Lipase-Catalyzed Ring-Opening Polymerization of Lactones: A Novel Route to Poly(hydroxyalkanoate)s

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Received November 29, 1995; Revised Manuscript Received March 26, 1996[⊗]

ABSTRACT: Lipases from porcine pancreas and from *Pseudomonas cepacia* catalyze the ring-opening polymerization of lactones. Polymerization of the four-membered β-butyrolactone yielded poly(3-hydroxybutyrate) in up to 89% yield with a degree of polymerization of 3−12. Polymerization of the four-membered β-propiolactone, the five-membered γ-butyrolactone, and the seven-membered ϵ-caprolactone also yielded the corresponding polyesters in good yields with degrees of polymerization up to 25. Coordination catalysts, such as alumoxanes, fail in the homopolymerization of γ-butyrolactone; thus this lipase route is the first homopolymerization of γ-butyrolactone. To characterize the product polyesters, we used the method of MALDI-TOF mass spectroscopy to determine both the true molecular weight of the components and the mass of the repeat unit.

Introduction

Many bacteria biosynthesize inclusions of poly(hydroxyalkanoate)s (PHAs) as a carbon reserve.[1−3] These high molecular weight polyesters are biodegradable and have attracted commercial interest as a possible replacement for polyolefins in some applications. The most frequently encountered PHA is poly(3-hydroxybutyrate) (PHB), which is a highly crystalline thermoplastic; however bacteria can make a wide variety of PHAs, as summarized in Figure 1. Researchers[4] have identified more than 90 different repeat units in naturally occurring PHAs. These PHAs differ from one another in the length and chemical composition of the side chain at the stereocenter and in the number of methylene groups in the polymer backbone.

A wide variety of PHA homopolymers and copolymers are being explored to find the ones best suited for commercial developments in packaging, coatings, adhesives, and drug delivery. Currently, the only commercial route to PHB is bacterial fermentation. Our aim was to prepare PHAs by a lipase-catalyzed ring-opening polymerization. Since lipases can accept a wide range of substrates, a lipase-catalyzed polymerization in an organic solvent may allow synthesis of a wide range of polymers, including chiral polymers.

Researchers have already demonstrated that lipases can catalyze polymerization to polyesters[5,6] by condensation,[7−11] by transesterification,[12−23] or by ring-opening polymerization.[19,24−28] In simple condensation reactions, researchers often condensed either a hydroxy acid or a diol with a diacid. For example, O'Hagan and Zaidi[9] polymerized 10-hydroxydecanoic acid with *Candida cylindracea* lipase to yield the corresponding polyester. Binns and co-workers[11] condensed adipic acid with butane-1,4-diol to form a polyester with an average of 20 repeat units.

Other researchers used transesterification reactions where an ester reacts with an alcohol to form a new ester and alcohol. For example, Geresh and co-workers[16−18] produced alkyd polyesters from fumarate diesters and diols. Although the molecular weights were less than 5000, the products had higher crystallinity and melting points than the corresponding chemically synthesized polymers. In another example, Wallace and Morrow[13] enantioselectively polymerized chiral,

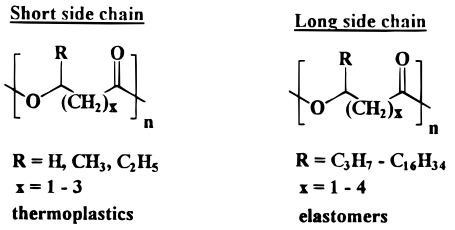


Figure 1. General structural formulas of bacterial poly(hydroxyalkanoate)s.

epoxide-substituted diesters with 1,4-butanediol, giving polyesters with 96% stereochemical purity and molecular weights of ∼5000. A disadvantage of both the simple condensation route and the transesterification route is the release of 1 equiv of water or alcohol in each condensation step. This release may limit the molecular weight of the polymers.

