Cyanophenoxy-Containing Microbial Polyesters: Structural Analysis, Thermal Properties, Second Harmonic Generation and In-Vivo Biodegradability

Richard A. Gross,* Oh-young Kim
University of Massachusetts Lowell, Department of Chemistry, One University Avenue, Lowell, Massachusetts 01854, USA

Denise R. Rutherford & Richard A. Newmark
3M Company, Corporate Research Laboratories, St Paul, Minnesota 55144-1000, USA

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Abstract: Pseudomonas putida KT 2442 was utilized as biocatalyst to form optoactive poly(β-hydroxyalkanoate) (PHAs) from a cosubstrate mixture of octanoate and the achiral polarizable carbon source 6(4-cyanophenoxy)hexanoate, CPH. COSY and heteronuclear multiplet quantum correlation experiments were used to assign 1H and 13C NMR signals of 3-hydroxy-6(4-cyanophenoxy)hexanoate (3HCPH) repeat units. The methine carbon of 3HCPH repeat units was sensitive to repeat unit sequence effects, indicating that a substantial fraction of 3HCPH centered triad sequences in the product contain neighboring 3-hydroxyoctanoate and 3-hydroxhexanoate repeat units. Comparing the thermal properties of 0 and 19.6 mol% 3HCPH samples by differential scanning calorimetry shows that 3HCPH incorporation results in melting at temperatures >64°C (not seen for the 0 mol% sample), more rapid crystallization and a new Tg transition at ~ -21°C. These characteristics indicate that chains and/or chain segments are formed that are enriched in 3HCPH which phase-separate and form a unique crystal structure. Measurements of second harmonic generation (SHG) intensities carried out using in-situ corona-poled samples showed weak SHG signals that increased by a factor of 8 for an increase in the 3HCPH content from 26 to 34 mol%. Comparatively higher SHG intensities (5 times) were found for PHAs which contained 5.1 mol% 3-hydroxy-6(4-nitrophenoxy)hexanoate (3HNPH) repeat units relative to a PHA with 17 mol% 3HCPH. In-vivo biodegradation studies of microbial polyesters prepared with and without 3HCPH repeat units showed that PHA chains with 3HCPH degraded to lesser extents (weight loss of ~20 and 50% over 72 h incubations). The large increase in polydispersity from 1.9 to 4.3 observed during in-vivo biodegradation of microbial polyesters containing 3HCPH repeat units was attributed to the existence of chains with highly variable contents of 3HCPH repeat units.

Key words: Pseudomonas putida, poly(β-hydroxyalkanoate), microbial polyesters, poly[3-hydroxy-6(4-cyanophenoxy)hexanoate], non-linear optics, in-vivo biodegradation.

* To whom correspondence should be addressed.
INTRODUCTION

A great deal of research has been carried out on the microbial production and characterization of poly(β-hydroxyalkanoate) (PHA) copolymers which contain variable repeat units. The microbial formation of PHAs that contain side chain functionalities has also been investigated. Recently, Lenz and coworkers obtained PHAs containing various functional groups such as halogen, nitrile, vinyl, and phenyl. Thus, it was envisioned that the flexibility inherent in many microbial PHA producing systems could be applied towards the development of exciting advanced materials that make use of the enantiopure [R]—stereocchemical configuration of PHA repeat units. For this purpose, we reported on the formation of PHAs by Pseudomonas putida and Pseudomonas oleovorans that contained side chain polarizable para-cyanophenoxyalkyl and para-nitrophenoxyalkyl ether (CP and NP, respectively) substituent groups. The mole% incorporation was found to be highly dependent on the spacer length between the chain terminal substituent and the 3-positions. Specifically, a spacer of three methylene units was needed to obtain substantial (> 5 mol%) incorporations of CP and NP side groups.

