Growth and Polyester Production by *Pseudomonas oleovorans* on Branched Octanoic Acid Substrates

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ABSTRACT: *Pseudomonas oleovorans* was grown on 2-methyl-3-hydroxyoctane, ethyl 2-methyl-3-hydroxyoctanoate, ammonium 2-methyl-3-hydroxyoctanoate, and methyl 2-methyl-3-hydroxyoctanoate in attempts to obtain poly(3-hydroxyalkanoate)s (PHAs), with methyl branches at the 2-position. The microorganisms did not grow on these carbon sources alone but survived and could be reactivated later with organic substrates which could be used for good cell growth. Futhermore, bacterial growth on the branched carbon sources was observed when a cofeeding technique was applied, in which case cell growth showed no lag time and formed a biomass which contained a high percentage of PHAs. In this manner a variety of copolymers were obtained, and these were investigated by NMR, methanolysis–gas chromatography, and thermal analysis. With nonanoic acid as the cosubstrate, the polymers obtained contained mainly 3-hydroxynonanoate and 3-hydroxyheptanoate units; this is the copolymer to be expected from the preferred consumption of nonanoic acid. In addition, however, a low percentage of units with 2-methyl substituents could be detected by NMR, and the incorporation of these unusual repeat units resulted in an observable change in the thermal properties of the PHAs.

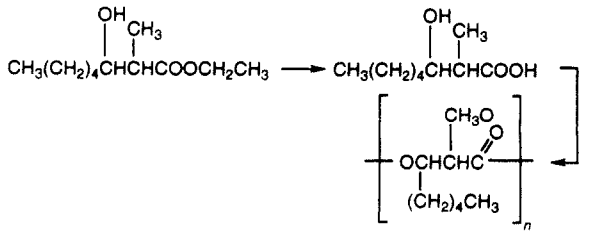
Introduction

Many different microorganisms are capable of producing poly(3-hydroxyalkanoate)s (PHAs) as internal carbon and energy storage materials.[1] Previous studies have shown that besides the commercially valuable poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), higher PHAs can also be produced by bacterial growth on appropriate substrates.[2-6] Furthermore, cell growth on organic compounds containing functional groups can result in the formation of functionalized PHAs.[7-10] During the conversion of these substrates to form PHAs, the functional groups[7-10] in the substrate can remain unattacked to a certain degree and will be present in the side chains of the polymers. In addition to their biodegradability, PHAs may be biocompatible so the polymers with functionalized groups could be of potential interest for biomedical applications.[11]

Fritzsche and co-workers[12] showed that the utilization of methyl-branched organic compounds by *Pseudomonas oleovorans* resulted in the formation of a copolymer which contained certain amounts of methyl branches in the side chain. Findlay and White[13] described bacterial polyesters recovered from marine sediments which contained iso-branched repeat units. They also discovered trace amounts of iso-branched repeat units in bacterial polyesters obtained from *Bacillus megaterium* monocultures. PHAs with methyl groups at the 2-position in the polymer backbone were first described by Satoh and co-workers.[14] While studying the anaerobic uptake mechanisms of acetate, propionate, and lactate by bacteria in activated sludge, they discovered the presence of storage polyesters containing, besides PHB and PHV, 3-hydroxy-2-methylbutyrate and 3-hydroxy-2-methylvalerate. However, the bacteria species which produced the methyl-branched polymers were not identified.

In the present study a monoculture of *P. oleovorans*, a well-investigated prokaryotic aerobic bacterium which has been proven to be capable of utilizing unusual carbon sources and transforming them into storage polyesters, has been evaluated in attempts to produce bacterial polyesters with methyl branches at the 2-position of the main chain.