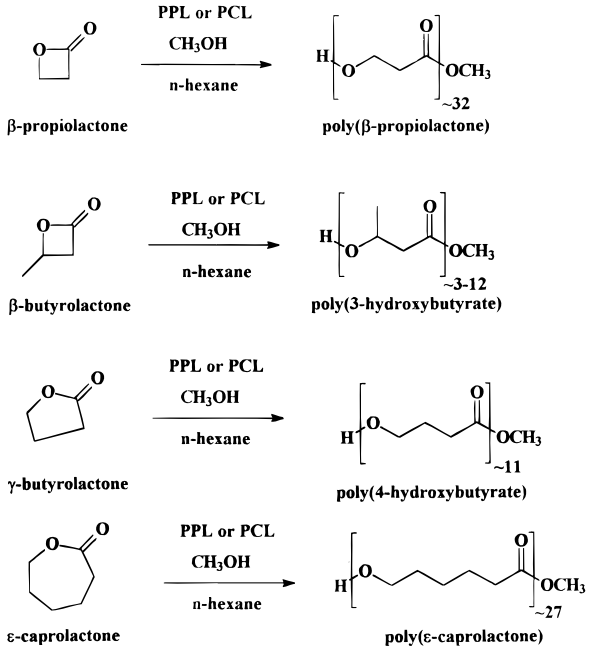
The ring-opening polymerization is a special case of transesterification polymerization which does not release a molecule of alcohol. Three groups, Knani et al.,[19] Uyama et al.,[24,25] and MacDonald et al.,[26] reported the polymerization of ϵ-caprolactone to the corresponding polyester with molecular weights of up to 7700. Uyama and Kobayashi also polymerized the six-membered δ-valerolactone to a low molecular weight polyester[24,25] and macrolides to polyesters.[27] In all cases, the alcohol portion is a primary alcohol.

In this paper, we report the first lipase-catalyzed ring-opening polymerization of the four-membered β-butyrolactone. In this case, the alcohol portion is the more hindered secondary alcohol. In addition, we examine the effect of ring size on the lipase-catalyzed polymerization and report the first polymerization of the four-membered β-propiolactone and the five-membered γ-butyrolactone (Scheme 1). To characterize the polyesters, we used matrix-assisted laser desorption and ionization time of flight mass spectroscopy (MALDI-TOF MS).[29−31] Biochemists often use this simple method for measuring the true molecular weights of unfragmented large biomolecules. Polymer chemists have just started to exploit this technique for the characterization of polymers. Recently, Bürger et al. characterized partially hydrolyzed bacterial PHB using MALDI-TOF MS.[32] MALDI is a soft ionization technique so the polymer is not fragmented by the ionization process. Analysis is

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1996.

S0024-9297(95)01774-8 CCC: $12.00

Scheme 1. Lipase-Catalyzed Ring-Opening Polymerizations Discussed in This Paper[a]



[a] PPL = lipase from porcine pancreas, PCL = lipase from *Pseudomonas cepacia*.

very rapid and yields true molecular weights and also shows the mass of the repeat unit.

Materials and Methods

Materials. Crude porcine pancreatic lipase (PPL; catalog no. L-3126, approximately 25 wt % protein), lipase from *Candida rugosa* (*C. cylindracea*), and lipase from *Pseudomonas* sp. were obtained from Sigma Chemical Co. (St. Louis, MO). *Pseudomonas cepacia* lipase (PCL; Lipase PS30) was obtained from Amano Enzyme USA Co. (Troy, VA). ϵ-Caprolactone, β-propiolactone (technical grade 90%), γ-butyrolactone, (±)-β-butyrolactone, and chloroform (HPLC grade) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Analytical grade *n*-hexane was obtained from Canlab-Baxter (Pointe-Claire, PQ). Anhydrous methanol was obtained from BDH Inc. (Ville St.-Laurent, PQ). All materials were used as received.

Lipase-Catalyzed Polymerization of (±)-β-Butyrolactone. Lipase from porcine pancreas (PPL, 3.00 g) was added slowly to a magnetically stirred solution of (±)-β-butyrolactone (1.63 mL, 0.020 mol) and methanol (0.016 mL, 0.40 mmol, 50:1 molar ratio) in *n*-hexane (20 mL). The resulting suspension was shaken in a rotary shaker at 300 rpm at 60 °C for 500 h. To monitor the progress of the reaction, we removed an aliquot of 2 mL and measured the decrease in the carbonyl stretch of the lactone at 1833 cm^{-1} by FTIR spectroscopy. The aliquot was returned to the reaction mixture after FTIR analysis. When the decrease slowed significantly, the enzyme was removed by filtration and washed with *n*-hexane (1 × 20 mL) and chloroform (2 × 20 mL). The combined filtrate and washes were diluted with methanol (4 °C, 800 mL) and concentrated by rotary evaporation to an off-white solid (1.34 g). MALDI-TOF MS showed this product to have a weight-average molecular weight of 779 with an average monomer repeat mass of 86, thus confirming the structure of PHB. In addition, peaks were seen from 274 (corresponding to trimer including the methoxy end group) to 1200 (corresponding to 14-mer).

