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Bacterial production of poly-3-hydroxyalkanoates containing arylalkyl substituent groups

Baki Hazer
Department of Chemistry, Karadeniz Technical University, Trabzon 61080 and TUBITAK-Marmara Research Centre, Gebze 41470 Kocaeli, Turkey

and Robert W. Lenz*
Department of Polymer Science and Engineering, University of Massachussets at Amherst, Amherst, MA 01003, USA

and R. Clinton Fuller
Department of Biochemistry and Molecular Biology, University of Massachussets at Amherst, Amherst, MA 01003, USA
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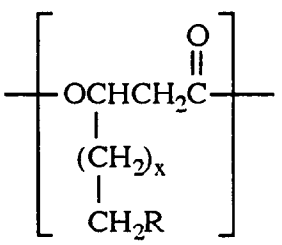
6-Phenylhexanoic acid (6PHxA), 7-phenylheptanoic acid (7PHpA), 9-phenylnonanoic acid (9PNA), 11-phenylundecanoic acid (11PUA), 9-*p*-tolylnonanoic acid (9TNA) and 9-*p*-styrylnonanoic acid (9SNA) were prepared and evaluated as substrates for cell growth and polyester production by *Pseudomonas oleovorans* and *Pseudomonas putida*. *P. putida* was more effective than *P. oleovorans* for producing polyesters from these aromatic substrates. Poly-3-hydroxyalkanoates, PHAs, were obtained from 6PHxA, 7PHpA, 9PNA and 11PUA. The PHAs produced from all of these substrates contained mostly 3-hydroxy-5-phenylvalerate (H5PV) and 3-hydroxy-6-phenylhexanoate (H6PHx) units. Polymer yields ranging from 3 to 47% of cell dry weight were obtained with molecular weights ranging from 156000 to 37000 and polydispersities from 2.3 to 2.9. Cofeeding of most of these substrates with nonanoic acid produced mixtures of two different PHAs with different glass transitions, one in the region of −8 to 12°C for the PHA with arylalkyl substituent groups, and one in the region of −14 to −35°C for the PHA from nonanoic acid. The PHA from 9TNA also had a crystalline melting transition. Copyright © 1996 Elsevier Science Ltd.

Introduction

Pseudomonas oleovorans can produce a variety of poly-3-hydroxyalkanoates, PHAs, of the general structure shown below when it is grown on n-alkanoic acids under nutrient limiting conditions[1–5]:



In this structure x can vary from 0 to at least 8 and R can be either H or a functional group depending on the substrate used.

The ability of bacteria to accumulate massive amounts of PHAs is a natural mechanism used to provide the cell with an organic reserve in a reduced form that is osmotically inert. For this purpose, the PHAs are accumulated as a result of nutrient imbalance when the environment still contains an excess of a suitable carbon source[5]. The polymer is formed in intracellular inclusion bodies, which are subsequently consumed by enzymatic depolymerization reactions under energy or carbon starvation conditions. In addition to their biodegradability both inside the cell and in many extracellular environments, these polymers are also biocompatible and of interest for biomedical applications[6,7].

Pseudomonas oleovorans can produce PHAs with long alkyl chains ($x = 2$ to at least 8) when this bacterium is grown on a wide variety of carbon substrates, including alkanes, alcohols and alkanoic acids which have chain lengths of between 6 and 14 carbon atoms[8–12]. PHAs containing aromatic pendant groups can also be produced from arylalkanoic acids[13–15]. For example, a PHA with a phenyl pendant group ($R = C_6H_5$) was obtained by feeding *P. oleovorans* with 5-phenylvaleric acid. This PHA was an amorphous homopolymer with a glass transition temperature (T_g) of 19°C[13]. In contrast, a crystalline PHA was obtained from 5-*p*-tolylvaleric acid. That PHA had a melting temperature (T_m) at 95°C. In the present study, this and other substrates were evaluated for the production of PHAs with arylalkyl substituents by both *P. oleovorans* and *P. putida*.