All naturally occurring bacteria accumulating PHAs presumably possess enzyme systems that degrade PHAs intracellularly under certain conditions. However, in contrast to extracellular depolymerases, little is known about the intracellular degradation of microbial polyesters. The majority of literature to date has used PHA granules and various cytoplasmic fractions to study this process. For example, in the case of Rhodospirillum rubrum, a granule-associated heat-labile factor, a poly(3-hydroxybutyrate) (P3HB) depolymerase, an oligomer hydrolase and a heat-stable activator protein were claimed to be requirements for intracellular polyester degradation. Doi and co-workers carried out investigations of PHA intracellular degradation by Alcaligenes eutrophus. They showed that, for example, P3HB accumulated in cells during a first stage of cultivation could be replaced by a poly(3HB-co-3-hydroxyvalerate) copolyester if the cells were transferred to a new nitrogen-free medium and incubated with valeric acid as the sole carbon source. Thus, it was concluded that polyester degradation and accumulation operate simultaneously in A. eutrophus under nitrogen-free conditions. In bioengineering new PHA structures, it is interesting to consider whether intracellular depolymerase systems can degrade the unusual synthesized products and, if so, whether these non-naturally occurring repeat units degrade at relatively slower rates.

In this paper, Pseudomonas putida KT 2442 was utilized as a biocatalyst to form PHAs which contain variable quantities of 3-hydroxy-6(4-cyanophenoxy) hexanoate (3HCPH) repeat units. COSY, heteronuclear multiplet quantum correlation (HMOC) and 1-D $^{13}$C NMR experiments were performed to establish peak assignments and to investigate the repeat unit sequence distribution of a 19.6 mol% 3HCPH-containing PHA. Effects of 3HCPH content on the product thermal properties were investigated by differential scanning calorimetry (DSC). The in-vivo biodegradability by P. putida of a PHA containing 17 mol% 3HCPH repeat units was also investigated. Finally, since CP substituents are hyperpolarizable, the non-linear optical properties of these first generation optoactive PHAs were studied by measuring the second harmonic generation (SHG) intensities.

EXPERIMENTAL

Polymer formation by batch fermentations

Two-stage batch cultivations of Pseudomonas putida KT 2442 were carried out as described previously by us. In summary, for the first stage, 40 mM sodium citrate was used as the carbon source in medium E, at 30°C, 250 rpm in a shake incubator for 20 h. For the second or PHA production stage, 6(4-cyanophenoxy) hexanoate (CPH), prepared as previously described, was used as a cosubstrate with sodium octanoate (OA) (total carbon source concentration equal to 15 mM). The relative mM concentrations of OA/CPH were 15/0, 7.5/7.5 and 5/10. Second stage cultivations were carried out for 24 or 48 h as specified below. Further procedures of harvesting, freeze-drying and lyophilizing of cells as well as polymer isolation and purification were exactly as previously reported.

In-vivo biodegradation studies

Batch cultivations were carried out similarly as described above and in Refs 12 and 13 except that a 1-liter fermentor (500 ml cultivation volume) was used in place of shake flasks. For this study two fermentations were carried out using OA/CPH ratios of 15/0 and 7.5/7.5 with second stage cultivation times of 1 and 2 days, respectively. Subsequent to the second stage periods the cultures were divided into 100 ml portions which were each harvested by centrifugation (Sorvall: 4°C, 8000 rpm), the cells were resuspended in 500 ml shake flasks containing 100 ml volumes of 0.02M sodium phosphate buffer (pH = 7.2) solutions and the flasks were placed in a shake incubator (New Brunswick Scientific Co., Inc., 250 rpm, 30°C). After incubation periods of 0, 12, 24, 48 and 72 h, the cells were harvested by centrifugation, washed with 0.02M sodium phosphate buffer solution (pH = 7.2), lyophilized and weighed. Finally, PHAs were isolated from the lyophilized cells by extraction with an excess of chloroform.
(15 ml per 1 g of biomass, 25°C, 48 h). Residual cellular material was then removed by filtering, the polymers formed were precipitated by addition of the chloroform solution into cold methanol (1:10 v/v), the precipitated polymers were washed with methanol and then dried in vacuo (30°C, 5 mmHg, 24 h).