The carbon sources for these experiments were selected on the basis of the following criteria or objectives: (1) Because *P. oleovorans* grows best on organic compounds containing eight or nine carbon atoms, derivatives of octanoic acid or octane were used. (2) The organic compounds with a 2-methyl substituent must retain that structure during the conversion to a PHA, which would then contain a 2-methyl branch in the polymer main chain. (3) For better solubility in the aqueous medium, an ammonium salt of the substituted octanoic acid was used. (4) When alkanoate esters are used as substrates, previous studies[15] have shown better and faster bacterial growth on the ethyl esters of octanoic acid compared to the methyl esters; consequently an ethyl 2-methyloctanoate was used. (5) To support the intracellular β-oxidation processes, organic compounds with 3-hydroxy substituents were used. Based on these considerations the following substrates were tested for PHA production by *P. oleovorans*: ethyl 2-methyl-3-hydroxyoctanoate (EtC_8), ammonium 2-methyl-3-hydroxyoctanoate (NH_4C_8), and 2-methyl-3-hydroxyoctane (C_8). It was hoped that, with these organic compounds as cosubstrates, monomer synthesis in the cell by the β-oxidation route should form the appropriate 2-methyl-substituted compound and its PHA units as shown below for the utilization of ethyl 2-methyl-3-hydroxyoctanoate:



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Experimental Section

Fermentations. Stock cultures of *P. oleovorans* (ATCC 29347) were used throughout the experiments. The strain was maintained at 4 °C on nutrient agar plates using modified E* medium with 10 mM concentration of either sodium octanoate or sodium nonanoate as the carbon source.[16] The bacteria were cultivated in a mineral medium (modified E* medium) consisting of 1.1 g/L $(NH_4)_2HPO_4$, 5.8 g/L K_2HPO_4, 3.7 g/L KH_2PO_4, 10.0 mL/L of a 100 mM $MgSO_4$ solution, and 1.0 mL/L of a microelement solution. The latter is a 1 N HCl solution containing 2.78 g/L $FeSO_4 \cdot 7H_2O$, 1.67 g/L $CaCl_2 \cdot 2H_2O$, 0.17 g/L $CuCl_2 \cdot 2H_2O$, 0.29 g/L $ZnSO_4 \cdot 7H_2O$, 1.98 g/L $MnCl_2 \cdot 4H_2O$, and 2.81 g/L $CoSO_4 \cdot 7H_2O$. The esters of the alkanoic acids were added at concentrations of 20 and 100 mM. The pH was adjusted to 7.0.

For the preculture, 200 mL of modified E* medium, using sodium octanoate as the carbon source, was autoclaved at 120 °C and 18 lb/in.^{2} for 20 min (Amsco Laboratory autoclave). After the culture solution was cooled to 30 °C, bacteria were inoculated from the plate by a sterile procedure. After 14–17 h the preculture was ready to use as an inoculum for the cultures. The growth of all cultures was examined by recording the optical density of the solution spectrometrically with a Spectronic 20 Bausch and Lomb at a layer thickness of 1 cm, referenced to distilled water.

Fermentations were carried out under aerobic, oxygen-limiting conditions in 1 L cultures using 2.8 L Fernbach flasks. The E* medium was autoclaved under the same conditions as described above for 30 min. After the flasks were cooled to 30 °C, the carbon source and inoculum were added. The carbon sources were not autoclaved to prevent hydrolysis of the ester groups or chemical rearrangements. The volume of the preculture solution added to the cultures was 1/10 of the culture volume. Oxygen-limiting conditions during the fermentation were achieved by using a cotton plug and aluminum foil to cover the flasks. To guarantee aeration of cultures, the flasks were continuously shaken in a Lab-line incubator shaker at 250 rpm and 30 °C.

Since the carbon sources under investigation have to be considered as poor growth and polymer production supporting carbon sources, the coefeeding technique as described in detail in ref 15 has been applied. An equimolar mixture of the branched substrate was cofed with a good growth and polymer production supporting carbon source, nonanoic acid.

The methyl-branched carbon sources 2-methyl-3-hydroxyoctane, ethyl 2-methyl-3-hydroxyoctanoate, ammonium 2-methyl-3-hydroxyoctanoate, and methyl 2-methyl-3-hydroxyoctanoate were synthesized via a Grignard reaction in our laboratory by Prof. B. Hazer.

