Conformational Analysis of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in Solution by ^{1}H NMR Spectroscopy

Naoko Kamiya, Yoshio Inoue,* Yasuhiko Yamamoto, and Riichirô Chûjô

Department of Biomolecular Engineering, Tokyo Institute of Technology, Meguro-ku, Tokyo 152, Japan

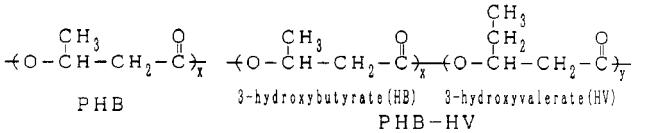
Yoshiharu Doi

Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Yokohama 227, Japan. *Received April 17, 1989; Revised Manuscript Received July 28, 1989*

ABSTRACT: It has been found that 3-hydroxybutyrate homopolymer (PHB) and its copolymers with 3-hydroxyvalerate (PHB-HV) are accumulated in some microorganisms. In this paper the conformational aspects of PHB and PHB-HV in chloroform were investigated on the basis of the vicinal coupling constants of the ^{1}H NMR spectrum. The two methylene proton resonances of PHB were assigned by using the two-dimensional ^{1}H-^{1}H NOESY spectrum. The fractional populations of gauche and trans conformers around the main-chain CH-CH_2 bond are estimated to be 0.6 and 0.4, respectively. The main-chain methylene resonances of PHB-HV are split by two factors, i.e., by the ABX spin-spin coupling between the methylene and methine protons and by the difference in the diad and/or triad monomer sequences. By separating these splitting factors, with the aid of the ^{1}H-^{13}C COSY spectra, the conformational structures of PHB-HV were determined. In every diad and/or triad monomer sequence, the fractional populations of gauche and trans conformers around the main-chain CH-CH_2 bond are also believed to be 0.6 and 0.4, respectively. It was confirmed that two kinds of monomer units, HB and HV, in PHB-HV assume almost the same conformation irrespective of HV mole fractions in chloroform solution. This result suggests the possibility that PHB-HV's with different HV mole fraction form isomorphic crystals.

Introduction

It is known that many kinds of bacteria accumulate poly(3-hydroxybutyrate) (PHB).[1] Recently, copolymers of 3-hydroxybutyrate with 3-hydroxyvalerate (PHB-HV)[2,3] and 4-hydroxybutyrate[4] were also found to be accumulated in some microorganisms. PHB and PHB-HV



are highly crystalline, optically active polyesters. They are receiving increased attention because of their biodegradability and biocompatibility.[5-8] PHB and its copolymers are environmentally hydrolyzed to soluble oligomers by extracellular enzymes of microorganisms and then finally to carbon dioxide and water by intracellular enzymes. PHB and its copolymers have much potential in medical applications utilizing their slow degradation rate. Their applications in drug release system, surgical sutures, etc. are increasing rapidly. PHB-HV's with HV mole fractions between 0 and 30% are now produced on an industrial scale and are available as commercial plastics.

The crystal regions of natural origin D-PHB[9,10] and synthesized isotactic D,L-PHV[11] in the solid state have similar structures. Both exist in 2_1 helices with fiber periods of 0.596 and 0.556 nm, respectively. PHB crystals consist of only left-handed helices. As PHV of ref 11 was a mixture of D- and L-PHV polymer chains, its crystals contains both right- and left-handed helices; D-PHV polymer chains are composed of only left-handed helices. The natural origin PHB-HV contains only D-HV units and was suggested to be composed of only left-handed helices. The conformation of PHB in solution has been investigated by Marchessault et al.,[12] Corniber et al.,[13] and Akita et al.[14] by viscometry, light scattering, and optical rotatory dispersion. Marchessault et al.[12] have found a helix-coil transition like that of polypeptides for PHB in solution when the solvent composition was varied. They concluded that PHB is helical in ethylene dichloride, trifluoroethanol, and chloroform and becomes a random coil in dichloroacetic acid and dimethylformamide. But Akita et al.[14] have reported that they could not reproduce the helix-coil transition. They concluded that the conformation of PHB in what Marchessault et al. called helicogenic solvents is random coil. Recently, Doi et al.[15] have reported the conformational structures of PHB in chloroform and dimethylformamide by analysis of vicinal coupling constants of ^{1}H NMR spectra. Although the two methylene proton resonances were assigned empirically, the fractions of gauche and trans conformers around the main-chain CH-CH_2 bond were suggested to be 0.6 and 0.4, respectively, over a temperature range of 20–100 °C. The chain dynamics have been also studied by means of ^{13}C NMR spectroscopy.[16] The ^{13}C spin-lattice relaxation time (T_1) and the nuclear Overhauser enhancement factor have indicated that PHB is a flexible linear polymer and does not have a rigid helix structure at 27 °C in chloroform.