The other lactones were polymerized using the same procedure. β-Propiolactone: monitored at 1717 cm^{-1} in the IR; ^{13}C-NMR ($CDCl_3$, 75.4 MHz) δ 170.3 (CO), 60.1 (OCH_2), 58.2 (OCH_3), 33.7 (CH_2); MALDI-TOF MS, a distribution of peaks was observed from 1870 (corresponding to 26-mer) to 4110 (corresponding to 57-mer), with a weight-average molecular weight of 2323 and an average monomer repeat mass of 72. γ-Butyrolactone: monitored at 1771 cm^{-1} in the IR; ^{13}C-NMR ($CDCl_3$, 75.4 MHz) δ 169.8 (CO), 68.5 (OCH_2), 63.6 (OCH_3), 27.8 (CH_2), 22.1 (CH_2); MALDI-TOF MS, a distribution of peaks was observed from 175 (corresponding to dimer) to 1370 (corresponding to 16-mer), with a weight-average molecular weight of 932 and an average monomer repeat mass of 86. ϵ-Caprolactone: monitored at 1730 cm^{-1} in the IR; ^{13}C-NMR ($CDCl_3$, 75.4 MHz) δ 173.5 (CO), 64.1 (OCH_2), 34.1 (CH_2), 28.3 (CH_2), 25.5 (CH_2), 24.5 (CH_2); ^{1}H-NMR (270 MHz) δ 1.37 (m, CH_2), 1.60 (m, CH_2), 1.67 (m, CH_2), 2.29 (t, CH_2), 3.65 (s, OCH_3), 4.04 (t, CH_2); MALDI-TOF MS, a distribution of peaks was observed from 1145 (corresponding to 10-mer) to 4910 (corresponding to 43-mer), with a weight-average molecular weight of 2902 and an average monomer repeat mass of 114. When PCL was used as catalyst, the amount of enzyme was decreased to 0.8 g.

Table 1. Polymerization of b-Butyrolactone Catalyzed by Mammalian and Microbial Lipases

enzyme[a]	mon:nuc ratio[b]	solvent	temp (°C)	reacn time (h)	% yield[d]	mol wt[e]
PPL	1:1	*n*-hexane	RT[c]	210	43	346
PPL	5:1	*n*-hexane	RT	547	43	391
PPL	10:1	*n*-hexane	RT	547	41	256
PPL	20:1	*n*-hexane	RT	547	30	437
PPL	30:1	*n*-hexane	RT	547	20	437
PPL	50:1	*n*-hexane	RT	368	71	532
PPL	200:1	*n*-hexane	RT	500	32	621
PPL	50:1	bulk	RT	500	33	1045
PPL	50:1	$CHCl_3$	RT	500	45	977
PPL	50:1	*n*-hexane	69	500	nil	
PPL	50:1	*n*-hexane	60	500	82	779
PPL	50:1	isooctane	60	500	33	634
PCL	50:1	bulk	60	240	61	681
PCL	50:1	*n*-hexane	60	500	89	643
PCL	50:1	isooctane	60	500	53	673
histidine	50:1	*n*-hexane	RT	500	nil	

[a] Enzymes: PPL = porcine pancreatic lipase (crude form); PCL = Lipase PS30 (from *Pseudomonas cepacia*). [b] Molar ratio of monomer (racemic mixture of (±)-β-butyrolactone) to nucleophile (methanol). [c] RT = room temperature (typically 23−25 °C). [d] Isolated yield of combined products. [e] Weight-average molecular weights determined by MALDI-TOF mass spectroscopy using gentisic acid as the matrix. [f] Histidine (2.01 g) with 0.05 mol of β-butyrolactone.

