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Structure elucidation of methylidene malonate 2.1.2 cyclic trimers with mass spectrometry, liquid chromatography and nuclear magnetic resonance investigations

A. Salvador [a], B. Herbreteau [a], P. Breton [b], N. Bru [b], M. Dreux [a,*]

[a] *Institut de Chimie Organique et Analytique, UPRES A 6005, Université d'Orléans, B.P. no. 6759, 45067 Orléans Cedex 2, France*
[b] *VIRSOL, 46 rue Boissière, 75116 Paris, France*

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Abstract

The anionic polymerization of methylidene malonate 2.1.2. is able to yield four different molecule types. The major product obtained at pH 5.5 corresponds to a trisubstituted cyclohexanone formed by a back-biting reaction. A mixture of four diastereoisomer couples due to the presence of three asymmetric carbon atoms was produced in the same proportion. These results were deduced from mass spectrometry (MS), liquid chromatography–MS (LC-MS) and ^{1}H nuclear magnetic resonance (NMR) investigations.

Keywords: Methylidene malonate 2.1.2; Diastereoisomers; NMR; Oligomerization; Mass spectrometry; Liquid chromatography–mass spectrometry

1. Introduction

Among the various systems developed in drug targeting strategies, the colloidal polymeric carriers represent along with liposomes and other hydrosoluble macromolecular vectors, one of the most advanced approaches for controlled drug delivery. Nanospheres and nanocapsules under the term nanoparticles were first described in the late 1970s [1,2] and soon poly(alkyl-2-cyanoacrylate) (PACA) nanoparticles [3] were extensively studied with the aim of pharmaceutical applications. However, some PACA properties, such as the low polymerization pH (around 2) required for their preparation and some in vitro cytotoxicity [4] represent evident drawbacks which led to the synthesis of a new methylidene malonate derivative, i.e. methylidene malonate 2.1.2 (MM 2.1.2) able to polymerize spontaneously in an aqueous buffer to form nanoparticles. The initial description of poly(methylidene malonate 2.1.2) (PMM 2.1.2) nanoparticles obtained at pH close to neutrality was done by Lescure et al. [5,6] and

* Corresponding author. Tel.: +33-2-38417074; fax: +33-2-38494777.
E-mail address: michel.dreux@univ-orleans.fr (M. Dreux)

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especially showed the molecular weight distribution of their constituents. Recently, Salvador et al. [7] described the chromatographic separation of PMM 2.1.2 nanoparticle-constituting oligomers and focused on the separation of the major diastereoisomers formed in the course of the anionic oligomerization in water. Previous reports [7,8] strongly suggested that these isomers could correspond to a 2,4,6 trisubstituted cyclohexanone trimer. In this paper, the structure elucidation of this product is confirmed by studies conducted in mass spectrometry (MS), liquid chromatography-MS (LC-MS) and proton ^{1}H nuclear magnetic resonance (NMR).

2. Experimental and methods

2.1. Reagents

Methanol, dichloromethane, (Hipersolv grade, BDH, Poole, UK), acetonitrile (ACN), heptane, dioxane (Mallinckrodt Baker, Noisy-le-Sec, France) and trifluoroacetic acid (TFA; Merck, Darmstadt, Germany) were analytical grade. Chloroform-d ($CDCl_3$) containing 1% (v/v) tetramethyl silane (TMS) was purchased from Janssen Chimica (Geel, Belgium). Purified water was purchased from Stalabo (Coopération Pharmaceutique Française, Melun, France). Finally, 1-ethoxycarbonyl-1-ethoxycarbonylmethylenoxycarbonyl ethene (Mw = 230) also referred to as methylidene malonate 2.1.2 (MM 2.1.2) was prepared according to Bru-Magniez et al. [9].

2.2. Nanoparticle preparation

PMM 2.1.2 colloidal suspensions were prepared under sterile conditions through anionic polymerization in phosphate buffer (KH_2PO_4/Na_2HPO_4 0.066 M, pH 5.5) supplemented with 1% dextran. Particle formation was followed by measuring suspension turbidity at 400 nm with a Model 25 spectrophotometer (Beckman, Gagny, France). After 24 h polymerization, nanoparticles were filtered, freeze-dried and finally dissolved in the solvent prior to filtration (Fluoropore filter, Millipore, Yonezawa, Japan) and analysis.