In this paper we report the assignments of the methylene proton resonances of PHB in chloroform by using the two-dimensional ^{1}H-^{1}H NOESY spectrum. If the assignments of the two methylene proton resonances are reversed in the previous paper,[15] the fractions of gauche and trans conformers are calculated to be 0.4 and 0.6, respectively. Therefore, the methylene proton resonances must be correctly assigned to analyze the conformational structure of PHB by means of ^{1}H NMR spectroscopy. We investigated the conformational aspects of PHB in chloroform on the basis of the ^{1}H-^{1}H NOESY

0024-9297/90/2223-1313$02.50/0

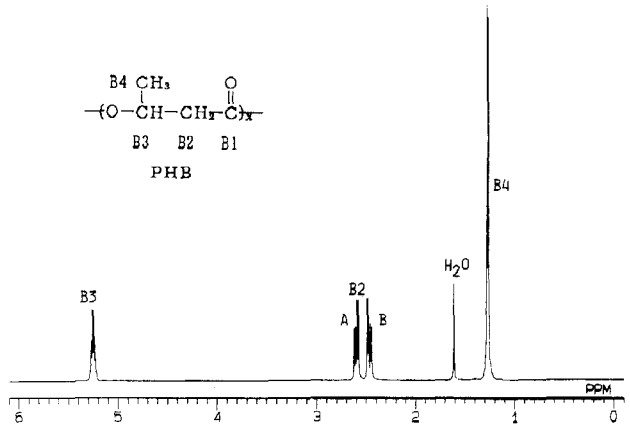


Figure 1. 500-MHz ^{1}H NMR spectrum of PHB in chloroform at 27 °C.

spectra. In addition, we report the conformation of PHB–HV determined by the ^{1}H–^{13}C COSY and ^{1}H–^{1}H NOESY spectra. The main-chain methylene resonances of PHB–HV are split by two factors, i.e., by the ABX spin–spin coupling between the methylene and methine protons and by the difference in the diad and/or triad monomer sequences. By separating these factors, the conformational structure of PHB–HV was determined.

Experimental Section

Materials. Samples of PHB and PHB–HV were isolated from *Alcaligenes eutrophus* (ATCC17699, NCIB11599) as described elsewhere.[17] As the carbon sources in cultivation, mixtures of valeric and butyric acids were used. The HV mole fraction of PHB–HV increases with increasing fraction of valeric acid in the mixture of carbon sources. One PHB and three PHB–HV samples with HV mole fractions 24, 58, and 93% were prepared. The PHB–HV samples were confirmed to be random copolymers, and not mixtures of random copolymers, by ^{13}C NMR analysis and DSC measurement.[17,18]

NMR Measurements. The 500-MHz ^{1}H NMR spectra were obtained at 27 °C in $CDCl_3$ (10–15 g L^{-1}) on a JEOL GX-500 spectrometer with 6-s pulse repetition, 4000-Hz spectral width, 32K data points, and 16 accumulations. The ^{1}H noise-decoupled 67.9-MHz ^{13}C NMR spectra were recorded at 30 °C in $CDCl_3$ (40–50 g L^{-1}) on a JEOL GSX-270 spectrometer with 5-s pulse repetition, 13 000-Hz spectral width, 64K data point, and 1500–2000 accumulations. The ^{1}H–^{13}C shift correlated spectra (COSY) were observed at 30 °C in $CDCl_3$ (40–50 g L^{-1}) on a JEOL GX-500 spectrometer with 2500-Hz spectral width and 512 data points for the ^{1}H axis and 10 000-Hz spectral width and 4096 data points for the ^{13}C axis, using the standard pulse sequence. The ^{1}H–^{1}H NOESY spectra were recorded at 30 °C in $CDCl_3$ (10–15 g L^{-1}) on a JEOL GX-500 spectrometer with 4000-Hz spectral width and 512 data points, using the $\pi/2$–t_1–$\pi/2$–t_m–$\pi/2$–t_2 pulse sequence with a mixing time t_m of 200 ms. The data were processed as a sine-bell squared function in both dimensions and symmetrized. The line-shape analysis and spin simulation were made on a NEC 9801Vm microcomputer.

