of the fractions relative to the SEC trace recorded using a refractive index detector.

**Table 4.** Experimental and Calculated Dyad Relative Peak Intensities for Samples 2 and 3

| | sample 2 | | | sample 3 | | |
|---|---|---|---|---|---|---|
| dyad | exptl[a] | 3HB/3MP[b] 64/36 | 38 mol % 3HB/3MP[c] 25/75 + 62 mol % poly(3HB) | exptl[a] | HB/MP[b] 43/57 | 67 mol % 3HB/3MP[c] 23/77 + 33 mol % poly(3HB) |
| 3MP-3MP | 0.212 | 0.130 | 0.212 | 0.391 | 0.325 | 0.391 |
| 3MP-3HB | 0.072 | 0.230 | 0.071 | 0.126 | 0.245 | 0.120 |
| 3HB-3MP | 0.070 | 0.230 | 0.071 | 0.114 | 0.245 | 0.120 |
| 3HB-3HB | 0.646 | 0.410 | 0.646 | 0.369 | 0.185 | 0.369 |

[a] Normalized experimental intensity of the $^{13}$C NMR carbonyl dyad resonances. [b] Normalized calculated dyad intensity assuming a random sequence and using the composition measured by $^1$H NMR. The 3HP unit was excluded from the calculation because of its low intensity (Table 1). [c] Normalized calculated dyad intensity assuming the presence of a blend of a random copolymer and of poly(3HB).

**Table 5.** Compositions of SEC Fractions of Poly(3HB-*co*-3MB) Samples 4 and 5 as Measured by $^1$H NMR Spectroscopy

| 3HB/3MB[a] | fraction 1 | fraction 2 | fraction 3 | fraction 4 | fraction 5 | fraction 6 |
|---|---|---|---|---|---|---|
| | | | Sample 4 | | | |
| 28/72 | 37/63 | 27/73 | 27/73 | 28/72 | 28/72 | 30/70 |
| area % | 10.0 | 26.1 | 27.0 | 21.9 | 11.5 | 3.5 |
| | | | Sample 5 | | | |
| 32/68 | 43/57 | 34/66 | 30/70 | 27/73 | 25/75 | |
| area % | 10.3 | 29.4 | 34.9 | 20.4 | 5.0 | |

[a] 3HB, 3-hydroxybutyrate; 3MB, 3-mercaptobutyrate. [b] Area of the fractions relative to the SEC trace recorded using a refractive index detector.

**Table 6.** Experimental and Calculated Dyad Relative Peak Intensities for Samples 4 and 5

| | sample 4 | | | sample 5 | | |
|---|---|---|---|---|---|---|
| dyad | exptl[a] | 3HB/3MB[b] 28/72 | 92 mol % 3HB/3MB[b] 23/77 + 8 mol % poly(3HB) | exptl[a] | 3HB/3MB[b] 32/68 | 85 mol % 3HB/3MB[b] 21/79 + 15 mol % poly(3HB) |
| 3MB-3MB | 0.547 | 0.518 | 0.547 | 0.526 | 0.462 | 0.526 |
| 3MB-3HB | 0.163 | 0.202 | 0.163 | 0.152 | 0.218 | 0.143 |
| 3HB-3MB | 0.162 | 0.202 | 0.163 | 0.134 | 0.218 | 0.143 |
| 3HB-3HB | 0.128 | 0.078 | 0.128 | 0.188 | 0.102 | 0.188 |

[a] Normalized experimental intensity of the $^{13}$C NMR carbonyl dyad resonances. [b] Normalized calculated dyad intensity assuming a random sequence and using the composition measured by $^1$H NMR. [c] Normalized calculated dyad intensity assuming the presence of a blend of a random copolymer and of poly(3HB).

demonstrating a substantial contamination of poly(3HB-*co*-3MP-*co*-3HP) with poly(3HB). In the case of sample 3 we did not isolate pure poly(3HB), but looking at the SEC fractions composition it was apparent that poly(3HB) contamination was present: Fractions 1−2 were 3HB-rich, fraction 3 had an intermediate composition, and fractions 4−7 showed an almost constant composition. In Table 4 we report the experimental dyad intensities of the $^{13}$C NMR carbonyl resonances together with the theoretical abundance for pure random copolymers having the same compositions as samples 2 and 3 and for mixtures of random copolymers and poly(3HB). Since the amount of 3HP units was too low to give accurate quantification in the $^{13}$C NMR spectra, this comonomer was not included in the calculations. An almost perfect match of the experimental data was obtained assuming that sample 2 is a blend of 38 mol % of poly(25 mol % 3HB-*co*-75 mol % 3MP) with 62 mol % of poly(3HB) and that sample 3 is a blend of 67 mol % of poly(23 mol % 3HB-*co*-77 mol % 3MP) with 33 mol % of poly(3HB). The DR and the 3HB and 3MB average block length ($L_{3HB}$ and $L_{3MP}$) values reported in Table 1 would indicate a microblock structure if the samples were pure copolymers; however, having demonstrated that this is not the case, this conclusion could be wrong.

