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Tailored Biosynthesis of Olefinic Medium-Chain-Length Poly[(*R*)-3-hydroxyalkanoates] in *Pseudomonas putida* GPo1 with Improved Thermal Properties

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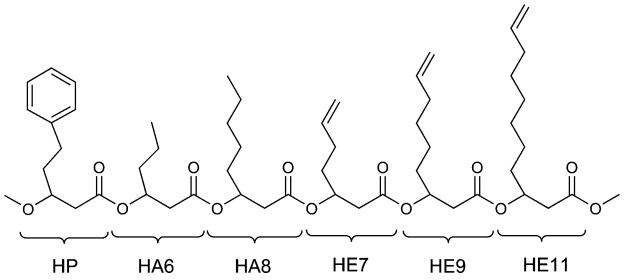
ABSTRACT: Mixtures of 5-phenylvalerate, octanoate, and 10-undecenoate were fed to a chemostat culture (dilution rate = 0.1 h^{-1}) of *Pseudomonas putida* GPo1 under well-defined dual-(C,N)-nutrient limited growth conditions. Five new, tailor-made copolymers were produced and consisted of poly(3-hydroxy-5-phenylvalerate-*co*-3-hydroxyalkanoates-*co*-3-hydroxy-*ω*-alkenoates), poly(HP-*co*-HA-*co*-HE), with increasing amounts of aromatic side chains (A, 0%; B, 3%; C, 19%; D, 42%; and E, 59%), approximately 10 mol % unsaturated side chains, and decreasing amounts of saturated side chains. On the basis of NMR analysis of polymer E, it was concluded that the incorporation of the substrates occurred randomly. The HP-content determined the glass transition temperature, which increased linearly from −38.7 °C for poly-(0%HP-*co*-90%HA-*co*-10%HE) to −6.0 °C for poly(59%HP-*co*-31%HA-*co*-10%HE).

Introduction

Poly-[(*R*)-3-hydroxyalkanoates] (PHAs) are biodegradable and biocompatible polymers of high molecular weight synthesized by a wide variety of microorganisms.[1,2] PHAs have received increased attention due to their potential applications in coatings, as medical implants, as controlled drug release systems or as a source of chiral monomers.[3−6] The mcl-PHAs (which contain medium-chain-length C_6−C_{12} alkanoate monomers) are mostly amorphous and soft-sticky with glass transition temperatures (T_g) between −44 and −30 °C and melting temperatures (T_m) between 39 and 61 °C.[7−9] Various mcl-PHAs with side chains containing functional groups, for example carbon−carbon double[10] and triple bonds,[11] acetoxy and ketone,[12] or aromatic groups[13−15] have been produced. Among the latter ones, mcl-PHAs containing phenyl groups, such as poly[(*R*)-3-hydroxy-5-phenylvalerate] (T_g = 13 °C),[13] or (*co*-)-polymers from 6-phenylhexanoic acid, 7-phenylheptanoic acid, and 8-phenyloctanoic acid (T_g between −14.8 and −1.3 °C)[16] were completely amorphous but showed an increase in the glass transition temperature.

The presence of functional groups in mcl-PHAs provides sites for chemical modifications, which may be useful to modify physical properties or to create chemical groups which cannot be directly introduced by biosynthesis. Polymer-analogous reactions on unsaturated mcl-PHAs have been studied in detail, and cross-linking,[17−19] epoxidation,[20] or conversion to carboxylic [21,22] and diol groups [23] have been reported so far.

Scheme 1. Structural Formula of Poly(HP-*co*-HA-*co*-HE)[a]



[a] Key: HP, 3-hydroxy-5-phenylvalerate; HA6, 3-hydroxyhexanoate; HA8, 3-hydroxyoctanoate; HE7, 3-hydroxy-6-heptenoate; HE9, 3-hydroxy-8-nonenoate; HE11, 3-hydroxy-10-undecenoate.

