(8) Yasuda, T.; Aida, T.; Inoue, S. *Macromolecules* **1983**, *16*, 1792.
(9) Yasuda, T.; Aida, T.; Inoue, S. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 3931.
(10) (a) Asano, S.; Aida, T.; Inoue, S. *J. Chem. Soc., Chem. Commun.* **1985**, 1148. (b) Inoue, S. *Makromol. Chem., Macromol. Symp.* **1986**, *3*, 295. (c) Inoue, S.; Aida, T. *Makromol. Chem., Macromol. Symp.* **1986**, *6*, 217.
(11) Adler, A. D.; Longo, F. R.; Finarelli, J. D.; Goldmacher, J.; Assour, J.; Korsakoff, L. *J. Org. Chem.* **1967**, *32*, 476.
(12) Inoue, S.; Takeda, N. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 984.
(13) Yasuda, T.; Aida, T.; Inoue, S. *J. Macromol. Sci., Chem.* **1984**, *A21*, 1035.

Biosynthesis of Copolyesters in *Alcaligenes eutrophus* H16 from ^{13}C-Labeled Acetate and Propionate

Yoshiharu Doi,* Masao Kunioka, Yoshiyuki Nakamura, and Kazuo Soga

Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 227, Japan. *Received March 27, 1987; Revised Manuscript Received July 8, 1987*

ABSTRACT: Copolyesters of 3-hydroxybutyrate (B) and 3-hydroxyvalerate (V) are isolated from *Alcaligenes eutrophus* H16 grown in nitrogen-free culture media containing the sodium salts of acetate and propionate. The content of V units in the copolyesters increases with increasing mole fraction of propionate to acetate in the culture medium. The copolyester is formed even when propionate is used as the sole carbon source, and the content of V units increases up to 45 mol % with increasing concentration of propionate in the medium. The biosynthetic pathway of the copolyester is investigated by using [1-^{13}C]acetate, [2-^{13}C]acetate, and [1-^{13}C]propionate as carbon sources. The use of ^{13}C-labeled acetate and propionate results in the formation of copolyesters specifically labeled with ^{13}C.

Introduction

Optically active poly(3-hydroxybutyrate) accumulates in a variety of bacteria and functions as a source of energy and carbon supply for the bacteria.[1] The biological function of poly(3-hydroxybutyrate) is similar to that of glycogen in mammals and starch in plants. Recently, the copolyester of 3-hydroxybutyrate (B) and 3-hydroxyvalerate (V) has been isolated from *Alcaligenes eutrophus* grown in a culture medium containing glucose and propionate.[2] This bacterial copolyester has attracted industrial attention as a possible candidate for large-scale biotechnological production, since the copolyester is environmentally degradable thermoplastic and has good mechanical properties, comparable to commercial thermoplastics such as isotactic polypropylene and poly(ethylene terephthalate).[2] The impact strength, flexural modulus, and melting temperature have been shown to be regulated by the content of V units in the copolyester.[2,3] It has been shown that the bacterial copolyester has a statistically random distribution of B and V units.[4,5] Marchessault et al.[5] found that the copolyester exhibits the unusual phenomenone of isodimorphism.

In this paper we report results on the biosynthesis of the copolyester in *Alcaligenes eutrophus* H16 from acetate and propionate. In addition, we determine the biosynthetic pathway of copolyester formation by using [1-^{13}C]acetate, [2-^{13}C]acetate, and [1-^{13}C]propionate as carbon sources.

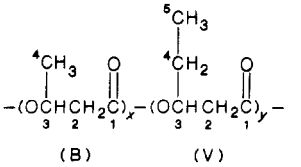
Experimental Section

Polyester Biosynthesis. Samples of the polyesters were isolated from *Alcaligenes eutrophus* H16 (ATCC 17699). The strain H16 was maintained on nutrient agar slants at 4 °C by monthly subculture. The bacteria were first grown at 30 °C in the nutrient-rich medium (100 cm^3) containing 10 g/dm^3 of yeast extract, 10 g/dm^3 of polypeptone, 5 g/dm^3 of meat extract, and 5 g/dm^3 of $(NH_4)_2SO_4$. The cells were harvested after 24 h, corresponding to the end of exponential growth, and washed with water. Under these culture conditions the accumulation of polyesters in the cells was not observed. To promote polyester synthesis, 0.20–0.15-g quantities of the washed cells were transferred into a nitrogen-free mineral medium[6] (100 cm^3) containing CH_3COONa and CH_3CH_2COONa. The cells were cultivated in this medium (pH 7.0) for 48 h at 30 °C, harvested by centrifugation, washed with acetone, and finally dried under vacuum at room temperature. Polyesters were extracted from the dried cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with hexane.