The SEC-NMR analysis was performed also on samples 4 and 5 showing a significant drift in the comonomer composition of the fractions obtained from both polymers (Table 5). Fractions with higher molecular weights exhibited higher 3HB and lower 3MB contents. This fact could be due to a "natural" compositional dependence on molecular weight or to the presence of a certain amount of poly(3HB) homopolymer in the samples. As for samples 2 and 3, we calculated the theoretical dyad intensities expected for a pure random copolymer and for a blend consisting of a random copolymer with poly(3HB). Again, an almost perfect match of the experimental data was obtained

assuming that sample 4 is a blend of 92 mol % of poly(23 mol % 3HB-*co*-77 mol % 3MB) with 8 mol % of poly(3HB) and that sample 5 is a blend of 85 mol % of poly(21 mol % 3HB-*co*-79 mol % 3MB) with 15 mol % of poly(3HB).

## Conclusions

ESI-MS analysis of the products generated by partial methanolysis or partial pyrolysis of the PTEs studied showed that they are true copolymers or terpolymers. Microstructure analysis was not applicable in the case of methanolysis due to partial selectivity of this reaction toward the oxoester and thioester bonds, the former being preferentially cleaved. Degradation by pyrolysis suffered from the same problem in the case of 3HB/3MP copolymers, as the two different ester bonds have dissimilar thermal stabilities. For 3HB/3MB copolymers, the thermal degradation proceeds at approximately the same temperature by the same mechanism ($\beta$-H transfer), and therefore it occurs randomly, but a Bernoullian model failed all the same to reproduce satisfactorily the experimental distribution of oligomers as measured by MS. $^{13}$C NMR analysis of the nondegraded samples indicated, through the analysis of the carbonyl dyads intensities and the calculation of the average block length and degree of randomness (Table 1), deviation from a random sequence. The MS and $^{13}$C NMR data, therefore, suggested that the samples could be blocky.

However, SEC fractionation of the original samples and subsequent $^{1}$H NMR analysis of the fractions obtained showed that samples 1, 2, and 3 contain high amounts of poly(3HB) and that samples 4 and 5 have a significant compositional drift (Tables 3 and 5). We calculated the theoretical dyad intensities expected for blends consisting of a random copolymer with poly(3HB) and found satisfactory matches between the theoretical values and the experimental data. The amounts of poly(3HB) hypothesized in the blends were 62, 33, 15, and 8 mol % for samples 2, 3, 5, and 4, respectively, which is consistent with the extent of deviation of the MS (pyrolysis of samples 4 and 5, Table 2) and NMR (Tables 4 and 6) data from a random sequence model. However, previous reports concluded that PTEs possess a microblock structure, based on NMR,[4] DSC,[15] and MALDI-MS[10] evidence. There is some possibility that the samples investigated in these papers contained poly(3HB) too; this would undermine to some degree the NMR and DSC data interpretation in refs 4 and 15. Elbanna et al.[10] found that a PHA depolymerase acting on a poly(3HB-*co*-3MP) sample cleaved exclusively the oxoester linkages and showed a MALDI mass spectrum of the water-insoluble fraction, remaining after enzymatic digestion, consisting of oligomers of 3MP with a single 3HB unit. This result does not necessarily mean that the sample investigated possesses a microblock sequence. In fact, homo sequences are statistically possible also in a random copolymer in amounts that are determined by the copolymer composition; the enzymatic hydrolysis cannot cleave these sequences that are accumulated in the water-insoluble fraction. Moreover, the oligomers quantitative distribution in MALDI spectra may deviate substantially from the real abundance in the sample, especially in the low mass range, where the MALDI matrix signals overlap or obliterate the sample signals. Our results suggest that PTEs are random copolymers, but the ultimate proof would require to purify our samples from poly(3HB) for $^{13}$C NMR analysis. This will be possible when sufficient amounts of PTEs become available.

It will be also an interesting point to investigate how the two biosynthesis pathways to PTEs and to PHAs do coexist and which chemical and/or biological factors do in fact influence discrimination of the PHA synthase between PHA and PTE biosynthesis.

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