Instrumental procedures

A UNITY-500 NMR spectrometer was used for all the NMR experiments described below. Proton (1H) NMR were recorded at 500 MHz. Chemical shifts in parts per million (ppm) were reported downfield from 0.00 ppm using tetramethylsilane (TMS) as an internal reference. The experimental parameters were as follows: 0.5% w/v polymer in chloroform-d, temperature 298 K, 2.4 μs (14°) pulse width, 3 s acquisition time, and 8000 Hz spectral width. The following characterizations by other NMR experiments were carried out on the PHA isolated from P. putida using OA/CPH 7.5/7.5 (mM) and a 2 day second stage cultivation period. The product of this fermentation had 19.6 mol% 3HCPH repeat units based on 1H NMR spectral integration (see Fig. 1, below). Carbon (13C) NMR spectra were recorded at 125 MHz with the following parameters: 2.0% w/v polymer in chloroform-d, 298 K, 7.4 μs (67°) pulse width, 0.4 s acquisition time, 26 400 Hz spectral width and continuous Waltz modulated proton decoupling. The observed 13C NMR chemical shifts in ppm were referenced relative to chloroform-d at 76.91 ppm. For the COSY experiment (0.5% w/v polymer in chloroform-d) the data were collected in a 1024 x 256 data matrix and zero-filled to 1024 x 1024 using eight scans per increment, a 4260 Hz sweep width, and a 1-s delay between transients. The data were processed using sinebell weighting. Two-dimensional reverse-detected HMOC spectra were obtained (2.0% w/v polymer in chloroform-d) with spectral windows of 4260 Hz (1H) and 12 771 Hz (13C). The data were optimized for a one-bond scalar coupling constant of 140 Hz and used 90° pulses of 9.8 μs (13C) and 14.8 μs (1H). Delay time between scans was 1-s. The data matrix was zero-filled to 1024 x 1024 and processed with a Gaussian weighting function.

All thermal characterizations were carried out using a DuPont 2910 differential scanning calorimeter equipped with a TA 2000 data station, using 3-0-10-0 mg of sample sealed in aluminum pans and a dry nitrogen purge. The polymer samples were heated at a rate of 10°C/min from room temperature to 100°C, rapidly quenched from the melt and then were analyzed during second heating scans from −100°C to 100°C. Data reported for the melting temperature (Tm) and enthalpy of fusion (ΔHf) were taken from the first heating scan. Where multiple melting transitions were observed, the reported Tm was the peak melting temperature of the largest endotherm transition. ΔHf values were taken as the cumulative value over the entire melting transition range. The reported glass transition temperature (Tg) values were the midpoint values measured during the second heating scans.

Molecular weights of PHAs were measured by gel permeation chromatography (GPC) using a Waters Model 510 pump, Model 410 refractive index detector and Model 730 data module with 500, 103, 104 and 105 Å Ultrastyragel columns in series. Chloroform (HPLC grade) was used as an eluant at a flow rate of 1-0 ml/min. The sample concentrations and injection volumes were 0-3% (w/v) and 150 μl, respectively. Polystyrene standards with a low polydispersity (Polysciences) were used to generate a calibration curve.

Corona-poled uniform polymer films which were spin-coated onto glass slides (2 mg/ml chloroform, 1000 rpm, 30 s spinning, at 25°C, 1.5 μm thickness) were characterized by SHG measurements. A p-polarized beam of a Q-switched Nd-YAG laser (wavelength = 1064 nm) was focused onto the sample. The fundamental wave was blocked using a CuSO4 solution and a 532 nm interference filter. The incident angle of the beam to the sample was 50°. The second harmonic signal was monitored by a photomultiplier tube (PMT) and averaged in a boxcar integrator. The incident fundamental intensity was held at a constant value during the course of these measurements.

RESULTS AND DISCUSSION

NMR analysis of 19.6 mol% 3HCPH-containing PHA

In a previous study, it was found that cultivations of P. putida using OA/CPH 7.5/7.5 (mM) as cosubstrates and 2 day second stage cultivation times allowed formation of a PHA containing approximately 19.6 mol% 3HCPH repeat units. Analysis of this product by a series of 1D and 2D NMR experiments was carried out to confirm product composition and to gain information on polymer microstructure (repeat unit sequence analysis). The 500 MHz 1H and 125 MHz 13C NMR spectra are shown in Figs 1 and 2, respectively. Assignments of 1H and 13C NMR signals based on comparisons with previously published spectra allowed determination that the product contained 3-hydroxyoctanoate (3HO) and 3-hydroxyhexanoate (3HH) repeat units. The 2D-COSY spectrum shown in Fig. 3 was used to make the assignments shown in Fig. 1 of 3HCPH 1H NMR signals. Specifically, correlations between the signals at 1.80 ppm (H or protons-f1) and 4.01 ppm (H-g1), 1.80 and 5.28 ppm (H-c1), 1.80 and 5.28 ppm (H-b1) were found that supported the assignments made. Also, correlations between the signals at 6.92 ppm (H-h1) and
Fig. 1. 500 MHz $^1$H NMR spectrum in chloroform-$d$ of the PHA containing 19.6 mol% 3HCPH repeat units produced by P. putida after a 2 day second stage cultivation time using OA/CPH 7.5/7.5 (mM) as cosubstrates.