Cells were harvested after their growth reached the stationary phase and separated from the supernatant by centrifugation (Sorvall RC-3, 4 °C, 4000*g*), and the biomass was frozen in liquid nitrogen to disrupt all cellular activity and to prevent the intracellular consumption of storage polyesters by the bacteria. Finally, the cells were lyophilized (Labonco Freeze Dry System −50 °C; 10–50 μmHg).

The polymer was extracted from the lyophilized cells by refluxing in 100–150 mL of chloroform for 12 h. After the cellular material was filtered, the solvent was removed by evaporation until a remaining residue of concentrated polymer solution of 1–5 mL was obtained. The polymer was precipitated into 50–70 mL of vigorously stirred methanol, collected by filtration, and dried at room temperature under vacuum for 12 h.

Polymer Characterization. For analysis of the repeating units of the polymers by gas chromatography (GC) after methanolysis, 3–4 mg of the polymers dissolved in 1.0 mL of chloroform was converted into the methyl esters with 1.0 mL of 15% H_2SO_4 in MeOH for 3 h at 100 °C. The solution was washed with 1.0 mL of distilled water by rapidly stirring for 20 s. The chloroform layer was separated and analyzed by GC (Perkin-Elmer 8500; D-B WAX; capillary column 15 m × 0.53 mm; carrier gas He, 20 mL/min; temperature program, 35 °C for 2.0 min, ramp 15 °C/min, 200 °C for 3.0 min).

The polymer was characterized using different NMR techniques. ^{1}H-NMR and ^{13}C-NMR DEPT spectra were obtained using chloroform-*d* solutions with a Bruker AC 2100 spectrometer at 200 MHz (^{1}H) and a Varian XL 300 spectrometer at 74.5 MHz, respectively. For further structure determination two-dimensional NMR investigations were carried out, applying COSY and HMQC techniques, using chloroform-*d* solutions. The ^{1}H-NMR spectra were referenced to tetramethylsilane (0 ppm) and the ^{13}C-NMR spectra to chloroform (77 ppm).

NMR data were acquired on a Bruker AMX500 spectrometer (^{1}H = 500 MHz) equipped with an inverse detection probe. ^{1}H NMR spectra were acquired using an excitation pulse of 3 μs (30°), an acquisition time of 2.0 s, a relaxation delay of 10 ms, a sweep width of 4032 Hz, 16K complex data sets, and 1024 transients. Spectra were Fourier transformed without apodization. ^{13}C-NMR spectra were acquired through the decoupler channel of the inverse probe using an excitation pulse of 10 μs (60°), an acquisition time of 0.6 s, a relaxation delay of 0.5 s, a sweep width of 27778 Hz, 32K complex data sets, and 11704 scans. Spectra were exponentially multiplied (line broadening 5 Hz) prior to Fourier transformation.

^{13}C DEPT spectra were acquired using a standard pulse sequence[18] and a 135° read pulse, an acquisition time of 0.6 s, a relaxation delay of 1.0 s, a sweep width of 27778 Hz, 32K complex data sets, and 9515 scans. Spectra were exponentially multiplied (line broadening 8 Hz) prior to Fourier transformation. COSY spectra were acquired in magnitude mode using the standard pulse sequence[18] (128 scans per increment) and were collected of 2048 complex data points each. The data were multiplied by a sine bell apodization function in each dimension and zero filled once in the t_1 dimension to form the resulting 1024 × 1024 transformed matrix.

The thermal analyses for determining glass transition temperatures (T_g), melting temperatures (T_m), and heats of fusion (ΔH) were carried out using a Perkin-Elmer DSC 7. The weight of each sample was between 6 and 10 mg. The polymer samples were heated at a rate of 20 °C from −30 to +100 °C, quenched, and scanned in a second run using the same parameters. Data used for T_g, T_m, and heat of fusion were taken from the first run, whereby the onset temperature exhibited T_g and the peak of the melting endotherm represented T_m.