Experimental

Substrate preparation. A Grignard reaction similar to that reported in the literature[11,12] was used to prepare

* To whom correspondence should be addressed

Table 1 ^{1}H n.m.r. chemical shifts of alkanoic acid substrates containing aromatic substituents

	δ, ppm				
Substrate[a]	1.25–1.45	1.45–1.75	2.30–2.45	2.52–2.65	7.10–7.35
6PHxA	m, 2H, H-4	m, 4H, H-3, -5	t, 2H, H-6	t, 2H, H-2	m, 5H, phenyl
7PHpA	m, 4H, H-4, -5	m, 4H, H-3, -6	t, 2H, H-7	t, 2H, H-2	m, 5H, phenyl
9PNA	m, 8H, H-4, -5, -6, -7	m, 4H, H-3, -8	t, 2H, H-9	t, 2H, H-2	m, 5H, phenyl
11PUA	m, 12H, H-4, -5, -6, -7, -8, -9	m, 4H, H-3, -10	t, 2H, H-11	t, 2H, H-2	m, 5H, phenyl
9TNA	m, 12H, H-4, -5, -6, -7	m, 4H, H-3, -8	s (2.35) 3H, H-10, m, 2H, H-9	t, 2H, H-2	m, 4H, phenyl
9SNA	m, 8H, H-4, -5, -6, -7	m, 4H, H-3, -8	t, 2H, H-9	t, 2H, H-2	5.2, d and 5.7, d, 2H, H-11 6.6–6.8, m, 1H, H-10 7.1–7.4, m, 4H phenyl

[a] See text for abbreviations

the arylalkanoic acid substrates as shown by the general equation below:

$$Ar(CH_2)_xBr + Br(CH_2)_yCOOH \longrightarrow Ar(CH_2)_{x+y}COOH$$

in which $x = 2, 4, 6$ and $y = 3, 4$.

As an example, the following procedure was used for the preparation of 6-phenylhexanoic acid (6PHxA): 40.08 g (0.24 m) of 4-bromobutyric acid was dissolved in 320 ml of anhydrous tetrahydrofuran, THF*. The solution was cooled to −20°C under a dry argon atmosphere, and 85 ml of a 3 molar solution of methyl magnesium chloride (0.255 m) in tetrahydrofuran (THF) (Fluka AG) was added dropwise while keeping the temperature below −15°C. After 15 min of stirring, 24 ml of a 0.1 molar solution of Li_2CuCl_4 (0.24 m) in THF[16] was added, followed by the dropwise addition of a solution of the Grignard reagent prepared from 51.80 g (0.28 mol) of 2-bromoethylbenzene and 6.8 g (0.28 mol) of magnesium in 300 ml anhydrous THF. The temperature was kept between −15 and −10°C. The mixture was allowed to warm to room temperature and stirring was continued overnight, after which the solution was poured into 0.5 l of an ice-cold 10% H_2SO_4 aqueous solution. The aqueous phase which separated was saturated with NaCl and extracted with ether. The combined organic phases were extracted with a solution of 50 g of KOH in 100 ml of water. After these extracts were acidified with 0.5 l of 10% H_2SO_4 solution, an oily layer was separated. This layer was dried over Na_2SO_4 and distilled under vacuum. Yield: 29.6 g (77%); b.p. of colourless liquid: 158–162°C/1 Torr. The ^{1}H nuclear magnetic resonance (n.m.r.) chemical shifts for this substrate and for the other arylalkanoic acids prepared in this manner are collected in *Table 1*. Six arylalkanoic acids were prepared by this procedure, including 9-phenylnonanoic acid (9PNA, 50% yield, b.p. 176–178°C/1 Torr, m.p. 29.5°C); 11-phenylundecanoic acid (11PUA, 54% yield, b.p. 215–216°C/1 Torr, m.p. 42.5°C); 9-*p*-tolylnonanoic acid (9TNA, 64% yield, m.p. 65.5°C) and 9-*p*-styrylnonanoic acid (9SNA, 32% yield, m.p. 65.5°C).