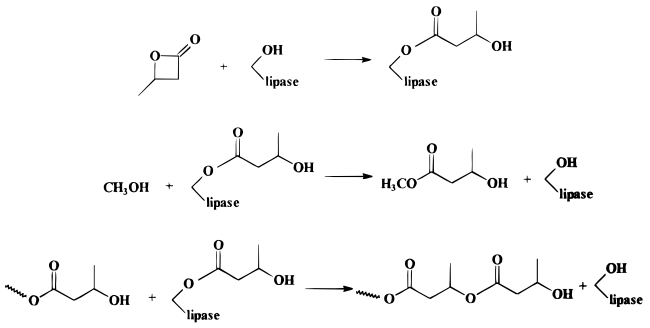
Characterization of Polymers. The crystallinity of poly(ϵ-caprolactone) was established by X-ray powder diffraction on a Philips PW 1730 instrument with a graphite monochromator. The molecular weight of poly(ϵ-caprolactone) was determined by end group analysis of the ^{1}H-NMR spectrum according to Shit and Maiti.[33]

Samples for MALDI were dissolved in chloroform to a concentration of less than 0.1 mM and mixed with an equal volume of matrix (gentisic acid dissolved in a 10:9:1 mixture of acetonitrile, ethanol, and water). An aliquot (<1 μL) was applied to the sample slide, the solvent was evaporated by vacuum, and the slide inserted into the spectrometer (MALDI III; Kratos Analytical).

Results

Both lipase from porcine pancreas (PPL) and lipase from *Pseudomonas cepacia* (PCL) catalyzed the ring-opening polymerization of β-butyrolactone to poly(3-hydroxybutyrate) under a variety of conditions (Table 1). Typical reaction times were several weeks using approximately equal weights of lactone and lipase. These rates are similar to those reported by other researchers for lipase-catalyzed polymerization of ϵ-caprolactone. Reactions were monitored by the decrease in the intensity of the lactone's carbonyl stretch. No reaction occurred in the absence of lipase. Histidine alone also gave no reaction, suggesting that the lipase active site, not a histidine on the surface of the protein, catalyzes the reaction. We also tested lipases from

Scheme 2. Proposed Mechanism for Lipase-Catalyzed Ring-Opening Polymerization of (±)-β-Butyrolactone[a]



[a] The first step involves reaction of the lactone with the lipase to form an acyl enzyme intermediate. In the second step, this acyl enzyme reacts with methanol. The third step involves reaction of the acyl enzyme with the hydroxy end of the growing polymer chain.

Candida rugosa and from *Pseudomonas* sp., but observed no polymerization.

The products were amber oils or off-white solids isolated in up to 89% yield. MALDI-TOF mass spectrometry revealed weight-average molecular weights ranging from 256 to 1045, corresponding to chain lengths of 3−12 (Table 1). In addition, the MALDI-TOF mass spectrum showed that the repeat unit has a mass of 86, which confirms the assigned structure. We did not examine the enantioselectivity of the polymerization reaction.

The presumed mechanism of this reaction (Scheme 2), relies on a nucleophile, methanol, to initiate a chain. As expected, decreasing the amount of methanol relative to lactone increased the molecular weight of the product, but the increase was only moderate. The highest molecular weight was obtained using neat β-butyrolactone as the solvent in place of hexane.

The propagation step of the mechanism in Scheme 2 involves attack of a secondary alcohol on the acyl enzyme intermediate. We hypothesized that increasing the rate of this step may increase the molecular weight of the product. One way to increase the rate of this step is to replace the secondary alcohol with a less hindered primary alcohol. For this reason, we examined the lipase-catalyzed reaction of unsubstituted lactones.

In agreement with these expectations, lipase-catalyzed reaction of β-propiolactone, γ-butyrolactone, and ϵ-caprolactone yielded higher molecular weight oligomers (Table 2). MALDI-TOF mass spectrometry showed weight-average molecular weights of 874−2323 for poly(propiolactone), corresponding to 12−32 repeat units; 888−972 for poly(γ-butyrolactone), corresponding to 10−11 repeat units; and 1364−2902 for poly(ϵ-caprolactone), corresponding to 12−25 repeat units. ^{1}H- and ^{13}C-NMR spectroscopy established the structure of the products and that the expected polymers were the sole products of the reaction. For the sample of poly(ϵ-caprolactone) with a molecular weight of 2902, we also measured a molecular weight of 3100 (±200) using end group analysis by ^{1}H-NMR. The agreement of the two methods confirms that the reaction yields oligomers with methyl ester end groups, as expected for the mechanism in Scheme 2. The X-ray powder diffraction patterns for poly(ϵ-caprolactone) showed sharp lines, indicating that the product was crystalline. The molecular weights, reaction yields, and reaction times and conditions agree with those obtained by other researchers.[19,24−26]

While the mechanism proposed in Scheme 2 relies solely on methanol as the nucleophile, to produce methyl ester end groups, NMR results show that, in the case of poly(3-hydroxybutyrate), the reaction products possess a significant degree of heterogeneity. We have found that the carboxyl end groups are between two-thirds and fully esterified. The remaining end groups are carboxylic acids, indicating that the methanol can be replaced by water in the mechanism in Scheme 2. In order to determine the extent of heterogeneity, we have used ^{31}P-NMR spectroscopy with a phosphitylation method for hydroxylic and carboxylic end groups described elsewhere.[34−36] Although this method has been reported to be effective for molecular weight determination, we have found it useful for determining the nature of the end groups of our polymer products.