NMR Analysis. The ^{1}H and ^{13}C NMR analyses of the polyester samples were carried out on a JEOL GX-500 spectrometer. The 500-MHz ^{1}H NMR spectra were recorded at 27 °C on a $CDCl_3$ solution of the polyester at a concentration of 0.01 g/cm^3 with a 45° pulse (3.5 μs), 6.0-s pulse repetition, 7000-Hz spectral width, 32K data points, and 100–400 accumulations. The proton-decoupled 125-MHz ^{13}C NMR spectra were recorded at 27 °C on a $CDCl_3$ solution of the polyester at a concentration of 0.02–0.05 g/cm^3 with a 45° pulse (10 μs), 5.0-s pulse repetition, 25 000-Hz spectral width, 64K data points, and 1000–5000 accumulations. Tetramethylsilane (Me_4Si, δ 0) was used as an internal chemical shift standard.

Results and Discussion

Biosynthesis of Copolyesters. Table I lists the results of the biosynthesis of polyesters from CH_3COONa and CH_3CH_2COONa by *A. eutrophus* H16. The ^{1}H NMR spectra of all copolyesters showed that the polymers contained the two monomeric units B and V as



(B) (V)

The mole fractions of the two monomeric units, F_B and F_V, were determined from the ^{1}H NMR spectra, based on the intensity ratio of the doublet resonance at 1.274 ppm of the (B4) methyl protons to the triplet resonance at 0.894 ppm of the (V5) methyl protons.[4]

Poly(3-hydroxybutyrate) (PHB) was obtained when acetate was used as a carbon source, and the polymer content in dry cells increased with the concentration of acetate in the culture medium. Copolyesters of B and V units are produced in the presence of acetate and pro-

0024-9297/87/2220-2988$01.50/0

Table I
Biosynthesis of Polyesters from Acetate and Propionate by *Alcaligenes eutrophus* H16 at 30 °C

run	carbon source[a] CH_3COONa, g	carbon source[a] CH_3CH_2COONa, g	cell dry wt, g	polyester content,[b] wt %	polyester comp,[c] mol % F_B	polyester comp,[c] mol % F_V	$[\eta]$,[d] dL g^{-1}
23-1	2.0	0	0.26	51	100	0	8.3
23-2	2.0	0.1	0.34	46	98	2	
23-3	2.0	0.2	0.39	52	95	5	5.8
23-5	2.0	0.4	0.46	51	91	9	6.6
27-3	0.5	0	0.30	13	100	0	
27-4	0.5	0.5	0.41	22	79	21	
27-5	0.5	1.0	0.54	38	74	26	
27-6	0.5	2.0	0.71	45	72	28	
21-1	0	0.2	0.26	12	78	22	
21-2	0	0.6	0.32	18	76	24	
21-3	0	1.0	0.39	28	72	28	
21-4	0	1.4	0.45	42	69	31	
21-5	0	1.8	0.48	56	73	27	3.9
21-6	0	2.2	0.57	31	70	30	
21-7	0	2.6	0.38	40	56	44	
21-8	0	3.0	0.50	35	55	45	3.1

[a] Carbon source in nitrogen-free culture media (100 cm^3). [b] Polyester content in dry cells. [c] Determined from ^{1}H NMR spectra. B and V represent 3-hydroxybutyrate and 3-hydroxyvalerate units, respectively. [d] The intrinsic viscosity $[\eta]$ of polyester is measured in chloroform at 30 °C.

pionate, and the content of V units increases to 28 mol % with increasing mole fraction of propionate to acetate. Copolyesters are obtained even when propionate is used as the sole carbon source. It is of interest that the content of V units increases from 22 to 45 mol % with increasing concentration of propionate. These results are discussed in the following sections on the basis of the biosynthetic pathway of the copolyester.