PHA production. Stock cultures of *P. oleovorans* (ATCC 29347) and *P. putida* (from Prof. S. C. Yoon, Gyeongsang National University, Korea) were used in all of the growth and polymer production experiments with the same E media as used for *P. oleovorans* in previous studies in this laboratory[4,11–15]. The arylalkanoic acid substrates were used both as the sole carbon source, at a concentration of 10 mM, and as cofeeds with nonanoic acid. In the latter case the total carbon substrate concentration was between from 4 to 20 mM. 1.5 l solutions (or dispersions) of the individual carbon sources, or mixtures of sources, were inoculated with 100 ml of an inoculum obtained from cultures obtained by growing *P. putida* or *P. oleovorans* on the E media containing 20 mM of nonanoic acid.

Polymer characterization. ^{1}H and ^{13}C n.m.r. spectra were obtained on chloroform-d solutions at 17°C with either a Varian XL 200 n.m.r. spectrometer at 200 MHz (1-H) or a Varian XL 300 n.m.r. spectrometer at 74.5 MHz (13-C). A duPont differential scanning calorimeter (d.s.c.) 2910 was used to determine the glass transition temperatures (T_gs), the melting transitions (T_ms) and the enthalpies of fusion, ΔH_m, of the PHAs produced. Samples were heated from −100 to +150°C in a nitrogen atmosphere at a rate of 20°C min^{-1}, quenched and heated a second time using the same range and heating rate.

Methanolysis–gas chromatography was used to determine the composition of the polymers produced. The polymer was converted into its constituent β-hydroxyalkanoate units in the form of their methyl esters by a previously described procedure[12]. The methyl esters were characterized using a Perkin Elmer 8500 gas chromatograph equipped with a Durabond Carbowax megabore capillary column (15 m × 0.54 mm, Carrier gas He, flow rate 20 ml min^{-1}). The program used was as follows: after injection, the sample was maintained at 80°C for 4 min, then heated at 8°C min to 160°C, then held for 11 min before cooling to 80°C.

Wide angle X-ray scattering (WAXS) patterns were obtained under reduced pressure using a Statton camera with a Siemens K710H generator operating at 40 kV and 30 mA. Nickel filtered CuKα radiation (λ = 1.5418 Å) was used. Polymer samples of approximately 0.5 mm thickness were exposed to X-rays for 8 h.

Molecular weights were determined by gel permeation chromatography (g.p.c.) with a Waters model 6000A solvent delivery system with a Mode 401 refractive index detector, and a Mode 730 data module, and with two Ultrastyragel linear columns in series. Chloroform was used as the eluent at a flow rate of 1.0 ml min^{-1}. Sample concentrations of 2–3 mg ml^{-1} and injection volumes of 150 μl were used. A calibration curve was generated with six polystyrene standards (M_Ws: 3M, 233K, 22K, 2150, 580, 92). The correlation coefficient was 0.994.

Results and discussion

P. oleovorans and *P. putida* were grown on each of six

* THF dried on MgO overnight was distilled on sodium under argon, taken middle fraction and freshly used