In this work, chemostatic culture conditions (also called continuous culture) were applied to produce tailored mcl-PHAs. A chemostat is essentially a perfusion reactor that is continuously supplied with sterile medium. The (culture) volume in the reactor is kept constant by the continuous removal of culture broth. Once such a system is in equilibrium (steady-state), cell number, nutrition, and productivity remain constant over time.[24] As we have shown previously,[25,26] *Pseudomonas putida* GPo1 can be grown under simultaneous limitation by carbon (C) and nitrogen (N) substrates in a chemostat. Under such growth conditions all fed C- and N-substrates are completely metabolized and therefore no toxicity of C-substrates is detected.[27]

In this study, different mixtures of octanoic, 10-undecenoic, and 5-phenylvaleric acid were used to produce mcl-PHAs (Scheme 1) in a chemostat under dual-(C,N)-limited growth conditions. This concept allowed the tailored synthesis of novel, olefinic PHAs

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with increased glass transition temperatures due to the integration of phenyl groups.

Materials and Methods

P. putida GPo1 (ATCC 29347) (formerly *Pseudomonas oleovorans*) was kept as frozen stock at -80 °C in 15% glycerol and was used for all experiments. For the preparation of inocula for continuous cultures, 1 mL frozen stock culture was added to 100 mL of minimal medium in 300 mL shake flasks and grown overnight at 30 °C. One liter of minimal medium contained 3.5 g of $NaNH_4HPO_4 \cdot 4H_2O$, 7.5 g of K_2HPO_4, 3.7 g of KH_2PO_4, and 2.9 g of Na_3(citrate)$\cdot 2H_2O$.[26] The pH was adjusted to 7.1 with 10 M NaOH. This medium was autoclaved and subsequently supplemented with filter sterilized $MgSO_4 \cdot 7H_2O$ (1 mL L^{-1}, 1 M) and 1 mL L^{-1} of MT (mineral trace element) stock solution which contained per liter: 2.78 g of $FeSO_4 \cdot 7H_2O$, 1.47 g of $CaCl_2 \cdot 2H_2O$, 1.98 g of $MnCl_2 \cdot 4H_2O$, 2.81 g of $CoSO_4 \cdot 7H_2O$, 0.17 g of $CuCl_2 \cdot 2H_2O$, and 0.29 g of $ZnSO_4 \cdot 7H_2O$ in 1 M HCl.[26] Fifty milliliters of shake-flask culture was used to inoculate the bioreactor.

For continuous cultivation the following medium was used (per liter): 1 g of KH_2PO_4, 0.71 g of $(NH_4)_2SO_4$, and 0.25 g of $MgSO_4 \cdot 7H_2O$. Further, 1 L of the medium was supplemented with 1 mL of 10 mM $FeSO_4 \cdot 7H_2O$ (in 1 M HCl) and 1 mL continuous culture mineral trace element (CCMT) stock solution containing per liter: 1.47 g of $CaCl_2 \cdot 2H_2O$, 1.98 g of $MnCl_2 \cdot 4H_2O$, 2.81 g of $CoSO_4 \cdot 7H_2O$, 0.17 g of $CuCl_2 \cdot 2H_2O$, 0.29 g of $ZnSO_4 \cdot 7H_2O$, and 10 g of EDTA at pH 4.[26] Forty liters of this medium was then filter sterilized into γ-sterilized 50 L medium bags (Flexboy, Stedim S.A., Aubagne Cedex, France). Specific mixtures of octanoic, 10-undecenoic, and 5-phenylvaleric acid were pumped directly into the culture vessel by using a dosimat (Metrohm, Herisau, Switzerland).

Cultivation Conditions. All continuous culture experiments were performed in a 3 L laboratory bioreactor (KLF 2000, Bioengineering, Wald, Switzerland) with a working volume of 2.5 L. The dilution rate (D) was set to 0.1 h^{-1}, which means that each hour 10% of the working volume is removed and continuously replaced with sterile minimal medium. The nitrogen content of the minimal medium was 150 mg of nitrogen L^{-1}. In contrast, the carbon feed rates of the continuous cultures were altered for each experiment in order to obtain particular carbon-to-nitrogen ratios (C_0/N_0) in the feed medium. The cultures were run at 30 °C, and the pH was maintained at 7.0 ± 0.05 by automated addition of either 2 M NaOH or 2 M H_2SO_4. The dissolved oxygen tension was monitored continuously with an oxygen probe (Mettler Toledo, Greifensee, Switzerland) and care was taken that it remained above 35% air saturation. The culture volume was kept constant with an overflow tube that was connected to a continuously running harvest pump. The culture was collected in a 10 L harvest tank, which was placed in a refrigerator (4 °C).