2.3. Mass spectrometry

Mass spectra were recorded from a Sciex API 300 triple quadripole mass spectrometer (Sciex, Toronto, Canada) working in an m/z range of 3000 a.m.u. equipped with an ionspray (IS) or an atmospheric pressure chemical ionization (APCI) ion source. Calibration was carried out with a polypropylene glycol (PPG) mixture (3.3×10^{-5} M PPG Mw = 425; 1.0×10^{-4} M PPG Mw = 1000 and 2.0×10^{-4} M PPG Mw = 2000), 0.1% acetonitrile and 2 mM ammonium formiate in 50% aqueous methanol. For IS or APCI, spectra were recorded in positive mode (dwell time, 2 ms; step size, 1 a.m.u.; data acquired to hard disk). For data acquisition, data processing and the control of the mass spectrometer, an Apple™ MacIntosh® system 7.5 was employed. For IS, the IS voltage was 5700 V and the orifice voltage was 60 V. For APCI, the current on the discharge needle was held at 3 μA, orifice voltage was 20 V, and temperature was set at 400°C. The curtain gas flow was 8 l min^{-1} and the nebulizer gas flow was 10 l min^{-1}. The solution was introduced into the IS source at a constant flow rate of 5 μl min^{-1}, using a medical infusion pump model 22 (Harvard Apparatus, South Natick, USA).

The sample of nanoparticle was dissolved in ACN/water (70:30, v/v) and concentration was 100 mg l^{-1}.

2.4. Liquid chromatography

LC-MS was carried out by using a Perkin–Elmer (Überlingen, Germany) solvent module coupled to a Rheodyne (Berkeley, CA, USA) Model 7125 injector connected to a 20-μl sample loop. Column was a Zorbax ODS 5 μm (150 × 4.6 mm I.D.) from Dupont (Wilmington, DE, USA) and eluent was a gradient of ACN/(water + 0.1% TFA), the composition of the eluent was 70/30 (v/v) for the first 10 min and then increased linearly from 70/30 to 100/0 in 5 min. Flow rate was 500 μl min^{-1}.

Preparative LC trimer purification was carried out by a Shimadzu preparative LC-8A system (Kyoto, Japan) connected to a 1-ml sample loop. Column was LiChrospher NH_2 10 μm (250 × 10.0

mm I.D.) (column A) from Merck, Hypercarb S 7 μm (250 × 10.0 mm I.D.) (column B) from Shandon (Astmoor Runcorn, UK). Eluent was CH_2Cl_2-heptane-dioxane (62:43:1, v/v/v) for column A and ACN/water (70:30, v/v) for column B and the flow rate was 4 ml min^{-1} [7]. Experi-