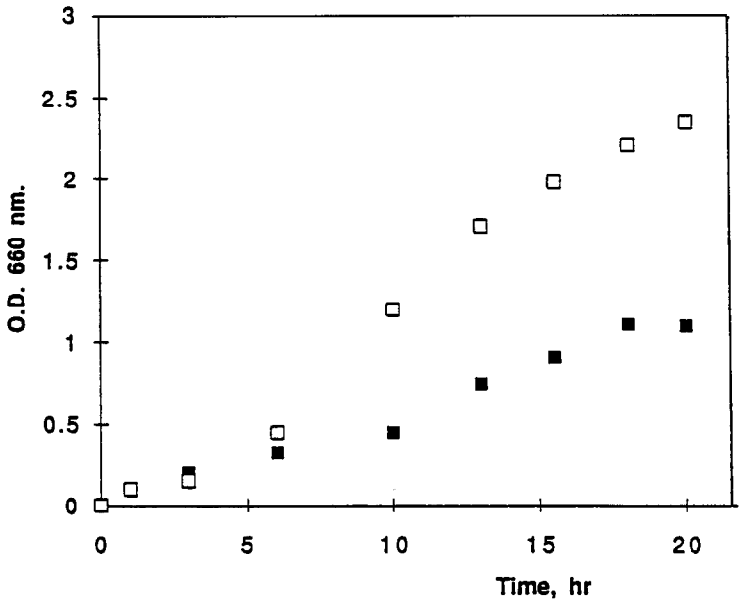


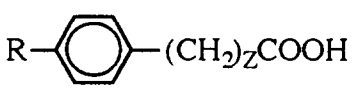
Figure 1 Growth curves obtained when *P. putida* was grown on the following substrates: □, 7 mM of 9-styrylnonanoic acid (9SNA); □, 7 mM 6-phenylhexanoic acid and 7 mM nonanoic acid

Table 2 Growth conditions and results from feeding either *P. oleovorans* or *P. putida* with arylalkanoic acids

PHA	Substrate conc. ($mol\,l^{-1}$)[a]	Bacterium	Cell harvest		Dry cell yield, $g\,l^{-1}$	Polymer yield		Molecular weights ($\times 10^3$)	
			o.d.	Time (h)		$g\,l^{-1}$	% DCW[b]	M_w	M_n
16	20 mM 6PHxA	*P. Putida*	2.6	34	1.29	0.61	47	142	30
15	20 mM 6PHxA	*P. Oleovorans*	2.4	34	1.11	0.35	32	169	91
14	20 mM 7PHpA	*P. Putida*	2.3	30	0.35	0.08	24	98	43
13	20 mM 7PHpA	*P. Oleovorans*	2.3	30	0.66	0.06	9	175	45
7-1	7 mM 7PHpA	*P. Putida*	2.3	22	0.47	0.24	35	130	59
18	10 mM 9PNA	*P. Putida*	2.6	27	0.32	0.11	35	93	38
9-1	7 mM 9PNA	*P. Putida*	2.0	22	0.67	0.18	27	113	48
17	10 mM 11PUA	*P. Putida*	2.4	27	1.12	0.27	24	100	40
11-4	7 mM 11PUA	*P. Putida*	2.1	23	1.6	0.57	35	119	53
10	10 mM 9TNA	*P. Putida*	1.7	30	0.51	0.04	8	113	48
11	10 mM 9TNA	*P. Oleovorans*	1.1	30	0.35	0.03	7	119	50
19	10 mM 9SNA	*P. Putida*	1.8	27	0.46	0.02	3	–	–
29	4 mM 9SNA	*P. Putida*	1.1	20	0.48	0.02	5	–	–

[a] See text for abbreviations
[b] DCW–dry cell weight

arylalkanoic acids having the general structure:



including the following: 6-phenylhexanoic acid (6PHxA) Z = 5, R = H; 7-phenylheptanoic acid (7PHpA) Z = 6, R = H; 9-phenylnonanoic acid (9PNA) Z = 8, R = H; 11-phenylundecanoic acid (11PUA) Z = 10, R = H; 9-*p*-tolylnonanoic acid (9TNA) Z = 8, R = CH_3; 9-*p*-styrylnonanoic acid (9SNA) Z = 8, R = $CH{=}CH_2$. Both *P. oleovorans* and *P. putida* grew very rapidly on all of these substrates, and cell densities reached a maximum o.d. in 22–34 h. *Figure 1* shows a typical example of the type of growth curve obtained, in this case when *P. putida* was grown on 7 mM 9SNA. As shown by the data in *Table 2*, the maximum o.d. achieved was between 2.0 and 2.6 with most of the phenyl-containing substrates, including 6PHxA, 7PHpA, 9PNA and 11PUA, and polymer yields were generally between 24 and 35%. In the preparation of PHA no. 13, the very low yield obtained from 7PHpA can be attributed to a delayed harvesting time[12], but for the PHAs from 9TNA and 9SNA, the polymer yield was only between 3 and 8% even at normal harvesting times. As can be seen from the data in *Table 2*, when *P. putida* and *P. oleovorans* were grown on the same concentrations of either 6PHxA (15 and 16) or 9TNA (10 and 11) the PHA amounts obtained for *P. putida* were higher than for *P. oleovorans*[14], so for the other substrates only *P. putida* was used.