Biosynthetic Pathway of PHB. The biosynthetic pathway of PHB was studied by NMR analysis of PHB samples derived from ^{13}C-labeled acetate. Here, two nitrogen-free culture media were used: medium A, 2.0 g of sodium [1-^{13}C]acetate (6.0% ^{13}C per C atom), and medium B, 2.0 g of sodium [2-^{13}C]acetate (6.0% ^{13}C per C atom). The 125-MHz ^{13}C NMR spectra of the isolated PHB samples are shown in Figure 1, together with the chemical shift assignments.[7] The PHB from [1-^{13}C]acetate exhibits specific enhancements in the intensities of the (B1) and (B3) carbon resonances (see Figure 1a). In contrast, the PHB from [2-^{13}C]acetate displays specific enhancements in the intensities of (B2) and (B4) carbon resonances (see Figure 1b). The enhancements of these carbon resonances were 5.5 ± 0.5 for the both PHB samples, which was determined from a comparison with the spectrum of an unenriched standard PHB sample recorded under identical conditions. The results indicate that the ^{13}C-labeled carbonyl carbon (6.0% ^{13}C) of acetate is selectively incorporated in the specific sites B1 and B3 of PHB without scrambling to other carbons and that the methyl carbon (6.0% ^{13}C) of acetate is introduced into the specific sites B2 and B4 of PHB. The selective incorporation of each ^{13}C-labeled carbon of acetate into PHB was also confirmed by 500-MHz H^1 NMR spectra which showed a fine structure due to ^{13}C-H coupling in the B2, B3, and B4 proton resonances. It was determined from the ^{1}H NMR spectra that the ^{13}C populations in these specific sites were about 6% (see Table II). The above results support the biosynthetic pathway[8-13] that PHB is formed by the condensation of D-(−)-(3-hydroxybutyryl)coenzyme A (CoA) which is synthesized from acetyl-CoA via acetoacetyl-CoA under the action of specific enzymes, as represented by the scheme in Figure 2.

Biosynthetic Pathway of Copolyesters. Table II lists the results for ^{13}C-labeled copolyesters derived from *A. eutrophus* H16 in different culture media (C to H) containing [1-^{13}C]propionate, [1-^{13}C]acetate, and [2-^{13}C]-acetate. The 125-MHz ^{13}C NMR spectra of typical copolyester samples (C, E, and G) are shown in Figure 3.

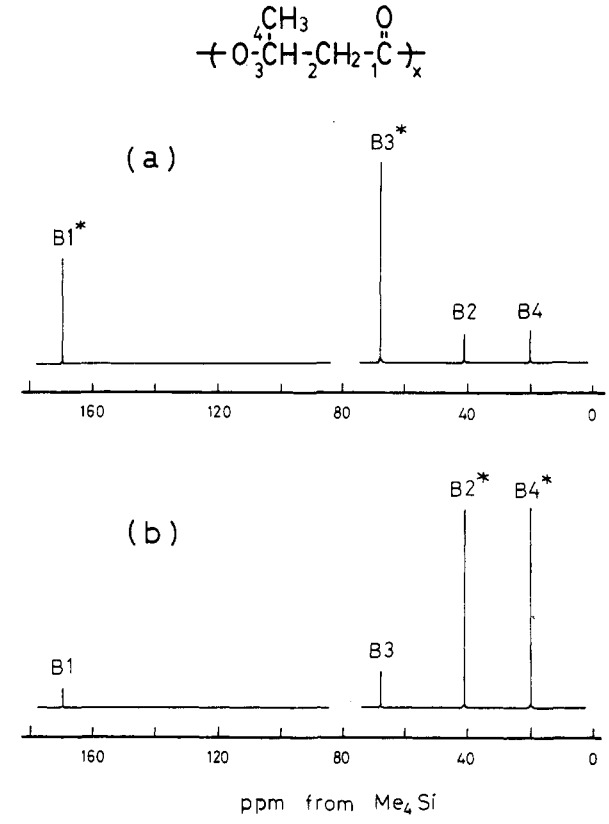


Figure 1. Proton-decoupled 125-MHz ^{13}C NMR spectra of ^{13}C-labeled PHB samples (A and B) in chloroform at 27 °C. (a) PHB from [1-^{13}C]CH_3COONa (6.0% ^{13}C) in medium A and (b) PHB from [2-^{13}C] CH_3COONa (6.0% ^{13}C) in medium B.