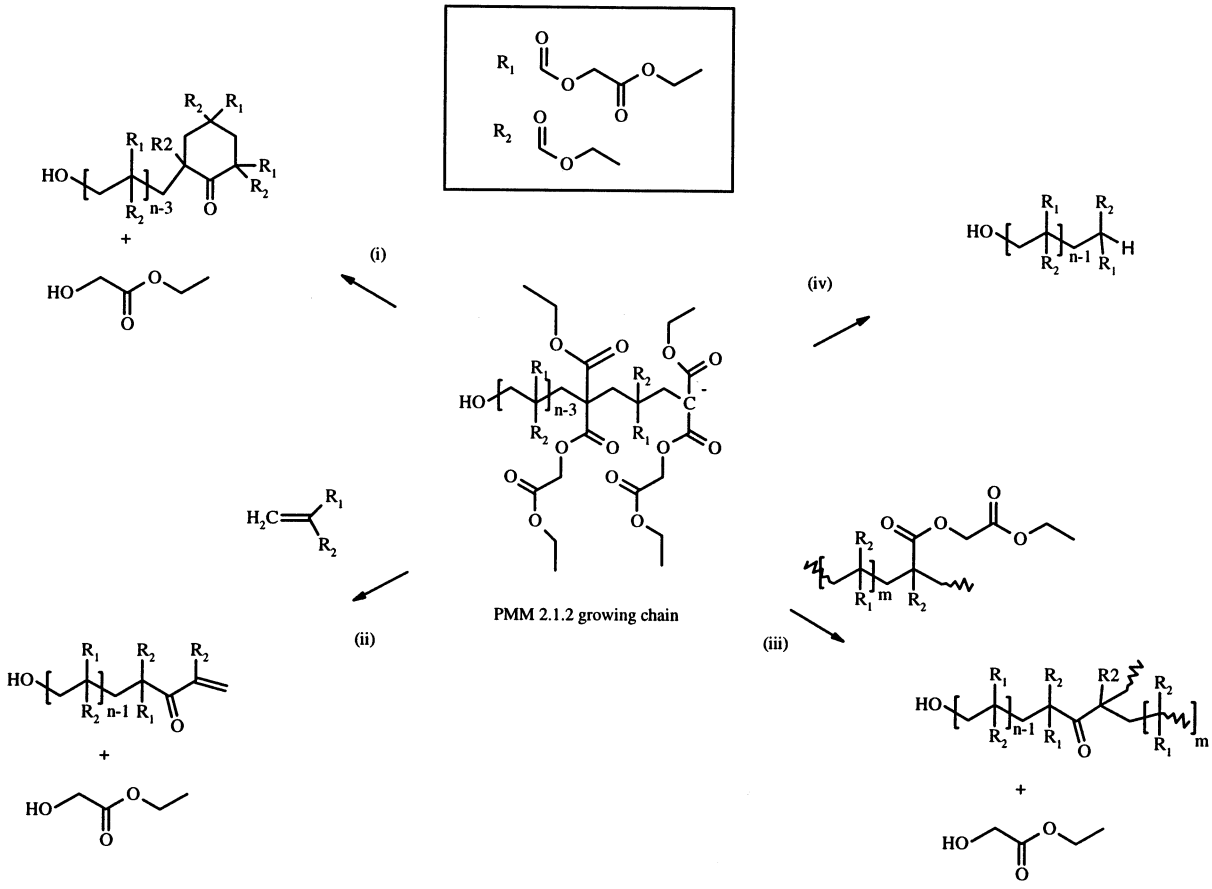


Fig. 1. The growing chain of PMM 2.1.2 and possible termination [10,11] of the anionic polymerization.

Table 1
Adduct molecular weights of ions generated by MS and resulting from termination pathways proposed on Fig. 1

n	Termination (i) or (ii): series (X_n)			Termination (iv): series (Y_n)		
	$[X_n+H]^+$	$[X_n+Na]^+$	$[X_n+K]^+$	$[Y_n+H]^+$	$[Y_n+Na]^+$	$[Y_n+K]^+$
3	606	628	644	709	731	747
4	836	858	874	939	961	977
5	1066	1088	1104	1169	1191	1207
6	1296	1318	1334	1399	1421	1437
7	1526	1548	1564	1629	1651	1667
8	1756	1778	1794	1859	1881	1897