Sample Preparation. Cells were spun down at 4500 × g for 15 min at 4 °C. The pellet was washed with nanopure water, lyophilized for 48 h, and stored in a desiccator. Samples of 20 mL of culture supernatant were stored at −20 °C before further analyses of residual nutrient concentrations.

Cell Dry Weight (CDW). Cells were collected on preweighed polycarbonate filters (pore-size 0.2 μm, Nuclepore, Sterico AG, Dietikon, Switzerland). The filters were first washed with 10 mM $MgCl_2$, dried overnight at 110 °C, cooled in a desiccator over silica gel, and weighed. An appropriate volume of cell suspension (5−10 mL) was then filtered through the preweighed filter. The filters were dried again at 110 °C overnight, and the weight difference was used to calculate the concentration of the biomass in the culture.

Analyses of the Substrate Concentrations in the Culture Supernatant. Ammonium was measured by using a photometric ammonium test (Spectroquant, Merck, Darmstadt, Germany). The detection limit of this method was 0.01 mg L^{-1} NH_4−N. The method was linear up to concentrations of 3.0 mg L^{-1} NH_4−N. If necessary, samples were diluted in nanopure water. Octanoate, 10-undecenoate, and 5-phenylvalerate were measured by a modified gaschromatographic method.[6,10] Samples were acidified by mixing an equal volume of 15% v/v sulfuric acid in ethanol after adding 2 g L^{-1} benzoic acid methylester as internal standard. They were directly injected (column: DB5, 30 m × 0.32 mm, MSP Friedli & Co, Koeniz, Switzerland) and measured with a flame-ionization detector.

PHA Extraction. PHA was extracted directly from the lyophilized cells. Cells were pulverized and transferred into pure methylene chloride (60 g CDW in 1 L methylene chloride). After the suspension was stirred overnight, the solution was filtered and concentrated by distillation at 60 °C until the solution became viscous. The polymer was then precipitated into ice-cold methanol (final ratio (v/v) of CH_2Cl_2/MeOH = 1:6). After removal of the solvents by filtration, the PHA was vacuum-dried (30 °C, 30 mbar) for at least 1 day.

PHA Analysis. The cellular content and the monomeric composition were determined by gas chromatography as well as by NMR spectroscopy. For gas chromatography a known amount of 4−9 mg of lyophilized cells were transferred into a 10 mL Pyrex tube with a Teflon cap. Two milliliters of chloroform containing 0.1 mg mL^{-1} methylbenzoate as internal standard and 2 mL of 15% H_2SO_4 v/v in methanol was added for the methanolysis at 100 °C for 150 min. Subsequently, the sample was cooled on ice, and 1 mL of nanopure water was added. The aqueous phase was removed and the resulting organic phase was dried by adding Na_2SO_4. This sample was used for injection in the GC (GC 8575 MEGA 2, Fisons Instruments, Rodano, Italy; column DB 5, 30 m × 0.32 mm, MSP Friedli & Co., Koeniz, Switzerland; split ratio 1:10, initial temperature 80 °C, 8 °C/min to 300 °C). The efficiency of the methanolysis was tested by using 1 mg of extracted PHA from octanoate which was treated in the same way and was used for calibration.

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ASX-400 NMR spectrometer at 300 K. For proton (^{1}H) NMR spectra 10 mg of polymer were dissolved in 0.7 mL chloroform-*d*, and 20−30 mg of polymer were used for carbon (^{13}C) spectra. Chemical shifts are given in parts per million (ppm) relative to the signal of chloroform as internal reference (^{1}H NMR, 7.26 ppm; ^{13}C NMR, 77.7 ppm). The ^{1}H (^{13}C) NMR spectra were recorded at 400.13 MHz (100.61 MHz) with the following parameters: 5 μs (3 μs) 45° pulse lengths, 32 (10K) transients, 3800 Hz (22400 Hz) spectral widths, 32K (64K) data points, and 10 s (5 s) relaxation delays. The ^{13}C NMR spectra were recorded in the inverse gated mode.