Results and Discussion

PHB. Figure 1 shows the 500-MHz ^{1}H NMR spectrum of PHB at 27 °C. The assignments of the signals have been reported.[15] The conformational structure around the CH_2–CH bond in solution can be determined by the analysis of the methylene proton resonances. The methylene protons (H_A, H_B) spin-couple to the methine proton (H_X), forming an ABX-type three-spin system. The methylene proton resonances split into an octet with coupling constants J_{AX}, J_{BX}, and J_{AB}. The optimum chemical shifts and coupling constants were determined by computer simulation of the methylene proton resonances using LAOCN98 and NMR98 programs.[19] The chemical shifts of the H_A and H_B resonances were estimated to be 2.47 and 2.60 pm, respectively, and the coupling constants J_{AX}, J_{BX}, and J_{AB} to be 5.67, 7.46, and −15.76 Hz, respectively. The coupling constants were analyzed under the reasonable assumption that the flexible polymer chain in solution undergoes rapid interconversion among the three minimum energy conformers, i.e., trans (T), gauche (G), and another gauche (Ḡ), as shown in Figure 2. The observed $J_{\alpha X}$ and $J_{\beta X}$ (J_{AX} and J_{BX}) values are related to the vicinal coupling constants weighted according to their populations as follows

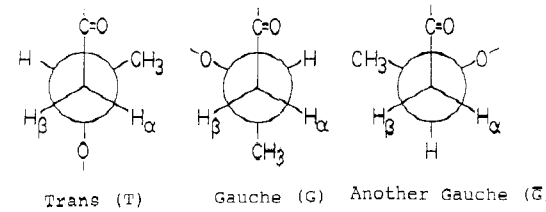


Figure 2. Newman projections of three rotational isomeric states.

$$J_{\alpha X} = P_T J_t + P_G J_g + P_{\bar{G}} J_g$$

$$J_{\beta X} = P_T J_g + P_G J_t + P_{\bar{G}} J_g$$

$$1 = P_T + P_G + P_{\bar{G}} \quad (1)$$

where J_g and J_t are the gauche and trans vicinal coupling constants and are assumed to be 2 and 11 Hz,[20] respectively. P_T, P_G, and $P_{\bar{G}}$ represent the fractional populations of conformers denoted by the respective subscripts in solution. Then they can be estimated by using the following relationship:

$$P_T = (J_{\alpha X} - J_g)/(J_t - J_g)$$

$$P_G = (J_{\beta X} - J_g)/(J_t - J_g)$$

$$P_{\bar{G}} = (J_t + J_g - J_{\alpha X} - J_{\beta X})/(J_t - J_g) \quad (2)$$

Since the correspondence of (H_A, H_B) resonances in Figure 1 to (H_α, H_β) protons in Figure 2 had not been known for PHB, the correspondence between (J_{AX}, J_{BX}) and ($J_{\alpha X}$, $J_{\beta X}$) values had not been established. Consequently, we could not estimate uniquely the values of P_T and P_G but only that of $P_{\bar{G}}$, which was found to be nearly zero. As the values of ($J_{\alpha X}$, $J_{\beta X}$) were (5.67, 7.46 Hz) or (7.46, 5.67 Hz), the values of (P_T, P_G) were (0.42, 0.58) or (0.58, 0.42).

In order to assign correctly the H_A and H_B resonances for PHB, the ^{1}H–^{1}H NOESY spectrum was observed, as shown in Figure 3. In Figure 3, one pair of cross peaks between main-chain methylene (2.4–2.7 ppm) and side-chain methyl (1.27 ppm) resonances reflects the nuclear Overhauser effect (NOE) and the other cross peaks represent the coupling. In this study we focused on the NOE. It is well-known that signal enhancement by NOE is inversely proportional to the sixth power of the ^{1}H–^{1}H internuclear distance. The shorter the proton–proton internuclear distance, the more significant the NOE enhancement appears. The distances between the main-chain methylene and side-chain methyl protons of the three conformers in Figure 2 were calculated with bond lengths and bond angles determined by X-ray analysis,[10] as shown in Table I. In the case of the G conformer, two methylene protons H_α and H_β are situated at equal distances from the methyl protons, while for the T conformer, the distance between the H_α and methyl protons is clearly shorter than that between the H_β and methyl protons. Considering that the fraction of another gauche con-