Results

Attempted Growth on Organic Compounds Containing 2-Methyl Substituents. *P. oleovorans* was grown on 2-methyl-3-hydroxyoctane, ethyl 2-methyl-3-hydroxyoctanoate, ammonium 2-methyl-3-hydroxyoctanoate, and methyl 2-methyl-3-hydroxyoctanoate. The substrates were used in concentrations of 20, 25, and 100 mM. No differences in the growth behavior as a function of the concentration were observed. *P. oleovorans* was inoculated from a strong preculture grown on sodium octanoate using 10 vol % as the inoculum. In the first 8 h of growth an increase in the optical density from 0.2 (t = 0, start of the growth) to 0.4 to 0.6 was observed. After 8–10 h of growth, the cultures went into a pseudostationary phase and remained in that phase over a period of 2 weeks at this level of optical density.

The cells were collected and the polymer analyzed after 10 h of growth. Only copolymers containing 3-hydroxyoctanoate (HO) and 3-hydroxyhexanoate, (HHx) units were accumulated. This result indicated that the growth observed and the polymer produced resulted only from the consumption of sodium octanoate, which was the carbon source used in the preculture that was transferred with the inoculum. *P. oleovorans* did not grow on the 2-methyl-branched organic compounds alone, so it did not produce a PHA with these units; that is, these organic compounds support neither cell growth nor polymer production.[17]

All of the 2-methyl-substituted organic compounds evaluated were insoluble in water, which resulted in forming a two-layer system with the carbon source on top. Thus, the accessibility of the organic compound to the bacteria was drastically reduced. To increase the solubility and to achieve a homogenous growth medium, 20 vol %

Table 1. Cell Growth and Polymer Production by Cofeeding 2-Methyl-Branched Organic Compounds and Nonanoic Acid[a]

carbon source[b]	growth time (h)	max OD	cell yield (g)	polymer yield (mg)	% DW[c]
EtC_8[d]	71.0	5.4	2.57	416	16.2
EtC_8	28.0	6.0	2.52	555	22.0
C_8	46.0	7.0	1.98	363	18.3
C_8NH_4	96.0	4.9	1.98	135	6.8

[a] Cofeeding concentrations: 25 mM nonanoic acid and 25 mM 2-methyl-branched organic compound. [b] EtC_8 is ethyl and 2-methyl-3-hydroxyoctanoate, C_8 is 2-methyl-3-hydroxyoctane, and C_8NH_4 is ammonium 2-methyl-3-hydroxyoctanoate. [c] Percent of dry cell weight. [d] Culture was harvested only after it had remained for 20 h in the stationary growth phase.

of ethanol was added as a solvent for the carbon source. This amount of ethanol usually does not deter the bacterial growth, and it was anticipated that the ethanol would help to dissolve the carbon source and make it available for the bacterial fermentation. By this method a homogenous growth medium was accomplished, but no bacterial growth was observed.

The same result was observed when specially-prepared 3-hydroxyalkanoic acids and esters were used as the potential substrate. Presumably, in both cases the branched structure of the substrate inhibited growth and polymer production. Fritzsche and co-workers[12] found that it was more difficult for the bacteria to utilize methyl-branched substrates if the branching was too close to the reactive center for the polymerization. In the present study the methyl branching of the substrates under investigation was at the 2-position and, therefore, the branch group was an immediate neighbor to the reactive centers, so it might have inhibited cell utilization and polymer production.

The results showed at least that the 2-methyl-branched substrates were apparently not toxic for *P. oleovorans*, so after leaving a bacterial culture for 2 weeks with 25 mM methyl 2-methyl-3-hydroxyoctanoate, 20 mM nonanoic acid was added to determine whether the bacteria were still viable. Within 24 h the growth medium developed activity visible by foam formation, and the optical density increased from 0.26 to 1.8. The cells were harvested and the copolymer obtained was found to consist of 3-hydroxynonanoate (HN) and 3-hydroxyheptanoate (HHp) units; this is the usual copolymer obtained from growth with nonanoic acid. Therefore, even though the bacteria did not grow, they survived in the presence of 2-methyl-branched substrates.