For the determination of T_g and T_m, differential scanning calorimetry (DSC) was used. Samples of 8−14 mg PHA were weighed into aluminum pans and analyzed with a DSC 30 (Mettler Toledo, Greifensee, Switzerland). The samples were cooled to −80 °C within 10 min. After temperature equilibration, the sample was heated to 100 °C at a heating rate of 10 °C/min. Molecular weights (number-average (M_n) and weight-average (M_w)) were determined by gel permeation chromatography (GPC, Waters 150, Milford MA) equipped with a RI detector. The system was calibrated by using 10 polystyrene standards with known M_w (2×10^3 to 2.13×10^6 g mol^{-1}) and low molecular weight distributions ($M_w/M_n \leq 1.09$). Forty milligrams of every sample were dissolved in 10 mL of THF for 2 h. Aliquots of 100 μL of the polymer solution were chromatographed with pure THF as the solvent phase through two GPC columns (Mixed-Bed, Viscothek, Houston, TX) at a flow rate of 1 mL min^{-1}.

Results and Discussion

For the determination of the dual nutrient limited growth regime, *P. putida* GPo1 was grown in a chemostat at a dilution rate of 0.1 h^{-1}. The C_0/N_0 ratio in the feed medium was changed by variation of the carbon concentration in the feed medium (a constant mixture of 55 mol % octanoate, 10 mol % 10-undecenoate, and 35 mol % 5-phenylvalerate) only; the nitrogen supply was kept constant at N_o = 150 mg nitrogen L^{-1}. The

Table 1. Compositions of the Carbon Mixtures Fed to the Continuous Cultures (D = 0.1 h^{-1}; C_0/N_0 = 15 g g^{-1}) and of the Resulting Polymers

Experimental conditions and results		A	B	C	D		E
C-source feed (mol %)	5-phenylvalerate		5	15	25		35
	octanoate	90	85	75	65		55
	10-undecenoate	10	10	10	10		10
cell and PHA production							
cell dry weight (g L^{-1})		1.05	0.98	1.02	1.05		1.06
PHA content during steady state (% CDW)		29	27	18	16		20
PHA composition							
poly(HP-*co*-HA-*co*-HE) (mol %)[a]	HP[b]	-	3	19	42		59
	HA = (HA6 + HA8)[b]	90	81	65	47	HA6 = 6.3[c]	31
						HA8 = 40.7[c]	
	HE = (HE7 + HE9 + HE11) [b]	10	16	16	11	HE7 = 2.5[c]	10
						HE9 = 6.8[c]	
						HE11 = 1.7[c]	

[a] From ^{1}H NMR spectra. [b] See Scheme 1. [c] Polymer D, from ^{13}C NMR spectra.

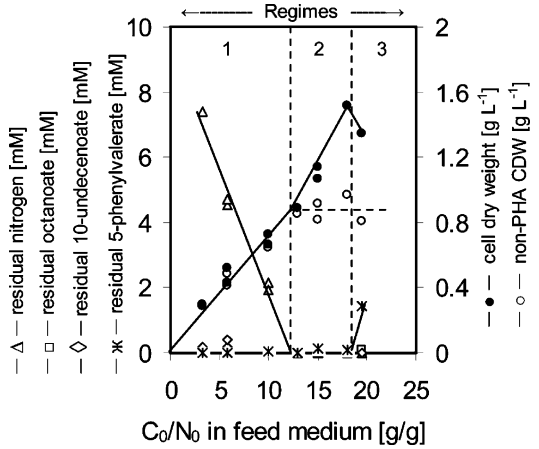

Figure 1. Growth and PHA content of *P. putida* GPo1 in a chemostat (D = 0.1 h^{-1}) at different C_0/N_0 ratios in the feed medium. A constant mixture of C source (55 mol % octanoate, 10 mol % 10-undecenoate, and 35 mol % 5-phenylvalerate) was used.

medium was designed in such a way that only nitrogen and carbon could limit growth, all other nutrients being in excess.