Fig. 2. 125 MHz $^{13}$C NMR spectrum in chloroform-$d$ of the PHA containing 19.6 mol% 3HCPH repeat units produced by P. putida after a 2 day second stage cultivation time using OA/CPH 7.5/7.5 (mM) as cosubstrates.

Fig. 3. COSY 500 MHz NMR spectrum in chloroform-$d$ of the PHA containing 19.6 mol% 3HCPH repeated units produced by P. putida after a 2 day second stage cultivation time using OA/CPH 7.5/7.5 (mM) as cosubstrates.
7.55 ppm (H-i1) were observed (see Figs 1 and 3). Spectral regions of the HMQC spectrum shown in Fig. 4(a) and (b) were used to make the assignments shown in Fig. 2 of 3HCPH $^{13}$C NMR signals. Aromatic protons of 3HCPH repeat units at 6.92 and 7.55 ppm (H-h1 and H-i1, respectively) correlated with signals at 115.0 and 133.7 ppm (see Fig. 4(b)) and therefore were assigned to carbon (C)-h1 and C-i1, respectively. Furthermore, the 4.01 ppm proton signal H-g1 correlated with the $^{13}$C NMR signal at 67.6 ppm (Fig. 4(b)) which was assigned to C-f1. Moreover, the 1.80 ppm proton signal H-f1 correlated with the 30.3 ppm carbon signal (see Fig. 4(a)) which was assigned to C-e1. Interestingly, the 5.28 ppm proton signal H-b1 correlated with the $^{13}$C NMR resonances at 70.1 and 70.3 ppm which were assigned to C-c1 carbons. The chemical shift dispersion of c1 carbons is believed to result from repeat unit sequence effects. If it is assumed that the effects of 3HO and 3HH repeat units on neighboring 3HCPH units are equivalent and directionality effects are not important, 3HCPH centered triad sequences which may be resolved are 3HO-3HCPH-3HO, 3HCPH-3HCPH-3HO and 3HCPH-3HCPH-3HCPH. Although sufficient information is not available herein to assign the signals at 70-1 and 70-3 ppm to triad sequences, these results indicate that the product formed is at least in part a copolymer of 3HO, 3HH and 3HCPH repeat units and not a mixture of homo-poly(3HCPH) and poly(3HO-co-3HH).

**Thermal properties of CP-containing PHAs**

Selected solution-precipitated PHAs containing CP side groups were characterized by DSC (see Experimental).
to determine whether the incorporation of CP side groups influenced product thermal properties. DSC thermograms for first heating scans were used to determine $T_m$ and $\Delta H_f$ values (see Table 1 and Fig. 5). Second heating scans after rapid quenching of samples from the melt were also recorded to determine $T_m$ values (see Table 1 and Fig. 6). Inspection of Table 1 shows that increased incorporation of 3HCPH in PHAs leads to a slight decrease in $\Delta H_f$ and $T_m$ values. For example, PHAs formed with 0 and 19.6 mol% 3HCPH repeat units had $\Delta H_f$ values of 4.6 and 3.6 cal/g, respectively. Comparison of the first heating scans for these two products shows that the sample containing 19.6 mol% 3HCPH showed a slightly decreased $T_m$ (by $\sim 2^\circ$C) and a relatively broader melting temperature range (35–80°C, see Table 1 and Fig. 5). Unlike the PHA formed from OA (15 mM) containing only HO and HH repeat units, the PHA containing 19.6 mol% 3HCPH shows melting at temperatures >64°C with a weak but distinct melting transition peak at $59.2^\circ$C (see Fig. 5, thermogram c). Also, unlike the PHA containing only HO and HC repeat units, the 6.8 and 19.6 mol% 3HCPH samples showed melting transitions during second heating scans (thermograms b and c, respectively, Fig. 6). Furthermore, the 19.6 mol% sample shows what appears to be a $T_m$ transition at approximately $-21^\circ$C that was not observed for the 0 to 6.8 mol% 3HCPH samples (see Fig. 6). The above results on the effects of 3HCPH incorporation on microbial polyester thermal transitions suggest the following: (1) a fraction of 3HCPH units exist in chain segments along with HO and HH repeat units and function to disrupt the crystalline organization of n-alkyl side groups resulting in depressed $T_m$ values; (2) chain segments and/or polymer chain enriched in 3HCPH repeat units were formed that phase-separate and have unique crystal structures that melt at higher temperatures and crystallize more rapidly than HO/HC copolymers. The additional $T_m$ observed for the 19.6 mol% sample further supports that the product formed is not a random copolymer but is heterogeneous, having chains and/or chain segments enriched in 3HCPH repeat units. X-ray diffraction studies as a function of temperature and annealing conditions are in progress to further investigate the hypothesis that a unique crystal organization was formed.