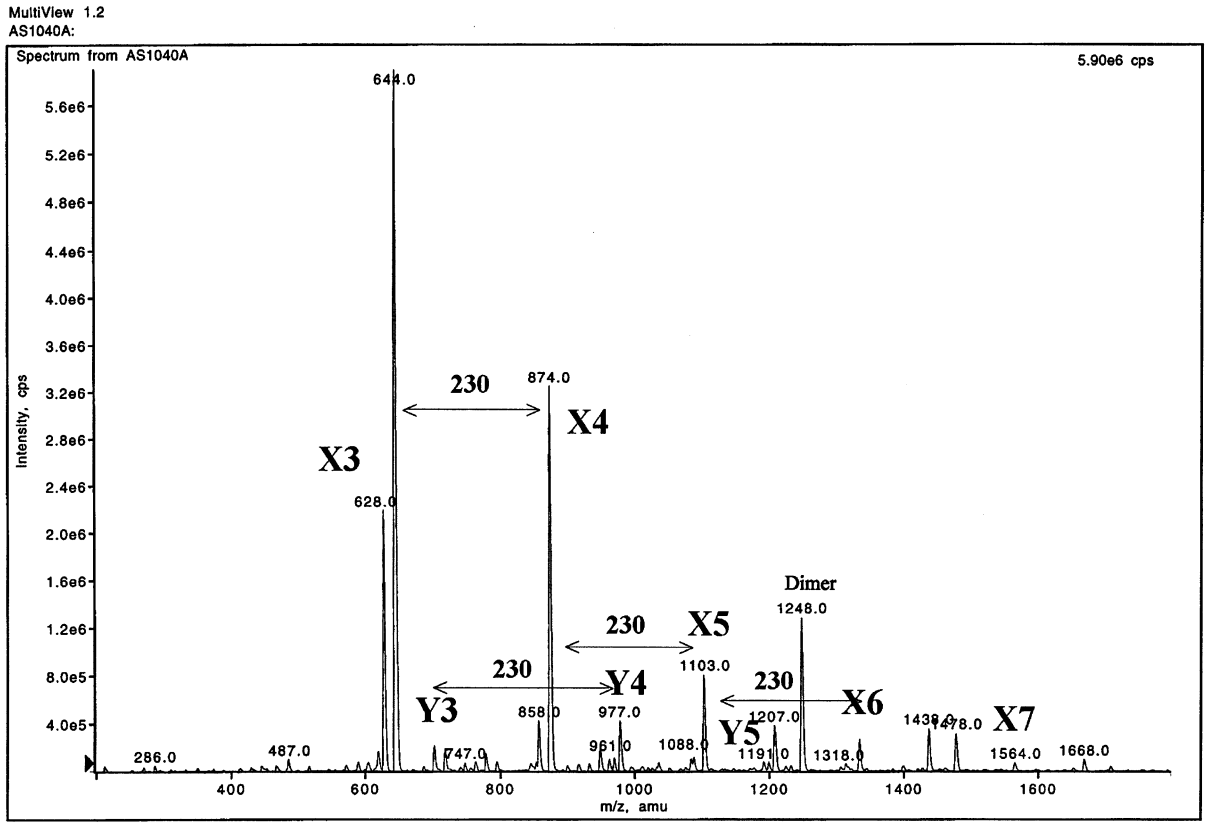


Fig. 2. Ionspray MS of PMM 2.1.2 oligomeric synthetic mixture.

ments were performed at room temperature and the detection was done by ultraviolet (Varian 2550, Palo, Alto, CA, USA) at 210 nm or by light scattering using an evaporative light scattering detector (ELSD) Model Sedex 55 (Sedere, Alftorville, France). The ELSD settings were photomultiplier, 7; drift tube temperature, 35°C; nebulizer gas (nitrogen), 2.2 bars. Data were processed with a Shimadzu integrator Model CR 5A.

2.5. Nuclear magnetic resonance

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded with a Brüker 250 spectrometer (Karlsruhe, Germany). Samples were dissolved in deuteriated chloroform ($CDCl_3$). Chemical shifts were expressed as part per million (ppm) from tetramethylsilane ($Me_4Si = 0.00$ ppm).

3. Results and discussion

As it has been proposed by Schreiber [10] and Gerner et al. [11] for methyl methacrylate anionic polymerization, the potential mechanisms for the termination of MM 2.1.2 polymerization are shown in Fig. 1. The carbanion could react according to four pathways described hereunder: (i) an intramolecular reaction with a carbonyl group resulting in the formation of a 2,4,6-trisubstituted cyclohexanone ring (back-biting reaction); (ii) a reaction with a carbonyl group of a monomer resulting in the formation of a vinyl ketone (monomer termination); (iii) a reaction with a carbonyl group of another polymer resulting in chain coupling (intermolecular polymer termination); and (iv) a protonation. Calculations of the different molecular weights expected in MS from Fig. 1 are shown in Table 1. Molecular weights

for the third termination type are not shown since the corresponding products were unlikely to occur and never observed on mass spectra (data not shown).

3.1. MS and LC-MS analysis

IS mass spectrometry was applied to PMM 2.1.2 reaction products dissolved in ACN/water (70:30, v/v) using direct infusion with a syringe pump. The resulting spectrum shown on Fig. 2 comprised several signals, regularly distributed from 600 to 1800. Two series of peaks, noted X and Y in Table 1, could be distinguished and inside each series, a constant mass deviation of 230 (molecular weight of MM 2.1.2. monomer) was noticed. Most of the time, adducts with K^+ or Na^+ were observed (e.g. X_3: $m/z = 628$ and 644 for $[X_3 + Na]^+$ and $[X_3 + K]^+$ adducts, respectively). As shown in Table 1, the peaks of series X and Y could, respectively, correspond to the termination (i) or (ii) and the termination (iv). All signals were identified and none was attributable to termination (iii). The signal at $m/z = 1248$ could correspond to a X_3 dimer $[2X_3 + K]^+$ with potassium. Dimers of other products of the series X and Y were also probably present, but too weak to be observed. These data agreed with the results published by Breton et al. [8]. In order to replace salt adducts by proton adducts APCI was used.