Growth Regimes. Three different growth regimes were found by increasing the carbon concentration in the feed medium, in agreement with earlier findings by Durner et al.[26] The first growth regime (Figure 1) went from a C_0/N_0 ratio of 0 to 12.1 g g^{-1}. In this regime, the cells grew under carbon limitation. A linear increase of the cell dry weight (CDW) was observed by increasing the carbon concentration in the feed medium. Thus, the concentration of bacteria in the culture broth increased with increasing C_0/N_0 ratios. A concomitant decrease of the residual nitrogen concentration in the culture supernatant occurred, since a higher concentration of microorganisms consumed more nitrogen. In the second regime, which began at C_0/N_0 = 12.1 g g^{-1} and went as far as C_0/N_0 = 18.3 g g^{-1}, no residual nitrogen was detected in the culture supernatant, indicating that growth was limited by carbon and nitrogen simultaneously. Thus, neither residual nitrogen nor residual carbon (octanoate, 10-undecenoate or 5-phenylvalerate) was found in the culture supernatant in this dual-(C,N)-limited regime. In fact, the cell dry weight continued to increase and even showed a higher yield in this second regime than under single carbon limitation: the biomass yield $Y_{X/C}$ (which is the yield coefficient of grams of biomass formed in the culture per grams of carbon substrate used) [24] was calculated and increased from $Y_{X/C}$ = 0.47 g g^{-1} in regime 1 to $Y_{X/C}$ = 0.58 g g^{-1} at a C_0/N_0 ratio of 18.0 g g^{-1}. This can be explained by the fact that there was very little PHA formation in regime 1. In this growth regime, 5-phenylvalerate was completely metabolized to 3-phenylpropionate, which has been reported not to be a growth substrate for *P. putida* GPo1.[13] Therefore, under C-limited growth conditions, only two carbon atoms from 5-phenylvalerate were used for biomass production. In regime 2, on the other hand, 5-phenylvalerate served not only as growth substrate, but was also incorporated into PHA as 3-hydroxy-5-phenylvalerate. Consequently, a higher fraction of the carbon atoms of this compound was used, and the carbon yield was higher in the dual-(C,N)-limited growth zone.

In regime 3, where the carbon input increased above a C_0/N_0 ratio of 18.3 g g^{-1}, unutilized 5-phenylvalerate was detected in the culture supernatant and the biomass concentration decreased slightly, which was also observed in a previous study.[25] This can be explained by a decreased cell viability due to a toxic effect of 5-phenylvalerate.[28]

The PHA-free biomass remained approximately constant in the dual-nutrient-limited (regime 2) and the nitrogen-limited growth regime 3. The cells accumulated significant amounts of mcl-PHA in growth regimes 2 and 3, increasing from about 4 wt % at a C_0/N_0 ratio of 13 g g^{-1}, to 36−40 wt % at the boundary of regimes 2 and 3.

Biosynthesis of Tailored, Olefinic mcl-PHAs. *P. putida* GPo1 was then grown on five different substrate mixtures of octanoate, 10-undecenoate, and 5-phenylvalerate in the feed medium at a constant C_0/N_0 ratio of 15 g g^{-1} (Table 1, experiments A−E), which is well within the dual limited growth zone (see Figure 1). The content of 10-undecenoate in the carbon feed was kept constant at 10 mol %, whereas the feed content of 5-phenylvalerate was gradually increased from 0 to 35 mol %, and octanoate was decreased from 90 to 55 mol %.

Because of the β-oxidation of the carbon sources to PHA precursors with one or more C_2-units less, PHA copolymers consisting of 3-hydroxyphenylvalerate (HP, Scheme 1), 3-hydroxyalkanoates (HA6, HA8), and 3-hydroxyalkenoates (HE7, HE9, HE11) were produced. No 3-hydroxy-3-phenylpropionate was found in the accumulated polymers. This agrees with the findings of Fritzsche et al.,[13] who showed that neither 3-phenylpropionate nor 3-hydroxy-3-phenylpropionate was a substrate for growth or PHA formation.