Growth with 2-Methyl-Branched Compounds and Nonanoic Acid Cofeeds. As shown by Fritzsche and co-workers,[12] it is possible to achieve incorporation of repeating units derived from nongrowth organic compounds by cofeeding, so nongrowth 2-methyl-3-hydroxy derivatives of octanoic acid were cofed with nonanoic acid as a good growth substrate to try to induce copolymer production with the former. The growth results of the cofeeding experiments are summarized in Table 1. The bacteria grew readily on these feed mixtures. The growth started immediately after the inoculation, and in none of the experiments was a lag time observed. In 1–4 days the amount of biomass obtained was between 1.98 and 2.57 g/L with polymer incorporation ranging between 6.8 and 22.0% of dry cell weight. With the ammonium salt of the 2-methyl-3-hydroxy-octanoate as a substrate, a comparatively longer growth time was needed, but eventually the same amount of biomass was obtained. However, the amount of polymer was considerably smaller than from the other carbon sources.

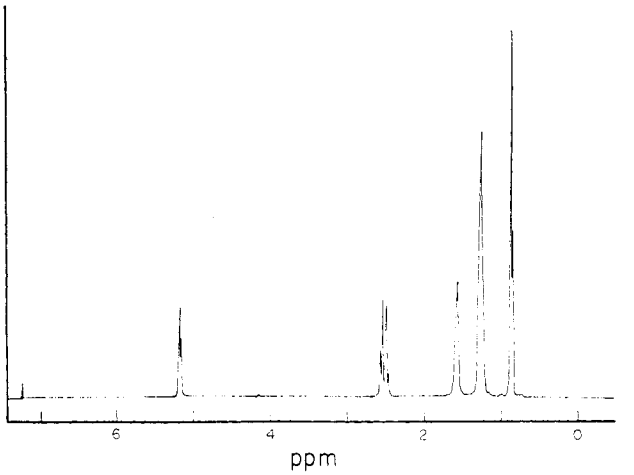
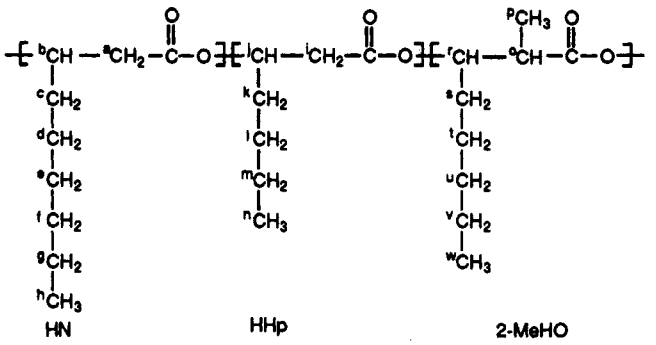


Figure 1. ^{1}H-NMR spectrum of the copolymer obtained from 2-methyl-3-hydroxyoctane and nonanoic acid by cofeeding.

Structure of PHAs Obtained. The PHA obtained contained mainly HN and HHp units as expected for the preferred consumption of the good carbon source nonanoic acid. To determine if the bacteria were capable of incorporating the unusual 2-methyl-branched units into the PHAs, the polymers were degraded by methanolysis to form the methyl 3-hydroxyalkanoates of the units present, and the esters were analyzed by gas chromatography. The chromatograms showed the expected components derived from the good growth substrate nonanoic acid, HN and HHp (65 and 30%), accompanied by small amounts of 3-hydroxyvalerate (HV) and 3-hydroxyundecanoate (HU) units, but the presence of units derived from branched substrates could not be observed by this method of analysis. The branched units were expected at higher retention times. However, at these higher retention times, 12 min and over, residues of the bacterial cells were observed. The amount of the unusual methyl-branched unit had to exceed 5% to be distinguishable from the signals of cell material.