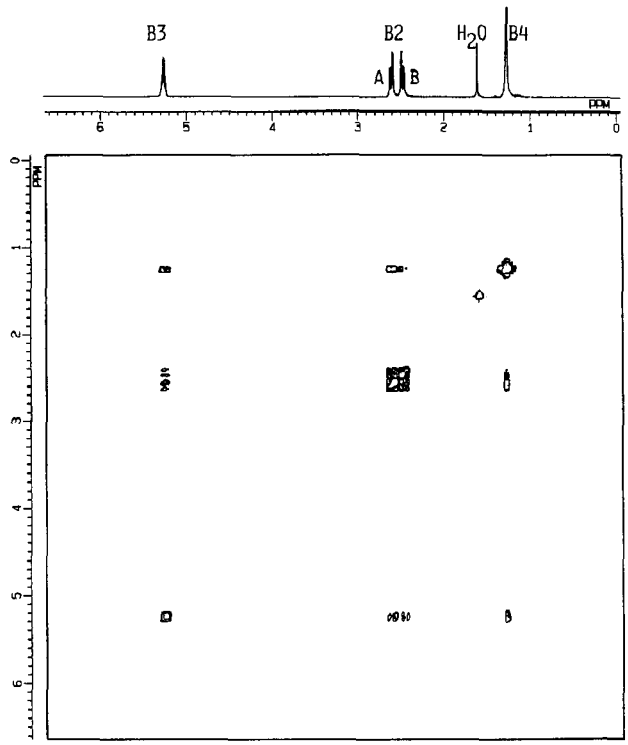


Figure 3. 500-MHz ^1H-^1H NOESY spectrum of methylene proton resonances of PHB.

Table I
Distance between Main-Chain Methylene and Side-Chain Methyl Protons in Three Preferred Conformers of PHB

	CH_3-H_α, nm	CH_3-H_β, nm
trans (T)	0.2910	0.3877
gauche (G)	0.2910	0.2910
another gauche (Ḡ)	0.3877	0.2910

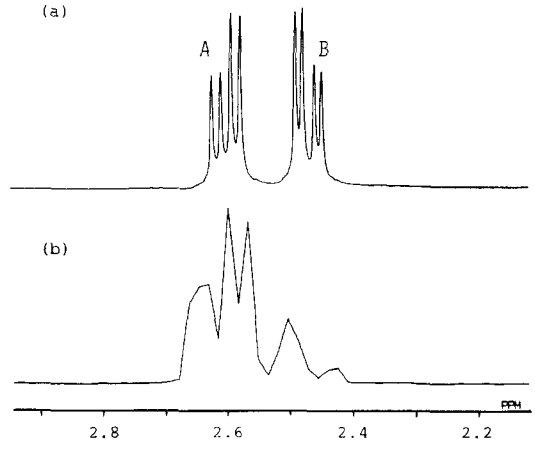


Figure 4. (a) 500-MHz ^1H NMR spectrum of methylene proton resonances of PHB. (b) Slice through the ^1H-^1H NOESY spectrum of PHB at 1.27 ppm (methyl resonances).

former is practically nil, the H_α resonance is expected to show more pronounced NOE enhancement than the H_β resonance. Figure 4 shows a slice through the NOESY spectrum at 1.27 ppm, which is the frequency of the methyl proton resonance. Since the NOE enhancement of the H_A resonance is obviously stronger than the H_B one, H_A and H_B are assigned to H_α and H_β, respectively, supporting the tentative assignments in the previous paper.[15] Hence, from eq 2, the values of P_G and P_T are unequivocally determined to be 0.58 and 0.42, respectively.

The gauche conformer is found to be the predominant conformation around the CH_2-CH bond of PHB in chloroform and the trans conformer is the next preference. The another gauche conformer is minimal. The conformation of this part of crystalline PHB is gauche in a 2_1 helix.[9,10] Conformational analysis based on intermolecular potential energies showed that this crystal structure is one of the minimum energy states.[9] Although PHB is reported to be very flexible in chloroform solution, the minimum-energy conformation of PHB is preferred in solution as well as in the solid.