Discussion

Our results show that lipases can catalyze the ring-opening polymerization of β-butyrolactone in spite of the more hindered secondary alcohol. In addition, the rate of polymerization of β-butyrolactone was comparable to that of the polymerization of β-propiolactone. This result means that lipases might be used to make a wide variety of poly(hydroxyalkanoate)s.

The ability of lipases to catalyze reactions of four-membered ring lactones has been reported only recently.[37] Koichi et al. reported the PPL-catalyzed enantioselective ring-opening of β-butyrolactone with benzyl alcohol. The ability of lipases to accept four-membered ring lactones as substrates is somewhat surprising because some lipase inhibitors contain a four-membered ring lactone as the key structural element. Tetrahydrolipastatin, a candidate for anti-obesity treatment, is one such inhibitor.[38,39] However, Koichi et al.'s and our results show that four-membered lactones can be substrates for lipases, although reaction rates are slow. The inability of the amino acid histidine to catalyze polymerization suggests that catalysis occurs at the lipase active site, not at a surface histidine residue.

Our results also show that lipases can catalyze polymerization of unstrained lactones such as γ-butyrolactone. This monomer does not polymerize when alumoxane catalysts are used. The only successful homopolymerizations of five-membered lactones are of derivatives of γ-butyrolactone, such as bicyclic bis(γ-butyrolactone)s and spirocyclic (γ-butyrolactone)s under anionic double ring-opening conditions.[40] Interestingly, Gutman et al. have reported a similar reverse reaction: the PPL-catalyzed formation of γ-butyrolactones from γ-hydroxy esters.[41] As mentioned in the Introduction, lipases also catalyze the polymerization of the relatively unstrained six-membered δ-valerolactone.

Ring strain in the lactones did not significantly affect the rate or the degree of polymerization. The four-membered β-propiolactone and β-butyrolactone have high strain energies,[42] while the five-membered γ-butyrolactone has a low strain energy, 8.7−8.8 kcal/mol, and the seven-membered ϵ-caprolactone has an intermediate strain energy, 10.7 kcal/mol.[43] Nevertheless, all polymerized similarly. This independence of ring strain and polymerization suggests that ring opening is not the limiting step in the polymerization.

Although the higher molecular weights obtained from primary versus secondary alcohols as nucleophiles are consistent with attack of the alcohol as the rate-determining step, it is also possible that a nonchemical

Table 2. Polymerization of Unsubstituted Lactones Catalyzed by Mammalian and Microbial Lipases

monomer[a]	enzyme[b]	mon:nuc ratio[c]	solvent	temp (°C)	reacn time (h)	% yield[e]	mol wt [f]
ϵ-CL	PPL	1:1	*n*-hexane	RT[d]	1100	81	1364
ϵ-CL	PPL	50:1	bulk	RT	1100	45	2902
ϵ-CL	PCL	50:1	bulk	60	240	56	1442
β-PL	PPL	50:1	*n*-hexane	60	430	44	2323
β-PL	PPL	50:1	*n*-hexane	60	430	16	874
γ-BL	PPL	50:1	*n*-hexane	60	430	25	932
γ-BL	PCL	50:1	*n*-hexane	60	430	42	888

[a] ϵ-CL = ϵ-caprolactone; β-PL = β-propiolactone; γ-BL = γ-butyrolactone. [b] PPL = porcine pancreatic lipase (crude form); PCL = Lipase PS30 (from *Pseudomonas cepacia*). [c] Molar ratio of monomer (lactone) to nucleophile (methanol). [d] RT = room temperature (typically 23−25 °C). [e] Isolated yield of combined products. [f] Weight-average molecular weights determined by MALDI-TOF mass spectroscopy using gentisic acid as the matrix.