To better determine the chemical structures of the PHAs obtained, the polymers were characterized using NMR techniques. The designations of the carbon atoms in of the anticipated copolymers containing HN and HHp units and 2-methyl-3-hydroxyoctanoate (2-MeHO) units are shown below:



The ^{1}H NMR spectra for such polymers showed the typical pattern for PHAs (Figure 1). The proton characteristic of the 2-methyl-branched structure, namely, the proton of the chiral methine group at the 2-position, could not be observed directly because the signal was hidden by the strong multiplet of the methylene groups in the HN and HHp units. The two protons of these methylene groups were split into an ABX multiplet, because they are nonequivalent due to hindered rotation, and, in addition,

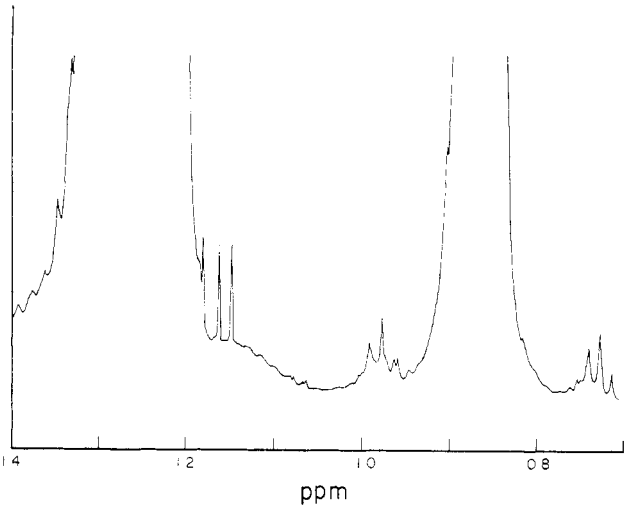


Figure 2. Expansion of the ^{1}H-NMR spectrum (0.7–1.4 ppm) of the copolymer obtained from 2-methyl-3-hydroxyoctane and nonanoic acid by cofeeding.

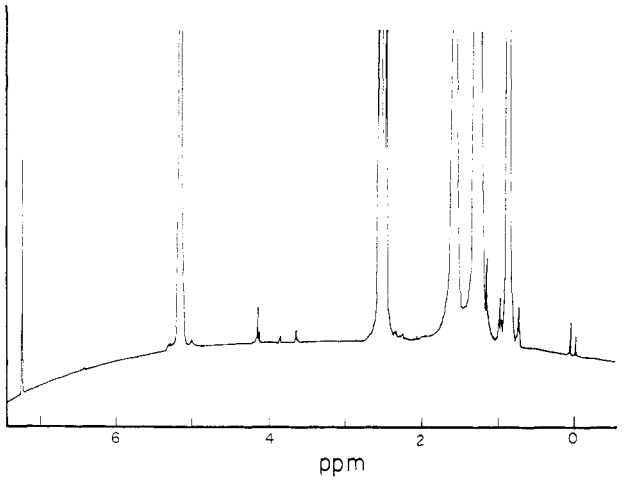


Figure 3. Expansion of the ^{1}H-NMR spectrum of the copolymer obtained from 2-methyl-3-hydroxyoctane and nonanoic acid by cofeeding.

each of the protons is coupled to the neighboring methine proton. Figure 2 shows an expansion of the ^{1}H-NMR spectrum in which a small doublet is observed at 1.16 ppm. This observation is consistent with the expected signal of protons of the methyl group (p), which has a neighboring methine (o), as it is expected in the proposed 2-MeHO structure.

Figure 3 contains another amplified view of this region and shows the presence of small quartets and other multiplets between 3.5 and 4.3 ppm. These signals are characteristic of PHAs in general. By applying correlated spectroscopy (COSY), a two-dimensional proton NMR spectrum was recorded. Figure 4 shows an expansion of this COSY spectrum, exhibiting the lower contours. One critical cross-peak, labeled "A", could be found in this spectrum. It indicates a correlation between the small doublet at 1.16 ppm and a signal buried underneath the methylene multiplet at 2.5 ppm. This observation is consistent with the expected branched polymer structure shown above.