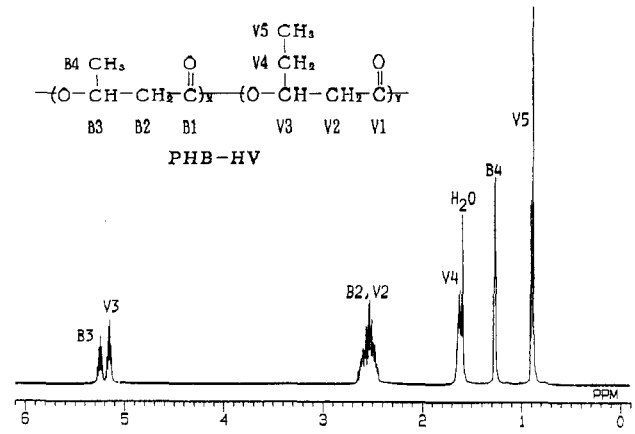


Figure 5. 500-MHz ^1H NMR spectrum of PHB-58%-HV.

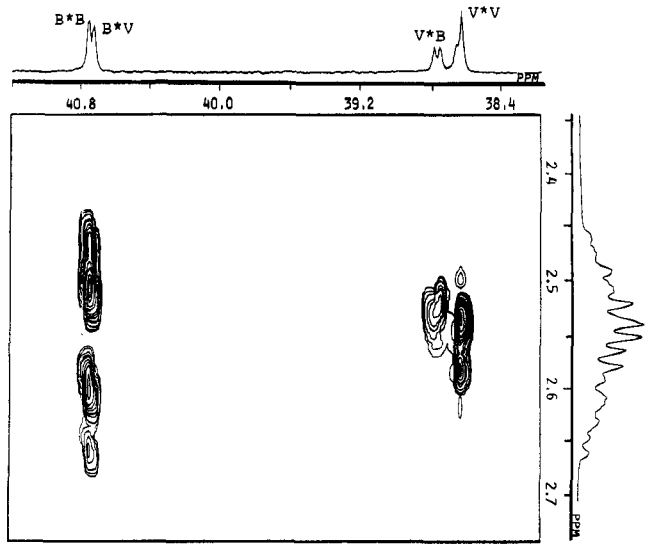


Figure 6. ^1H-^{13}C COSY spectrum of methylene resonances of PHB-58%-HV.

PHB-HV. The 500-MHz ^1H NMR spectrum of PHB-HV with 58% HV mole fraction (PHB-58%-HV) is shown in Figure 5. The assignments of the resonances have been reported.[16] The methylene and methine protons of PHB-HV also form an ABX spin system, but the appearance of methylene resonances in the ^1H NMR spectrum is more complex than that expected from an ABX spin system. In addition to methylene-methine spin couplings, there are splittings due to the differences in monomer sequence structures. The 2D ^1H-^{13}C COSY spectrum of PHB-HV was obtained. The expanded ^1H-^{13}C COSY spectrum of methylene resonances region of PHB-58%-HV is shown in Figure 6. The assignments of the ^{13}C NMR spectra have been reported for PHB-HV.[16,21] The splittings of the methylene carbon resonance due to the difference in diad sequences, i.e., the V*B and V*V sequences, have been known in the 1D ^{13}C NMR spectrum. In the COSY spectrum, the splittings were found not only in the ^{13}C column but also in the ^1H column. The proton resonances of the V*B sequence appear at higher mag-

netic field and over a narrower range than those of the V*V sequence of the HV unit. Here, V*B indicates a HV–HB diad sequence. In the case of the HB unit, we could not determine by using only the ^1H–^{13}C COSY spectrum whether the splittings due to the difference in the B*B and B*V sequences appear or not. The splittings due to the two sequences were too narrow in ^{13}C NMR spectrum. But the resonances may also split owing to the presences of B*B and B*V sequences in ^1H column, since there are too many split peaks to be explained only by spin couplings.