All of the arylalkanoic acid substrates were also cofed with nonanoic acid (NA) and the data for harvest time,

Table 3 Growth conditions and results from cofeeding *P. putida* with arylalkanoic acids and NA

PHA	Substrate conc. (mol l^{-1})[a]	Harvest		Dry cell yield $g\,l^{-1}$	Polymer yield		Molecular weights ($\times 10^3$)	
		o.d.	Time (h)		$g\,l^{-1}$	% DCW[b]	M_w	M_n
22	7 mM 6PHxA 7 mM NA	1.9	23	0.97	0.15	15	130	32
30	7 mM 6PHxA 7 mM NA	2.4	20	1.2	0.51	41	117	43
23	7 mM 7PHpA 7 mM NA	1.9	23	1.2	0.39	33	120	62
24	7 mM 9PNA 7 mM NA	1.9	23	1.2	0.63	52	128	53
25	7 mM 11PUA 7 mM NA	1.9	23	1.1	0.43	41	110	41
20	7 mM 9pTNA 7 mM NA	1.9	23	1.1	0.35	31	113	39

[a] See text for abbreviations
[b] DCW–dry cell weight

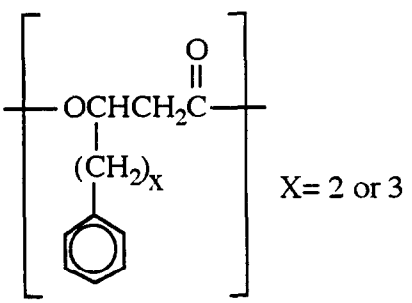
Table 4 Gas chromatographic analysis of aromatic polyesters obtained when *P. putida* was fed an arylalkanoic acid either alone or in combination with NA

Substrate[a]	Wt% of PHA units in polymer mixture[a]					
	H6PHx	H5pTV	H5PV	Other[b]	HN	HHp
6PHxA	75			5	15	5
6PHxA + NA	25				58	17
7HpA			67	3	23	7
7PHpA + NA			60	5	28	7
9PNA			70	15	11	4
9PNA + NA			36	10	48	6
11PUA			78	12	7	3
11PUA + NA			8	17	55	20
9pTNA		15		22	38	25
9pTNA + NA		10		20	50	30

[a] See text for abbreviations; HN–3-hydroxynonanoate, HHp–3-hydroxyheptanoate
[b] Unit structure unknown but believed to be 3-hydroxy-7-phenyl-heptanoate

o.d. at harvest, cell yield, polymer yield are given in *Table 3*. All growth studies resulted in moderate polymer yields except for polymer 22, which had a delayed harvesting time. From gas chromatography and n.m.r. analysis the amount of arylalkyl units in the PHA product was between 40 and 78% as shown by the data in *Table 4*.

^{1}H n.m.r. characterization of the PHA obtained from 6PHxA showed the characteristic peaks of both 3-hydroxy-6-phenyl-hexanoate (H6PHx) and 3-hydroxy-5-phenylvalerate (H5PV) units as shown below:



All of the products obtained from arylalkanoic acids alone also contained small amounts of the PHA from nonanoic acid (PHN) because the preculture used for inoculation was prepared from nonanoic acid.

Figures 2 and *3* show typical ^{1}H and ^{13}C n.m.r. spectra of the PHAs produced. The signal at 1.9 ppm in the ^{1}H