The ^{13}C-NMR spectra were characterized by the presence of signals of the HN and HHp units, which were the main component of this PHA (Figure 5). In addition to these signals, a sharp distinguishable peak was observed at 9.8 ppm. This peak is exactly in the position expected for a methyl carbon atom at the 2-position, which was

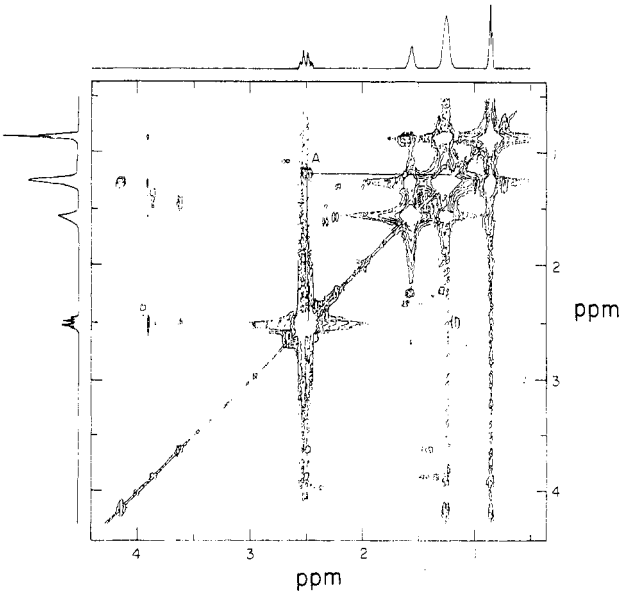


Figure 4. COSY spectrum of the copolymer obtained from 2-methyl-3-hydroxyoctane and nonanoic acid by cofeeding.

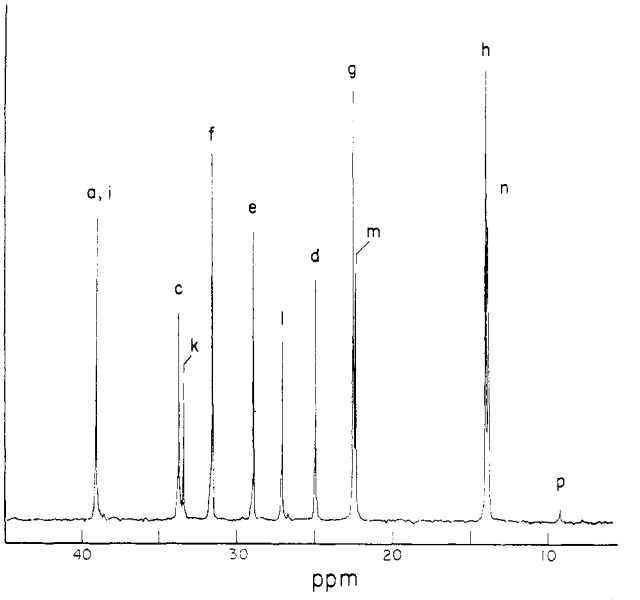


Figure 5. ^{13}C-NMR spectrum of the copolymer obtained from 2-methyl-3-hydroxyoctane and nonanoic acid by cofeeding.

calculated to be shifted to 9.2 ppm. The calculation is based on the equation for the estimation of the ^{13}C chemical shifts δ in aliphatic compounds: $\delta = -2.3 + \sum z_i + S$, in which z_i represents the increments for the substituents in the α, β, γ, and δ positions and S represents the steric correction.[19]

The DEPT spectrum (distortionless enhancement by polarization transfer) distinguishes between methyl, methylene, and methine groups by applying different pulse lengths. Figure 6 exhibits the 135°-pulse spectrum which verifies that this particular signal was derived from a methyl group. This characteristic peak was present in all the spectra of the PHAs obtained in the cofeeding experiments, regardless of the 2-methyl-branched carbon source. Furthermore, this signal could not result from contaminations with organic substrate, because this particular carbon signal occurred at 10.6 ppm in these compounds. In addition, the signal at 9.8 ppm remained at this position with the same intensity even after several purification processes.