The split peaks of the methylene proton resonances of PHB–HV were analyzed by taking account of the two factors, i.e., different monomer sequences and spin couplings. To analyze these complex spectra, we assumed that the chemical shifts and the coupling constants for HB and HV units in each diad and triad sequence do not depend on the HV contents of PHB–HV. The values of chemical shifts and coupling constants for the protons in the B*B diad sequence were estimated from the spectrum of PHB. Similarly, those for the V*V diad were estimated from PHB–93%-HV. The reasonableness of the estimated values was examined by the spectrum simulation with the aid of LAOCN98 and NMR98 programs. By comparison of the observed and simulated spectra, the splittings due to HB-centered triad sequences were found to be present, although they were too small to determine the exact coupling constants for the triad sequence. In the simulations, the coupling constants for BB*B and VB*B triad sequences and those for BB*V and VB*V triads were assumed to be equivalent, and only the values of the chemical shifts were varied. The spectra calculated with the optimum values of spectrum parameters for PHB–24%-HV are shown in Figure 7. Figure 7a shows the calculated methylene resonances corresponding to respective diad or triad sequences. The relative intensities of the diad and triad sequences were based on the sequence distributions derived from ^{13}C NMR spectra and Bernoullian statistics. Figure 7b shows the spectrum synthesized by summing up all subspectra of part a. Figure 7c is the experimentally observed methylene ^1H resonances. Figures 8 and 9 show the calculated and observed ^1H spectra of PHB–24%-HV and PHB–93%-HV, respectively. The calculated spectra reproduced the observed spectra well. Thus the two prior assumptions are obviously justified. The values of chemical shifts and coupling constants for HB and HV units in each diad and triad sequence do not depend on the HV content of PHB–HV. The coupling constants for BB*B and VB*B sequences and those for BB*V and VB*V are almost equivalent. Although the resonances of the methylene carbons in the 67.9-MHz ^{13}C NMR spectrum show the splittings reflecting only the diad sequences, those of the methylene protons in the 500-MHz ^1H NMR spectrum show triad splittings. The optimum values of chemical shifts and coupling constants are listed in Table II. By using eq 2 with observed J_{AX} and J_{BX} values, P_G is also estimated to be practically zero for every sequences in PHB–HV. The correspondence between ($J_{\alpha X}$, $J_{\beta X}$) and (J_{AX}, J_{BX}) of PHB–HV, as in the case of PHB, could not been determined only by 1D ^1H NMR and 2D ^1H–^{13}C COSY NMR spectra.

In Figure 2, the side-chain methyl group must be replaced with an ethyl group to analyze the conformation of the HV unit in PHB–HV. When the $\bar{G}$ conformer does not exist, the distance between H_α and side-chain methylene protons is smaller than that between H_β and side-chain methylene protons. Figure 10 shows a slice of the NOESY spectrum at the side-chain methylene resonance (1.61 ppm) of PHB–93%-HV, indicating the presence of NOE enhancement in the main-chain methylene resonance (2.4–2.6 ppm) arising from the side-chain methylene protons. The extent of NOE enhancement for H_A resonance is greater than that for H_B resonance. Consequently, H_A is assigned to H_α and H_B to H_β, similarly to the case of PHB. The values of P_G, P_T, and $P_{\bar{G}}$ calculated with eq 2 are also shown in Table II. For all HB and HV units in every sequences in copolymers as well as in PHB, P_G is about 0.6, P_T is about 0.4, and another gauche does not exist. Thus, the different sequences and/or different monomer units do not affect the short-range chain conformations in solution.

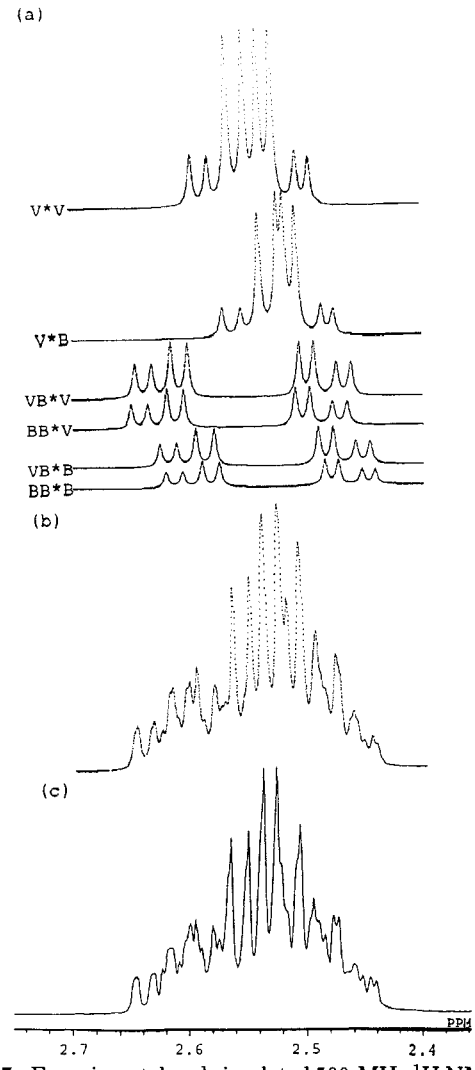


Figure 7. Experimental and simulated 500-MHz ^1H NMR spectra of methylene resonances of PHB–58%-HV. (a) Component curves generated by LAOCN98; (b) total curve of (a); (c) experimental curve.