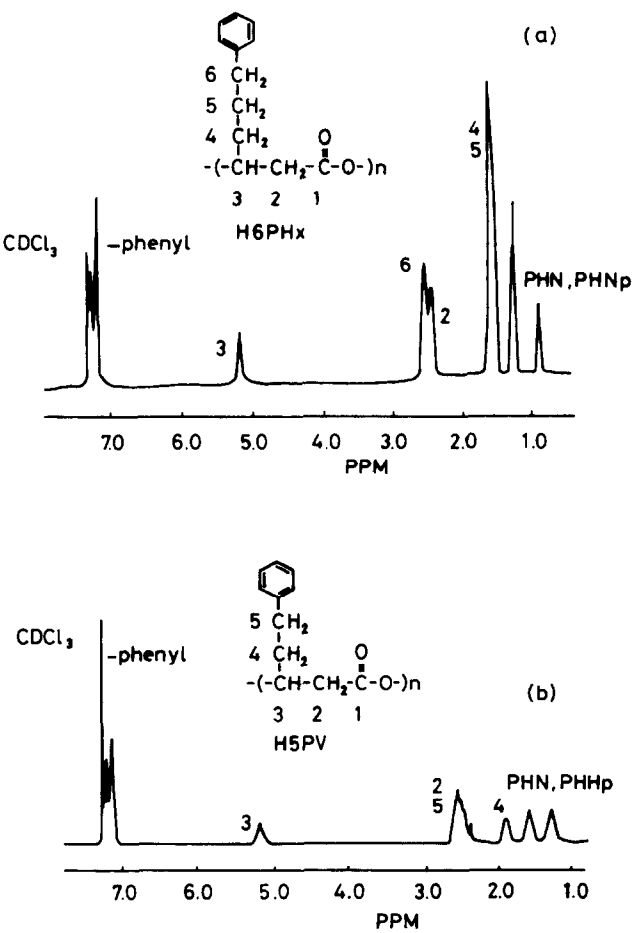


Figure 2 ^{1}H n.m.r. spectra of PHAs obtained from 11PUA (a) and 6PHxA (b)