**Table 1.** Thermal analysis by DSC measurements of PHAs containing variable 3HCPH contents

<table>
<thead>
<tr>
<th>3HCPH (mol%)</th>
<th>Culture conditions</th>
<th>$T_q$ (°C)</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H_f$ (cal/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15/0 (1)</td>
<td>-35.4</td>
<td>55.5</td>
<td>4.6</td>
</tr>
<tr>
<td>6.8</td>
<td>5/10 (2)</td>
<td>-37.0</td>
<td>54.5</td>
<td>4.3</td>
</tr>
<tr>
<td>19.6</td>
<td>7.5/7.5 (2)</td>
<td>-37.5</td>
<td>53.5</td>
<td>3.6</td>
</tr>
</tbody>
</table>

*Microbial polyesters were obtained by solution precipitation (see Experimental section). DSC scans were performed using a 10°C/min heating rate.

* Measured by $^1$H NMR spectral integration (see Fig. 1).

* Second stage conditions for polymer formation, see the Experimental section and Refs 12 and 13 for additional detail.

* Measured at the midpoint of the step transition during the second heating scan after quenching from the melt.

* Measured during the first heating scan where the value reported is the peak melting temperature for the largest endotherm transition.

* Measured during the first heating scan where the value reported is the cumulative heat of fusion taken over the entire melting range.
Non-linear optical properties of CP-containing PHAs

To characterize the non-linear optical (NLO) properties of microbially synthesized PHAs which contain hyperpolarizable CP side groups, the relative second harmonic signal intensities were measured. Figure 7 shows the relative SHG intensities of in-situ corona-poled PHA films as a function of the mol% CP incorporation. The wide range of samples having 3HCPH incorporations ranging from 0 to 34 mol% was obtained by variation in the mole ratio of OA to CPH as well as the second stage cultivation times (see the Experimental section and Ref. 12). With an increase in the mol% incorporation of 3HCPH units to 26 mol% there was little change in the SHG intensities. A further increase in the 3HCPH incorporation from 26 to 34 mol% resulted in a corresponding increase in the SHG signals than PHAs with equivalent or higher 3HCPH contents. For example, the SHG intensity of a PHA with 5.1 mol% 3HNP was I07 which is approximately five times greater than a PHA with 17 mol% 3HCPH repeat units. Unfortunately, to date, we have not been successful in preparing PHAs with >5.1 mol% 3HNP using either P. oleovorans or P. putida. As would be anticipated, the SHG intensities for these optoactive PHAs were much less than the SHG intensities measured for advanced NLO organic polymeric materials synthesized by traditional chemical methods. For example, an azo dye containing polymer prepared by conventional synthetic techniques and having comparable thickness has SHG activity which is 1000 times greater. Nevertheless, our work has demonstrated a unique biosynthetic route to NLO-active polymers. In the future we plan to redesign optoactive PHAs for improved performance.