The cell dry weights during steady-states did not significantly differ from experiments A to E, whereas

the PHA content of the cells decreased slightly with increasing 5-phenylvalerate content in the feed medium. We propose that this PHA decrease was caused by a shift of the dual-(C,N)-limited growth regime, because 5-phenylvalerate cannot be metabolized completely under carbon limitation. This is in contrast with the results obtained for pure *n*-alkanoic acids.[29] As a result, more carbon equivalents are required in order to yield the same biomass. In fact, Durner and co-workers [25] observed in a chemostat culture ($D = 0.1$ h^{-1}) of *P. putida* GPo1 grown on octanoic acid only that the lower boundary of the dual-(C,N)-limited growth regime was at 10.6 mol mol^{-1} (9.1 g g^{-1}) and the yield of biomass ($Y_{X/C}$) was 0.83 g g^{-1}. In the present work, we determined for the same growth conditions but a different carbon feed mixture (55 mol % octanoate, 10 mol % 10-undecenoate, and 35 mol % 5-phenylvalerate) a lower boundary at $C_0/N_0 = 12.1$ g g^{-1}, and for $Y_{X/C}$ a value of 0.47 g g^{-1}. Therefore, with increasing content of 5-phenylvalerate, the dual-(C,N)-limited growth regime shifted toward higher C_0/N_0 values. In practice, this means that for identical C_0/N_0 ratios but increasing 5-phenylvalerate contents in the feed medium, the PHA content has to decrease. The limited data set of our experiments supports this explanation (compare Table 1).

PHA Composition. The monomeric composition of poly(HP-*co*-HA-*co*-HE) was determined from ^{1}H and ^{13}C NMR spectra. The aromatic [HP], saturated [HA = (HA6 + HA8)] and unsaturated [HE = (HE7 + HE9 + HE11)] fractions were readily obtained from ^{1}H NMR spectra by integration of the aromatic signals resonating at $\delta = 7.15$ ppm, the methyl groups at 0.87 ppm, and the olefinic protons at 5.76 ppm (Table 1). The signals of the different saturated and unsaturated units overlapped in the ^{1}H NMR spectrum, but the resonances of HA6 and HA8, and these of HE7, HE9, and HE11 were separated in the ^{13}C NMR spectra (for example $\delta(-CH_3)$ of HA6 = 14.4 ppm, of HA8 = 14.6 ppm; $\delta(=CH_2)$ of HE7 = 115.9 ppm, of HE9 = 115.2 ppm, of HE11 = 114.9 ppm). Integration of these (small) resonances yielded the ratios HA6:HA8 and HE7:HE9:HE11, and with the data from ^{1}H NMR, the complete composition could be estimated. As an example, the composition of poly(HP-*co*-HA-*co*-HE) from experiment D is shown in Table 1.

The fraction of HE units (10−16 mol %) in the polymers from experiments A−E approximately reflected the constant 10 mol % 10-undecenoate of the feed media. However, the content of HP was larger compared to the content of 5-phenylvalerate in the feed. For example, cells fed with 35 mol % 5-phenylvalerate, 55 mol % octanoate, and 10 mol % 10-undecenoate produced a polymer with 59 mol % HP units, 31 mol % HA units, and 10 mol % HE units. The increase of the HP content was accompanied by a strong decrease of the HA units. Figure 2 illustrates these changes in polymer composition.

The increase of the HP unit, the decrease of the HA units and the almost constant amounts of HE units may be explained by the different substrate affinity to the carbon sources used in these experiments. It is known that octanoic acid is a good growth substrate for *P. putida* GPo1, whereas this strain grows only slowly on 5-phenylvaleric acid.[13,26] Thus, the consumption of octanoic acid during steady-state conditions is faster than the consumption of 5-phenylvaleric acid, leading to the observed changes of polymer composition from experiments A−E.