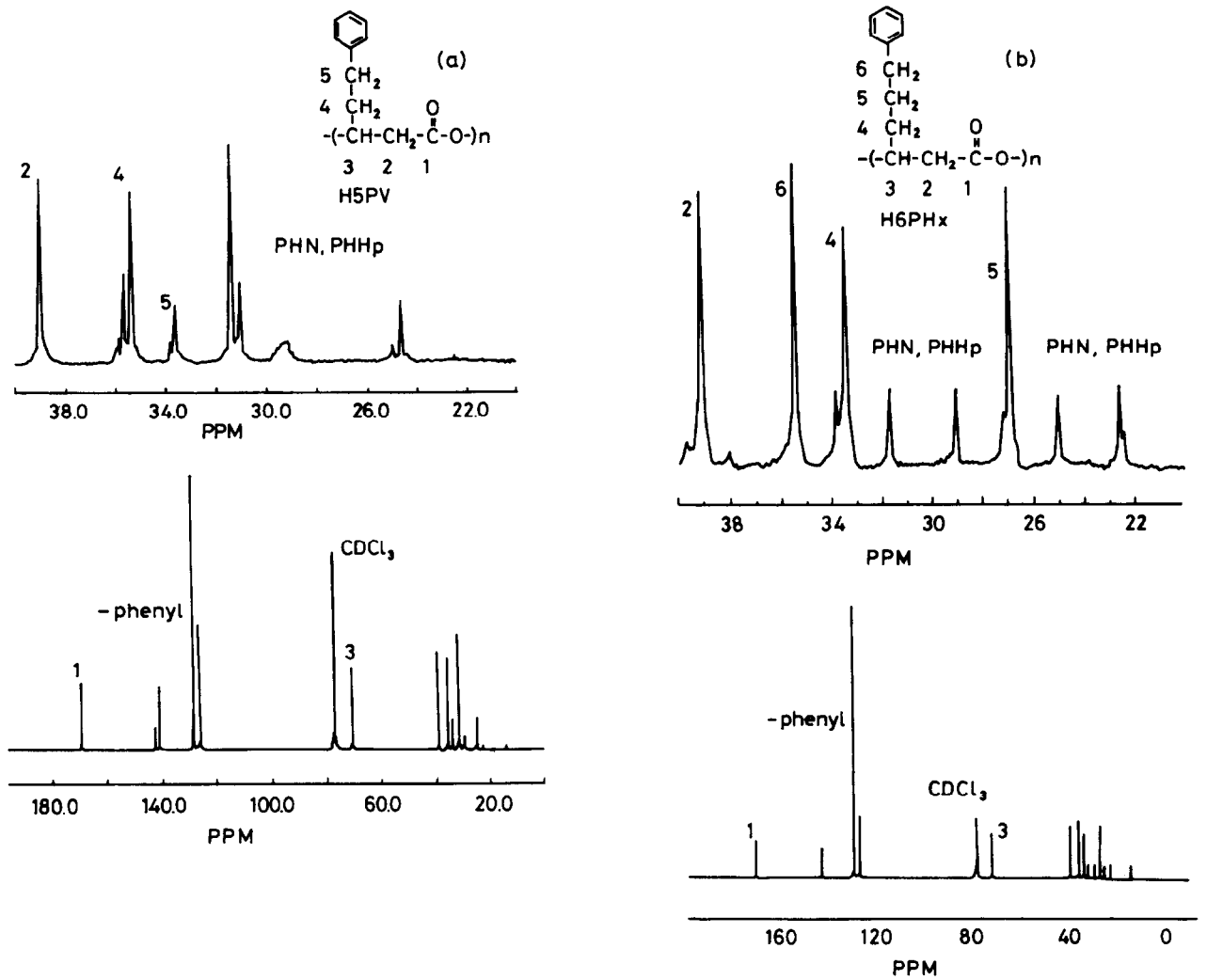


Figure 3 ^{13}C n.m.r. spectra of PHAs obtained from 11PUA (a) and 6PHxA (b)

Table 5 ^{13}C n.m.r. chemical shifts of PHAs containing arylalkyl groups

		Chemical shifts												
PHA	Substrate	1 −C=O	Phenyl		3 OCH	2 O ‖ CH_2C	6 CH_2 	4 CH_2 OCH	5 CH_2 CH_2 OCH	PHN	PHHp		10 CH_3 	
15	6PHxA	169.3	141.8	125.8	70.8	39.0	35.4	33.8	27.0	29.0	25.0	22.5	14.1	
				128.3				33.4	26.8					
7-1	7PHpA	169.4	140.9	126.0	70.7	39.0	35.9	33.8	27.1	29.0	25.0	22.5	14.1	
			142.2	129.1			35.4	33.6			24.9	22.4		
9-3	9PNA	169.2	140.9	126.0	70.9	39.0	35.6	33.8		29.7	25.0	22.5	14.1	
			142.2	129.1			35.3	33.6		29.2	24.6	22.4		
										29.0				
17	11PUA	169.4	140.9	125.7	70.7	39.0	35.9	33.6		29.2	24.6			
			142.2	129.7			35.6							
							35.4							
10	9TNA	169.4	137.4	129.1	70.8	39.1	35.5	33.8	27.1	29.0	25.0	22.5	14.1	20.9
			137.8	129.4				33.5		29.0	24.7	22.4		

n.m.r. spectrum is typical for PH5PV (*Figure 2a*). Characteristic bands of PH5PV can also be seen in this ^{13}C n.m.r. spectrum of the PHA obtained from 11PUA in *Figure 3a*. *Figure 2b* shows an ^{1}H n.m.r. spectrum of the aromatic polyester obtained from 6PHxA. The absence of a signal at 1.9 ppm in the ^{1}H spectrum and presence of a signal at 29 ppm in the ^{13}C n.m.r. spectrum in *Figure 3b* confirms the presence of H6PHx units. The

Table 6 Thermal analysis of PHAs produced from arylalkanoic acids as determined by d.s.c.[a]

		Thermal transitions					
		1st Heating cycle			2nd Heating cycle		
PHA	PHA[a]	T_g(°C)	T_m(°C)	ΔH (J g^{-1})	T_g(°C)	T_m(°C)	ΔH (J g^{-1})
16	6PHxA	+4					
22	6PHxA + NA		49	0.2	−34	23	0.1
					1		
14	7PHpA	−8	48	0.1	+9	68[b]	0.2
		+6					
23	7PHpA + NA	−14		0.2	−14	6.1	0.1
		0			+7		
9-3	9PNA	+6			+7	68[b]	0.1
14	9PNA + NA	−24	35		−25		
17	11PUA	2					
25	11PUA + NA	−35	41	0.9	−37		
11	9TNA	−37	45	0.4	−38	44	0.1
			60	0.1	+14		
20	9TNA + NA	−22	35	0.1	−23		
		1	46	0.1			
29	9SNA	−16	64	0.1	−39	52[c]	
						65[c]	