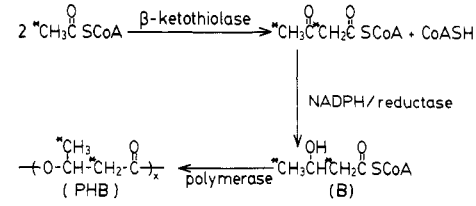


Figure 2. Biosynthetic pathway of poly(3-hydroxybutyrate).[8-13]

Table II
Biosynthesis of ^{13}C-Labeled Polyesters from [1-^{13}C]Acetate, [2-^{13}C]Acetate, and [1-^{13}C]Propionate by *Alcaligenes eutrophus* H16 at 30 °C

medium	carbon source[a]	polyester comp.,[b] mol % F_B	F_V	^{13}C-labeled sites[c]	^{13}C enrichment at the labeled sites[b]
A	[1-^{13}C]CH_3COONa (6.0% ^{13}C, 2.0 g)	100	0	B1,B3	5.8
B	[2-^{13}C]CH_3COONa (6.0% ^{13}C, 2.0 g)	100	0	B2,B4	6.0 ± 0.2
C	[1-^{13}C]CH_3CH_2COONa (10% ^{13}C, 1.0 g)	66	34	V3	9.5
D	[1-^{13}C]CH_3CH_2COONa (10% ^{13}C, 1.0 g) + CH_3COONa (0.5 g)	73	27	V3	9.8
E	[1-^{13}C]CH_3COONa (21% ^{13}C, 0.5 g) + CH_3CH_2COONa (1.0 g)	70	30	B1, B3, V1	7.0
F	[2-^{13}C]CH_3COONa (21% ^{13}C, 0.5 g) + CH_3CH_2COONa (0.5 g)	82	18	B2, B4, V2	7.2 ± 0.2
G	[2-^{13}C]CH_3COONa (21% ^{13}C, 0.5 g) + CH_3CH_2COONa (1.0 g)	72	28	B2, B4, V2	7.3 ± 0.2
H	[2-^{13}C]CH_3COONa (21% ^{13}C, 0.5 g) + CH_3CH_2COONa (2.0 g)	74	26	B2, B4, V2	4.3 ± 0.2

[a] Carbon source in nitrogen-free culture media (100 cm^3). [b] Determined from ^1H NMR spectra. [c] Determined from ^{13}C NMR spectra.

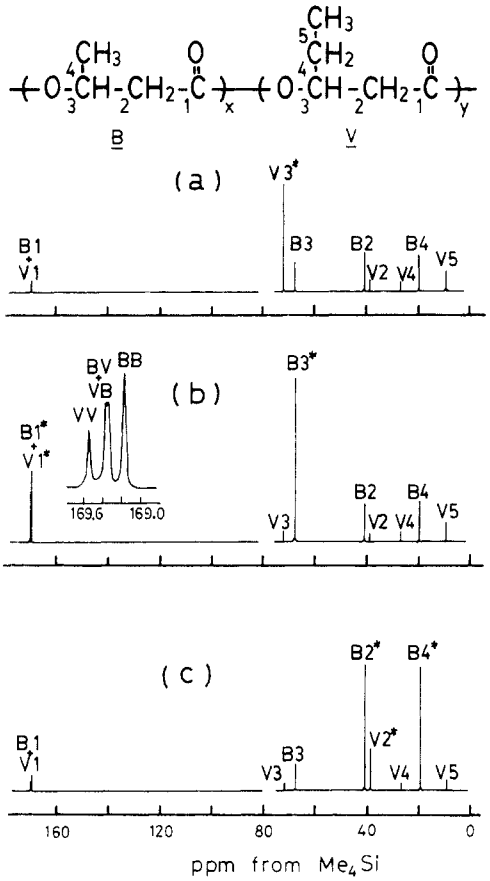


Figure 3. Proton-decoupled 125-MHz ^{13}C NMR spectra of ^{13}C-labeled copolyester samples (C, E, and G) in chloroform at 27 °C: (a) copolyester from [1-^{13}C]CH_3CH_2COONa (10% ^{13}C) in medium C; (b) copolyester from [1-^{13}C]CH_3COONa (21% ^{13}C) and CH_3CH_2COONa (1.1% ^{13}C) in medium E; (c) copolyester from [2-^{13}C]CH_3COONa (21% ^{13}C) and CH_3CH_2COONa (1.1% ^{13}C) in medium G. The BB, BV, VB, and VV represent the different diad sequences of connecting 3-hydroxybutyrate (B) and 3-hydroxyvalerate (V) units.[4]

The copolyester sample C from [1-^{13}C]propionate (10% ^{13}C per C atom) shows a specific enhancement for the resonance of the V3 carbon (see Figure 3a). It was determined from the 500-MHz ^1H NMR spectrum that the ^{13}C population in the V3 site was 9.5% and there was no significant labeling of other carbons. The selective incorporation of the ^{13}C-labeled carbonyl carbon (10% ^{13}C) of propionate into the specific site (V3) of the copolyester was also observed in the presence of natural abundance acetae (1.1% ^{13}C) (see medium D in Table II).