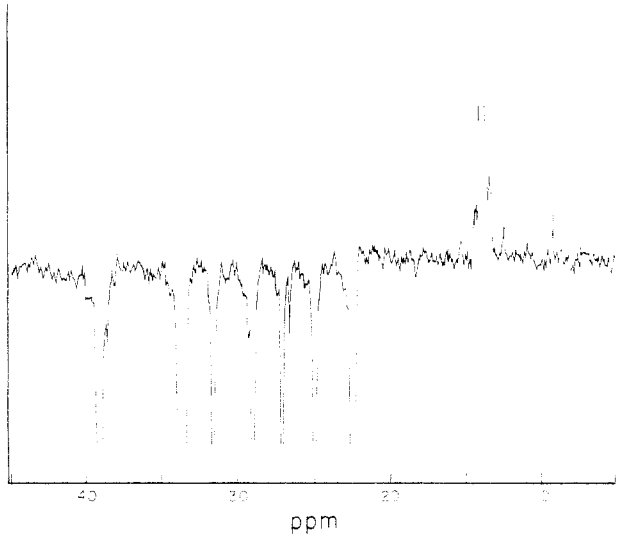


Figure 6. ^{13}C-NMR DEPT spectrum (135° pulse) of the copolymer obtained from 2-methyl-3-hydroxyoctane and nonanoic acid by cofeeding.

Table 2. Thermal Analysis of PHAs with 2-Methyl-Branched Repeating Units in the Backbone and the PHA from Nonanoic Acid

substrate for PHA[a]	glass transition (°C)	melting point (°C)	heat of fusion (J/g)
PHN/PHH	38	45	12
EtC_8	41	48	16
C_8	40	49	16

[a] See footnote in Table 1.

Unfortunately, the signal of the methyl-carbon atom in the polymer backbone (o), to which the α-methyl group (p) is attached, could not be found in the carbon NMR spectrum. It was calculated to occur at 40.8 ppm, but it is likely that the signal was shifted and hidden beneath the signals a and i of the HN and HHp units of the copolymer. It was hoped by applying heteronuclear correlated spectroscopy, which shows the cross-peaks between carbon atoms and the directly bound protons, that more evidence could be revealed for the structure in question. A heteronuclear multiple quantum correlated spectrum (HMQC), which is an inverse detected proton/carbon experiment, was recorded. However, the signal-to-noise ratio was not sufficient to see cross-peaks other than from the main components of the copolymer.

Thermal Properties of PHAs Containing Branched Units. The polymers were characterized by differential scanning analysis (DSC) to determine whether the incorporation of small amounts of 2-methyl-branched units in the PHAs influenced their thermal properties. The polymers obtained using ethyl 2-methyl-3-hydroxyoctanoate (EtC_8) and 2-methyl-3-hydroxyoctane (C_8) as cofeeds were investigated. The results, which are summarized in Table 2, showed that the heat of fusion was slightly increased compared to a HN/HHp copolymer, and the melting points were also increased. These changes in the thermal properties of the PHAs can be taken as an additional indication of the incorporation of repeating units with 2-methyl branches because in another study in this laboratory, a PHA with units having methyl branches at the 6-position was found to have crystalline property enhancements.[20]

Summary

In conclusion, evidence was obtained which supported the presence of 2-methyl-branched units. That is, the presence of a doublet at 1.16 ppm in the proton spectrum and a cross-peak between this doublet and another peak buried at 2.50 ppm in the COSY spectrum indicated the presence of the branched structure. The carbon spectrum showed clearly a signal for a carbon atom of the 2-methyl-branching type in a DEPT spectrum, and it was verified to be a methyl group. The signals of the branched units were very weak because of the small amounts of their incorporation, which were estimated to be 5% in all cases. The thermal properties of the PHAs were affected due to the incorporation of the branched units.

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