It has been confirmed in this paper that two kinds of monomer units, HB and HV, assume almost the same conformation in chloroform solution. PHB–HV's with any HV mole fractions assume quite similar conformations in chloroform solution. Therefore, the copolymers of HB and HV units with different HV mole fractions have a higher possibility of forming isomorphic crystals. It has been reported that PHB–HV's in the crystalline state are isomorphic, with both HB and HV units being included in the same crystalline phase.[21] The HB and HV units in the crystalline phase form quite similar structures, with geometrical parameters that differ little between

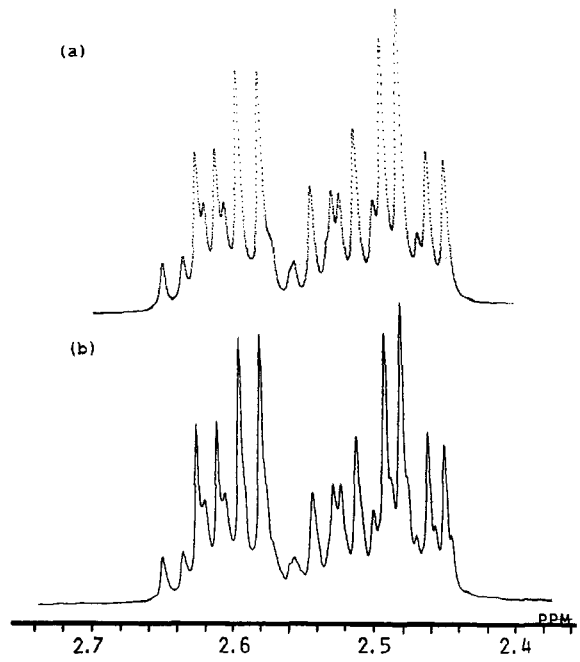


Figure 8. Experimental and simulated 500-MHz ^{1}H NMR spectra of methylene protons of PHB–24%-HV. (a) Simulated; (b) experimental.

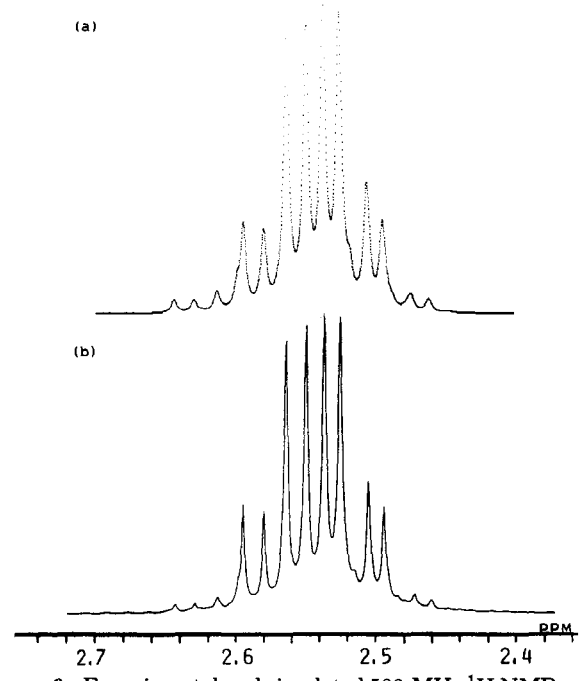


Figure 9. Experimental and simulated 500-MHz ^{1}H NMR spectra of methylene protons of PHB–93%-HV. (a) Simulated; (b) experimental.

Table II
Parameters along CH_2–CH Bonds in Different Sequences

	chemical shift, ppm		coupling const, Hz			fraction		
sequence	H_A	H_B	J_{AX}	J_{BX}	J_{AB}	P_T	P_G	P_G
V*V	2.520	2.567	5.3	7.5	−15.6	0.363	0.606	0.031
V*B	2.506	2.544	5.3	7.6	−15.6	0.354	0.616	0.030
VB*V	2.484	2.616	5.9	7.2	−15.6	0.427	0.573	0.000
BB*V	2.488	2.620	5.9	7.2	−15.6	0.427	0.573	0.000
VB*B	2.470	2.599	5.8	7.3	−15.6	0.416	0.584	0.000
BB*B	2.476	2.605	5.8	7.3	−15.6	0.416	0.584	0.000

HB and HV units. The NMR results obtained in this work are compatible with the previous report[21] that a series of PHB–HV's with different HV contents form isomorphic crystals.