[a] See text for abbreviations
[b] Very small peak
[c] Very broad peak

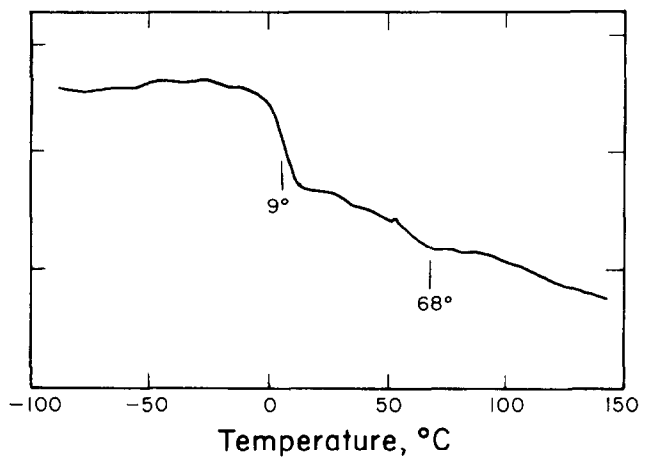


Figure 4 D.s.c. thermogram of the PHAs obtained from 7PHpA, no. 7-1

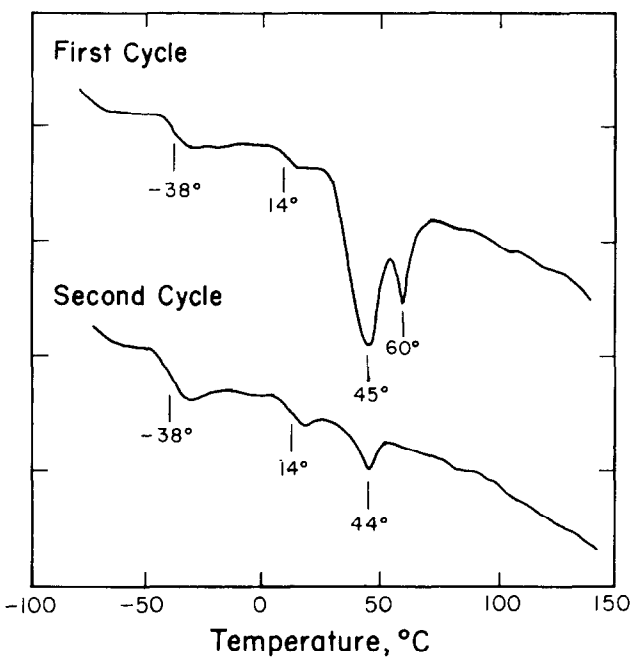


Figure 5 D.s.c. thermogram of PHAs obtained from 9TNA, no. 10

^{13}C n.m.r. chemical shifts for the PHAs containing arylalkyl groups are listed in *Table 5*. Molecular weights of the polyesters obtained varied from 37 000 to 156 000 with polydispersities between 2.3 and 2.9.

Thermal analysis of the PHAs was performed with a d.s.c. The PHAs with arylalkyl substituents had T_g values which ranged from +2 to +14°C but no melting peaks except for the polyester obtained from 9TNA, which had a melting peak, T_m, at 60°C (see *Table 6*). The melting transitions observed at around 45°C and all of the T_g transitions below 0°C were from PHN present. The T_m observed at 65–68°C in the second cycles were also from PHN. As typical examples of the d.s.c. results, *Figures 4* and *5* show the thermograms of the PHAs obtained from 7PHpA and 9TNA, respectively.

The X-ray diffraction pattern of the PHA sample obtained from 9TNA contained four sharp lines corresponding to $d_1 = 18.67$, $d_2 = 4.85$, $d_3 = 4.47$, $d_4 = 4.13$. These values are quite different from those of PHN[12,14,15].

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