From these results we have reached the following conclusion on the mechanism of copolyester biosynthesis in

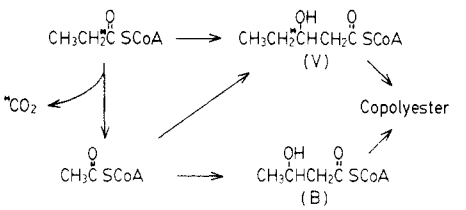


Figure 4. Biosynthetic pathway of copolyesters containing 3-hydoxybutyrate (B) and 3-hydroxyvalerate (V) units.

A. eutrophus. When propionate is used as the sole carbon source, (3-hydroxybutyryl)-CoA is formed from two molecules of acetyl-CoA which is generated by a selective elimination of ^{13}C-labeled carbonyl from propionyl-CoA, and 3-hydroxyvaleryl-CoA is formed by the reaction of propionyl-CoA with the acetyl-CoA, resulting in the formation of a random copolyester under the action of PHB polymerase, as shown schematically in Figure 4. As pointed out in Table I, the content of V units in the copolyester increased with the concentration of propionate in the culture medium. This result suggests that the mole ratio of (3-hydroxyvaleryl)-CoA to (3-hydroxybutyryl)-CoA increases with the concentration of propionyl-CoA in a cell. When acetate coexists with propionate in the culture medium, acetyl-CoA in a cell arises from the both carbon sources, resulting in a decrease in the content of V units in the copolyester. The incorporation of acetate into the copolyester was confirmed by the following experiments using ^{13}C-labeled acetate with natural abundance propionate (media E to H in Table II).

The ^{13}C NMR spectrum of the copolyester sample E from [1-^{13}C]acetate (21% ^{13}C) and natural abundance propionate (1.1% ^{13}C) shows specific enhancements in the intensities of the B1, B3, and V1 carbon resonances (see Figure 3b). It was determined from the ^1H NMR spectrum that the ^{13}C population in the B3 site was 7.0%. The ^{13}C enrichment is lower than the ^{13}C enrichment of [1-^{13}C]-acetate (21% ^{13}C), which indicates that acetyl-CoA is formed from both carbon sources of ^{13}C-labeled acetate and natural abundance propionate. The selective incorporation of the ^{13}C-labeled carbonyl carbon of acetate into the B1, B3, and V1 sites of the copolyester can be understood on the basis of the biosynthetic pathway in Figure 4.

The ^{13}C NMR spectra of the copolyester samples F, G, and H from [2-^{13}C]acetate (21% ^{13}C) and natural abundance propionate showed specific enhancements in the intensities of the B2, B4, and V2 carbon resonances. (See Figure 3c and Table II). The ^1H NMR spectra showed that the ^{13}C population in the B2, B4, and V2 sites decreased with increasing mole fraction of natural abundance propionate to [2-^{13}C]acetate, and that there was no significant ^{13}C-labeling on other carbons. Again this result supports

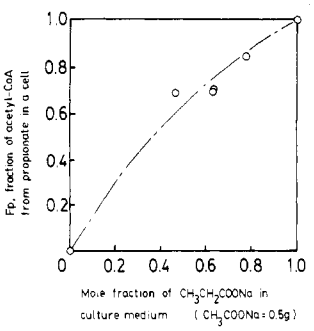


Figure 5. Relationship between F_p and mole fraction of propionate in the culture medium. F_p represents the fraction of acetyl-CoA from propionate to total acetyl-CoA in a cell.

the copolyester biosynthetic pathway shown in Figure 4. The fractions of acetyl-CoA arising from acetate and propionate as carbon sources were determined by using the values of ^{13}C enrichment at the specific sites of the copolyesters and the following relations:

$$21F_a + 1.1F_p = F(^{13}C) \quad (1)$$

$$F_a + F_p = 1.0 \quad (2)$$

Here, F_a and F_p represent the fractions of acetyl-CoA from ^{13}C-labeled acetate (21% ^{13}C) and natural abundance propionate (1.1% ^{13}C) to total acetyl-CoA in a cell, respectively, and $F(^{13}C)$ is the ^{13}C enrichment at the labeled sites of the copolyester. The results are given in Figure 5. The fracton F_p of acetyl-CoA from propionate is slightly higher than the mole fracton of propionate in the culture medium.

Acknowledgment. We thank Professor I. Karube for helpful discussions.