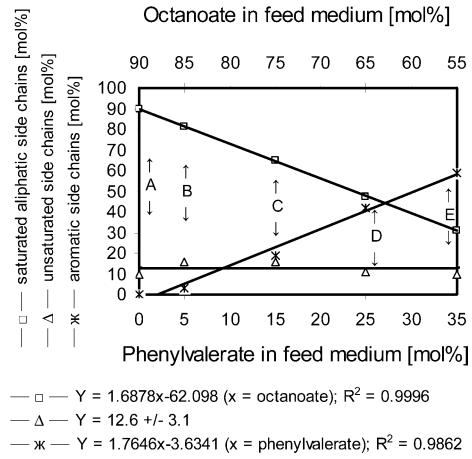

Figure 2. Linear regressions of the copolymer contents of poly(HP-*co*-HA-*co*-HE) produced in continuous cultures ($D = 0.1$ h^{-1}; $C_0/N_0 = 15$ g g^{-1}) with 10-undecenoate (10 mol %), 5-phenylvalerate (0−35 mol %), and octanoate (90−55 mol %), respectively.

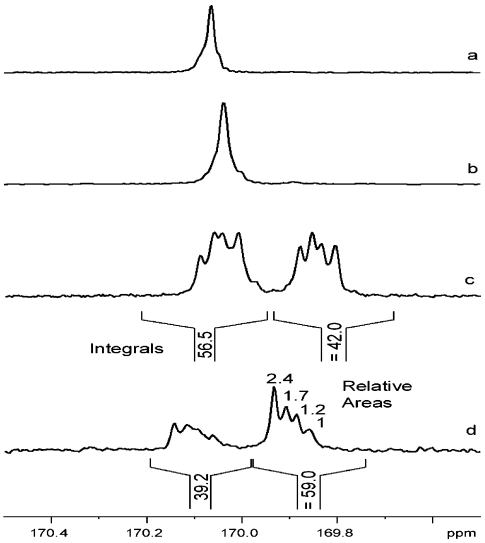

Figure 3. ^{13}C NMR carbonyl chemical shift region of mcl-PHAs from (a) feed 100 mol % octanoate, (b) experiment A, (c) experiment D, and (d) experiment E.

^{13}C NMR spectra of the carbonyl region of various mcl-PHAs are shown in Figure 3. Figure 3a shows a single carbonyl resonance at 170.07 ppm for poly(HA6-*co*-HA8) (a polymer that was produced from octanoate as the only carbon source). The same is true for the carbonyl carbons (170.04 ppm) of poly(90%HA-*co*-10%HE) from experiment A, as shown in Figure 3b. For polymers containing 5-phenylvalerate units, such as poly(42%HP-*co*-47%HA-*co*-11%HE) and poly(59%HP-*co*-31%HA-*co*-10%HE) from experiments D and E, the carbonyl region was split into two groups of four peaks (Figure 3, parts c and d). The splitting can be related to the sensitivity of the carbonyl carbons to their chemical environment and reflects the sequence distribution of the monomeric units along the polymer chain. By comparison with the chemical shifts of poly(HA6-*co*-HA8) and poly(90%HA-*co*-10%HE), the group of four lines at 170.05 ppm (Figure 3c) and 170.10 ppm (Figure 3d) were assigned to the HA and HE units, while the four lines at 169.84 ppm (Figure 3c) and 169.9 ppm (Figure 3d) came from HP monomeric unit. This assignment was confirmed by the area of the NMR signals; in Figure 3c, the intensity of the HP unit was set to 42.0

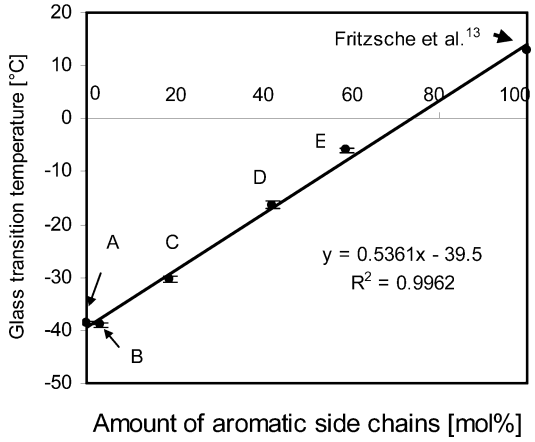

Figure 4. Glass transition temperature (T_g) as a function of the monomeric composition.