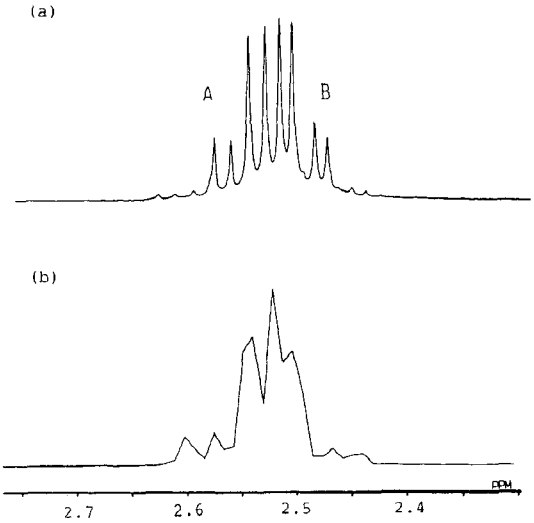


Figure 10. (a) 500-MHz ^{1}H NMR spectrum of methylene proton resonances of PHB–93%-HV. (b) Slice through ^{1}H–^{1}H NOESY spectrum at 1.61 ppm (side-chain methylene resonances) of PHB–93%-HV.

References and Notes

(1) Lemoigne, M. *Ann. Inst. Past.* **1925**, *10*, 135.
(2) (a) Holmes, P. A.; Wright, L. F.; Collins, S. H. Eur. Pat. Appl. 0052459, 1982. (b) Holmes, P. A.; Wright, L. F.; Collins, S. H. Eur. Pat. Appl. 0069497, 1983.
(3) Doi, Y.; Tamaki, A.; Kunioka, M.; Nakamura, Y.; Soga, K. *Appl. Microbiol. Biotechnol.* **1988**, *28*, 330.
(4) Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1988**, *21*, 2722.
(5) Holmes, P. A. *Phys. Technol.* **1985**, *16*, 32.
(6) Howells, E. R. *Chem. Ind.* **1982**, *7*, 508.
(7) Miller, N. D.; Williams, D. F. *Biomaterials* **1987**, *8*, 129.
(8) Holland, S. J.; Jolly, A. M.; Yasin, M.; Tighe, B. J. *Biomaterials* **1987**, *8*, 289.
(9) Cornibert, J.; Marchessault, R. H. *J. Mol. Biol.* **1972**, *71*, 735.
(10) Yokouchi, M.; Chatani, Y.; Tadokoro, H.; Teranisi, K.; Tani, H. *Polymer* **1973**, *14*, 267.
(11) Yokouchi, M.; Chatani, Y.; Tadokoro, H.; Tani, H. *Polym. J.* **1974**, *6*, 248.
(12) Marchessault, R. H.; Okamura, K.; Su, C. J. *Macromolecules* **1970**, *3*, 735.
(13) Cornibert, J.; Marchessault, R. H.; Benoit, H.; Weill, G. *Macromolecules* **1970**, *3*, 741.
(14) Akita, S.; Eigata, Y.; Miyake, Y.; Fujita, H. *Macromolecules* **1976**, *5*, 774.
(15) Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1986**, *19*, 1274.
(16) Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1986**, *19*, 2860.
(17) Kamiya, N.; Yamamoto, Y.; Inoue, Y.; Chûjô, R.; Doi, Y. *Macromolecules*, in press.
(18) Inoue, Y.; Kamiya, N.; Yamamoto, Y.; Chûjô, R.; Doi, Y. *Macromolecules*, in press.
(19) Satake, K. *Kagaku* **1983**, *No. 9*, 10.
(20) Bovey, F. A. *High Resolution NMR of Macromolecules*; Academic: New York, 1972; Chapter IX.
(21) Bluhm, T. L.; Hamer, G. K.; Marchessault, R. H.; Fyfe, C. A.; Veregin, R. P. *Macromolecules* **1986**, *19*, 2871.

Registry No. PHB (homopolymer), 26063-00-3; PHB (SRU), 124479-32-9; (PHB)(HV) (copolymer), 80181-31-3.