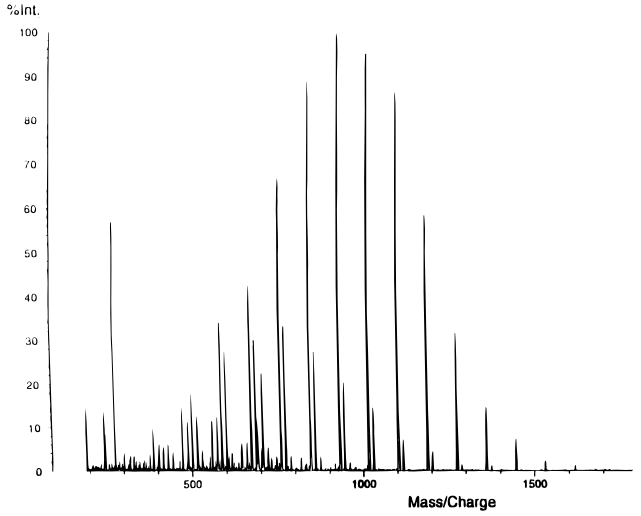


Figure 2. Positive ion MALDI-TOF mass spectrum of poly-(3-hydroxybutyrate) obtained from the lipase-catalyzed ring-opening polymerization of (±)-β-butyrolactone. The weight-average molecular weight is 977, corresponding to a degree of polymerization of about 11.

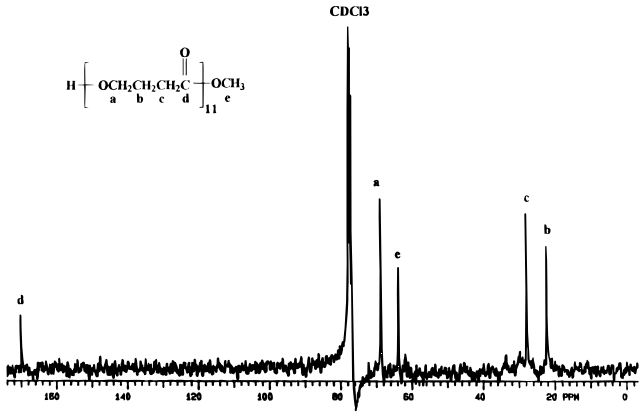


Figure 3. ^{13}C-NMR spectrum (74.5 MHz in $CDCl_3$) of poly-(4-hydroxybutyrate) obtained from the lipase-catalyzed ring-opening polymerization of γ-butyrolactone. The weight-average molecular weight is 932, corresponding to a degree of polymerization of about 11.

step limits the rate. For example, the acyl enzyme may undergo a slow conformational change between the ring-opening and the deacylation steps. Unfortunately, our understanding of the polymerization mechanism is not sufficient to suggest a strategy to further increase the rate of reaction or the molecular weights of PHAs from lipase-catalyzed reactions.

The lipase route to PHAs has several advantages over existing chemical routes. First, chemical routes use alumoxane catalysts,[44] which must be handled and used in a dry, oxygen-free atmosphere, while the lipase reactions occur on the bench with no special precautions. Second, the alumoxane route leaves catalyst residues in the polymer, while the lipase catalyst is removed by simple filtration at the end of the reaction. Third, the lipase route may be an enantioselective catalyst, although we are still examining this point. Another important advantage of the lipase route is the ability to polymerize unstrained lactone like the five-membered γ-butyrolactone.

The lipase route to PHAs has several advantages over the microbial route. First, avoiding microbial cultures eliminates problems with cell viability under conditions of depleted nutrients. Second, the PHAs are much easier to isolate from the lipase reaction. However, the microbial route yields higher molecular weight PHAs (typically >100 000) which are highly crystalline. Using the lipase route, only poly(ϵ-caprolactone) was highly crystalline. The crystallinity in the other polyesters is difficult to ascertain since the low molecular weights lead to low T_m, which affects the physical nature of the product at room temperature. However, it may also be true that the very low molecular weight material (less than 600) is noncrystalline.

Recently, the PHB synthase enzyme has been cloned and is now available in a recombinant form. This has led to the development of an in vitro polymerization of 3-hydroxybutyryl-CoA leading to PHB.[45] The resulting polymer is optically pure and is high molecular weight. While reaction times are less than those in our lipase reaction, the scale of the reaction is several orders of magnitude smaller and only PHB can be prepared by this method at this time.

Acknowledgment. We thank Ilie Saracovan for the X-ray diffractometer scans and molecular mechanics calculations and Apostolos Spyros for assistance in acquiring the NMR spectra and useful discussions. This work was supported by Xerox Corp. and the Natural Sciences and Engineering Research Council of Canada (NSERC).

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MA951774G