In-vivo biodegradation studies

It is interesting to consider whether intracellular microbial PHA depolymerase systems show similar flexibility to enzymes involved in the polymerization of unusual carbon sources. Therefore, studies were initiated to compare the in-vivo intracellular degradability of microbial polymers formed by P. putida that contained 0 and 17 mol% 3HCPH. The polymers were prepared from 15/0 and 7.5/7.5 (mM) OA/CPH mixtures after 1 and 2 day second stage cultivation times, respectively. At the end of the cultivations, the % PHA of the cellular dry weights were 12.0 and 2.8%, respectively. In-vivo degradability was studied by isolation of polymer-containing cells by centrifugation, resuspending these cells in 0.02 M sodium phosphate buffer (pH = 7.2), and incubation of cells for up to 72 h (see Experimental). The % PHA in cells as a function of the incubation time for these two systems is shown in Fig. 8. From Fig. 8 it is clear that the PHA containing 3HCPH repeat units was degraded more slowly than PHAs having no 3HCPH repeat units. When the degradation is measured as % loss of the initial PHA in cultures, the 0 and 17.0 mol% 3HCPH samples lost ~50 and 20% by weight over 72 h incubation periods. Furthermore, the recovered 3HCPH microbial polyester...
after an incubation period of ~48 h showed a substantial increase from 17 to ~25 mol% 3HCPH repeat units. Thus, these results indicate that the incorporation of the unusual repeat unit 3HCPH in PHAs slows intracellular PHA degradation. Also, degradation of the 17 mol% 3HCPH product occurred by preferential consumption of n-alkanoate repeat units. It may be that 3HCPH repeat units are poor substrates for the intracellular depolymerase system. Other possible explanations are that the intracellular accumulation of CP degradation products inhibits depolymerase activity and/or are toxic.

Figure 9 shows changes in microbial polyester number-average molecular weight ($M_n$, Fig. 9(a)) and polydispersity ($M_w/M_n$, Fig. 9(b)) as a function of the incubation time for cells resuspended in buffer without added carbon source (see Experimental section, in-vivo degradation experiment). For the 0 mol% 3HCPH sample (OA/CPH = 15/0) and incubation times of 0 and 72 h, the $M_n$ values steadily decreased by a factor of 1-6 (124 000 to 77 800) while $M_w/M_n$ values increased slightly from 1-8 to 2-7. The $M_n$ and $M_w/M_n$ values of the 17 mol% 3HCPH sample (OA/CPH = 7-5/7-5) for identical incubation time periods decreased by a factor of 2-4 (75 200 to 31 900 g/mol) and increased from 1-9 to 4-3, respectively. The increased dispersity of the 3HCPH-containing products with increased incubation time was particularly striking. The GPC chromatographs of these samples were unusually broad, and apparently unimodal. Increased polydispersities may result from preferential molecular chain scission of PHA chains enriched in 3H0/3HH repeat units. Thus, if the product is indeed heterogeneous as was suggested above from the DSC results and has chains with variable amounts of 3HCPH, variable degradation rates of the heterogeneous intracellular product may result, which would cause the dispersity of the residual intracellular material to increase.

**SUMMARY OF RESULTS**

COSY and HMQC spectra of the microbial polyester formed by *P. putida* containing 19-6 mol% 3HCPH repeat units facilitated assignments of 3HCPH $^1$H and $^{13}$C NMR signals. The two signals observed for the methine carbon c1 of 3HCPH units were attributed to effects of repeat unit sequence distribution. In other words, the product formed had to some extent 3HCPH centered triads that contained 3HO and 3HH neighboring repeat units. Results of DSC on the 19-6 mol% product indicated that the sample was heterogeneous. This was concluded based on the observation that an increase in the 3HCPH content of the microbial polymers resulted in a depression of the $T_m$ that was also accompanied by sample melting at elevated temperatures, more rapid crystallization and an additional $T_g$ transition. NLO properties of *in-situ* corona-poled microbial polysteres containing either 3HCPH or 3HNPH repeat units were evaluated by measurement of SHG intensities. Even though only weak SHG intensities were measured for these bacterial polysteres, nevertheless, it was demonstrated that chiral NLO-active products can be prepared from achiral precursor molecules by this microbial catalytic route. In-vivo biodegradation studies of microbial polysteres prepared with and without 3HCPH repeat units showed that PHA chains with 3HCPH degraded more slowly. The large increase in polydispersity observed during in-vivo biodegradation of microbial polysteres containing 3HCPH repeat units was attributed to the existence of chains with highly variable contents of 3HCPH repeat units.
Cyanophenoxy-containing microbial polyesters units. Therefore, both DSC and in-vivo degradation studies support the hypothesis that the 3HCPH-containing products formed were not simply random copolymers but were heterogeneous having highly variable contents of 3HCPH units in different chains and/or chain segments.

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