Table 2. GPC Data of the Five Polymers (A−E) Produced in Continuous Cultures

polymer	M_w	M_n	M_w/M_n
A	191 000	86 000	2.2
B	246 000	120 000	2.1
C	227 000	104 000	2.2
D	214 000	83 000	2.6
E	358 000	124 000	2.9

(mol %, Table 1), with a resulting intensity of 56.5 (mol %) for HA and HE, close to the expected value of 58 (mol %) for the sum of the fractions of HA (47 mol %) and HE (11 mol %). The same was true for poly(59%HP-*co*-31%HA-*co*-10%HE) in Figure 3d. The splitting into four lines was attributed to triads HP(HA/HE)*HP, HP(HA/HE)*(HA/HE), (HA/HE)(HA/HE)*HP, and (HA/HE)(HA/HE)*(HA/HE) at high frequency, and to HPHP*HP, HPHP*(HA/HE), (HA/HE)HP*HP, and (HA/HE)HP*(HA/HE) at low frequency. The resolution of the (HA/HE) centered triads was poor, but the HP unit of poly(59%HP-*co*-31%HA-*co*-10%HE) could be fit to a sum of four Lorentzian lines with relative intensities of approximately 2.4:1.7:1.2:1. The calculated intensities for a statistically random copolymer are 2.1:1.4:1.4:1. The experimental intensities deviate only slightly from the values expected for a random copolymer, and the differences may be the result of the poor spectral resolution and the rather low NMR signal-to-noise ratio. Therefore, the observed line splitting in the NMR spectra let us conclude that *P. putida* GPo1 incorporated 5-phenylvalerate, octanoic acid, and 10-undecenoic acid without any preferential sequence, producing a statistically random copolymer.

Physical Properties. Molecular weights (number-average (M_n) and weight-average (M_w)) as well as the molecular weight distribution (M_w/M_n) of the different polymers A−E are shown in Table 2. M_w and M_n values were typical for molecular weights reported for mcl-PHAs,[9] with the weight-average value for sample E, containing the largest fractions of HP units, close to M_w = 350 000 for the homopolymer poly(3-hydroxy-5-phenylvalerate).[13]

Depending on the amount of 3-hydroxy-5-phenylvalerate, the polymers showed different thermal properties (Figure 4). T_g increased linearly from − 38.7 °C in polymer A to −6.0 °C in polymer E. An extrapolation to 100% aromatic side chains would lead to a glass transition temperature of about 14.1 °C for poly(3-hydroxy-5-phenylvalerate). This is in good agreement with the literature value of T_g = 13 °C for this homopolymer.[13] This means that the glass transition temperatures of mcl-PHAs with 10 mol % olefinic side chains can be predicted also for HP fractions larger than 59 mol % (as reported in this work) over a total range of approximately 50 °C. The dependence of T_g on the fraction of phenyl groups parallels the findings for several copolymers of poly(3-hydroxybutyrate-*co*-X) with X = 3-hydroxypropionate, 4-hydroxybutyrate, 3-hydroxyvalerate, and 3-hydroxyhexanoate, where the values of T_g of all copolymers decreased linearly with the increase of the second monomer unit content.[30]

Polymer A showed a melting point at 40.1 °C. In contrast, polymers of experiments B−E containing phenyl groups did not display any crystalline melting endotherm. It is known that the homopolymer poly(3-hydroxy-5-phenylvalerate) as well as phenyl group bearing (co-)polymers from 6-phenylhexanoate, 7-phenylheptanoate, and 8-phenyloctanoate are amorphous.[14,16] We found here that the incorporation of as little as 5 mol % phenyl groups leads to completely amorphous olefinic mcl-PHAs. On the other hand, crystalline mcl-PHAs have been produced from 5-(4-tolyl)valeric acid and 8-(*p*-methylphenoxy)octanoic acid.[14,15] As a natural extension of this work, it would be interesting to use such aromatic containing substrates for the production of polymers of type poly(3-hydroxy-5-(4-tolyl)valerate-*co*-HA-*co*-HE). This would enable the production of crystalline mcl-PHAs with olefinic side chains, where not only T_g, but also T_m can be adjusted in a predictable way over a wide temperature range.

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