Registry No. (B)(V) (copolymer), 80181-31-3; PHB (SRU), 26744-04-7; PHB (homopolymer), 26063-00-3; $H_3CCH_2CO_2Na$, 137-40-6; H_3CCO_2Na, 127-09-3.

References and Notes

(1) Dawes, E. A.; Senior, P. J. *Adv. Microb. Physiol.* **1973**, *10*, 135.
(2) (a) Holmes, P. A. *Phys. Technol.* **1985**, *16*, 32. (b) Holmes, P. A.; Collins, S. H. *Japan Kokai* 150393, 1982.
(3) Owen, A. J. *Colloid Polym. Sci.* **1985**, *263*, 799.
(4) Doi, Y.; Kunioka, M; Nakamura, Y.; Soga, K. *Macromolecules* **1986**, *19*, 2860.
(5) Bluhm, T. L.; Hamer, G. K.; Marchessalt, R. H.; Fyfe, C. A.; Veregin, R. P. *Macromolecules* **1986**, *19*, 2871.
(6) Repaske, R.; Repaske, A. C. *Appl. Environ. Microbiol.* **1976**, *32*, 585.
(7) Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1986**, *19*, 1274.
(8) Senior, P. J.; Dawes, E. A. *Biochem. J.* **1971**, *125*, 55.
(9) Senior, P. J.; Dawes, E. A. *Biochem. J.* **1973**, *134*, 225.
(10) Oeding, V.; Schlegel, H. G. *Biochem. J.* **1973**, *134*, 239.
(11) Fukui, T.; Yoshimoto, A.; Matsumoto, M.; Hosokawa, S.; Saito, T.; Nishikawa, H.; Tomita, K. *Arch.* Microbiol. **1976**, *110*, 149.
(12) Saito, T.; Fukui, T.; Ikeda, F.; Tanaka, Y.; Tomita, K. *Arch. Microbiol.* **1977**, *114*, 211.
(13) Nishimura, T.; Saito, T.; Tomita, K. *Arch. Microbiol.* **1978**, *116*, 21.

Mechanism of Thermal Decomposition of Nylon 66

Alberto Ballistreri,† Domenico Garozzo,† Mario Giuffrida,‡ and Giorgio Montaudo*†

Dipartimento di Scienze Chimiche, Università di Catania, Viale Andrea Doria, 6, 95125 Catania, Italy, and Istituto per la Chimica e la Tecnologia dei Materiali Polimerici, Consiglio Nazionale delle Ricerche, Viale Andrea Doria, 6, 95125 Catania, Italy. *Received January 27, 1987*

ABSTRACT: The thermal decomposition mechanism of nylon 66 [poly(hexamethyleneadipamide)] has been investigated by direct pyrolysis into the mass spectrometer. Several methods have been used in order to identify compounds present in the pyrolysis mixtures: comparison of electron impact and chemical ionization spectra, accurate mass measurements, comparison with the mass spectra of authentic compounds, and tandem mass spectrometry (daughter and parent ions spectra). The results show that the thermal decomposition mechanism of nylon 66 occurs via a C–H hydrogen-transfer reaction to nitrogen with formation of compounds bearing amine and ketoamide end groups. These primary thermal products further decompose or react with formation of cyclopentanone, aminohexamethylene isocyanate, and compounds bearing amine and/or Schiff base groups. The synthesis of some key thermal decomposition compounds of nylon 66 has been performed, allowing comparison of authentic samples with pyrolysis products.

Introduction

The thermal decomposition of nylon 66 [poly(hexamethyleneadipamide)] has been the subject of several publications over the past years.[1,2] Formation of hexamethylenediamine (HMDA) and cyclopentanone (CP) in the pyrolysis was recognized already in earlier studies,[3–5] whereas the presence of the cyclic monomer I was later detected by Peebles et al.[6] among the volatile products originating from the pyrolysis of nylon 66.

$$\underbrace{NH\text{–}(CH_2)_6\text{–}NH\text{–}CO\text{–}(CH_2)_4\text{–}CO}_{I}$$

It is now understood[7] that nylon 66, and many other condensation polymers as well, contains cyclic oligomers formed during the polymerization reaction, so that compound I cannot be considered a pyrolysis product without further scrutiny.

Since the advent of modern pyrolysis mass spectrometry techniques,[8–10] the thermal degradation of nylon 66 has been intensively investigated, and several mass spectro-

† Università di Catania.
‡ Consiglio Nazionale delle Ricerche.

0024-9297/87/2220-2991$01.50/0