The APCI analysis revealed the expected X series with proton adducts as reported in Table 1 and an unexpected series (data not shown). This unexpected series did not correspond to the $[Y + H]^+$ series which is probably fragmented. Conse-

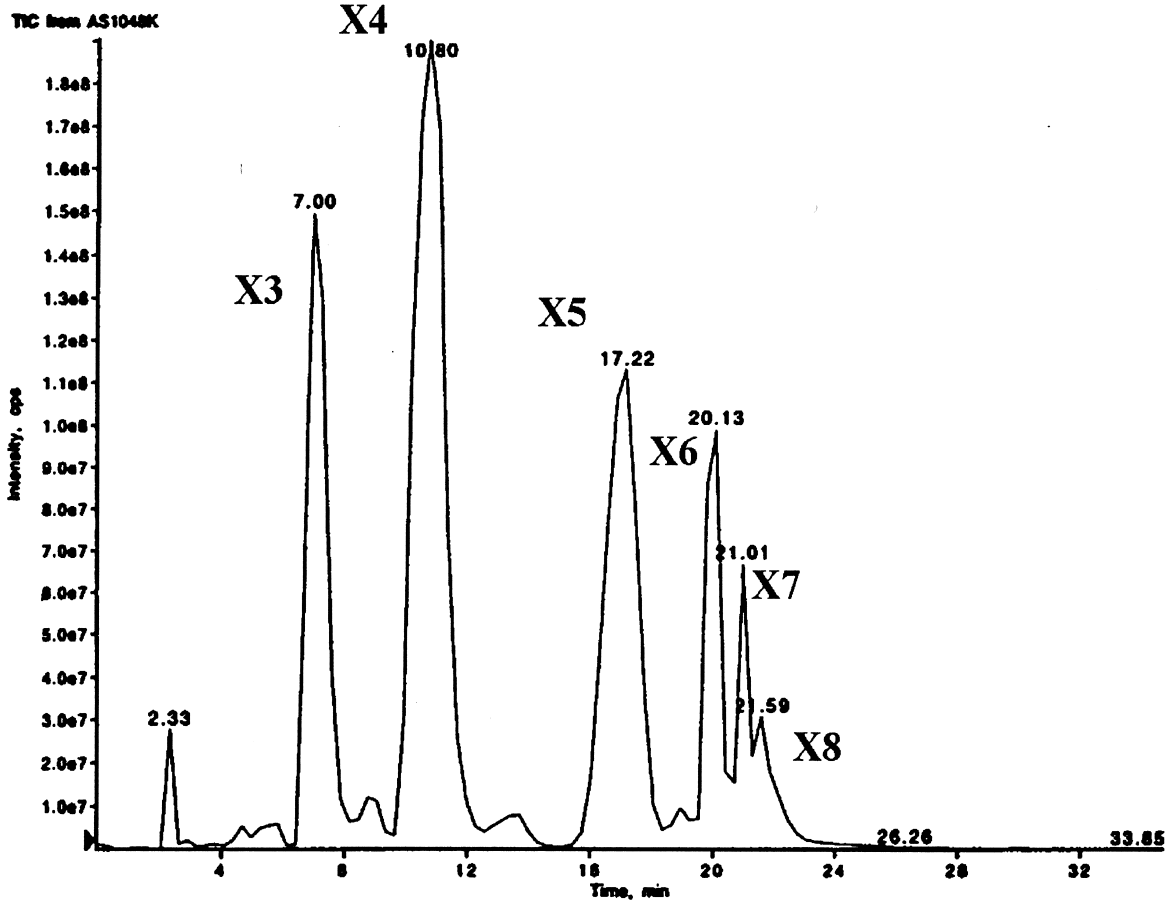


Fig. 3. APCI LC-MS of PMM 2.1.2 oligomers (TIC).

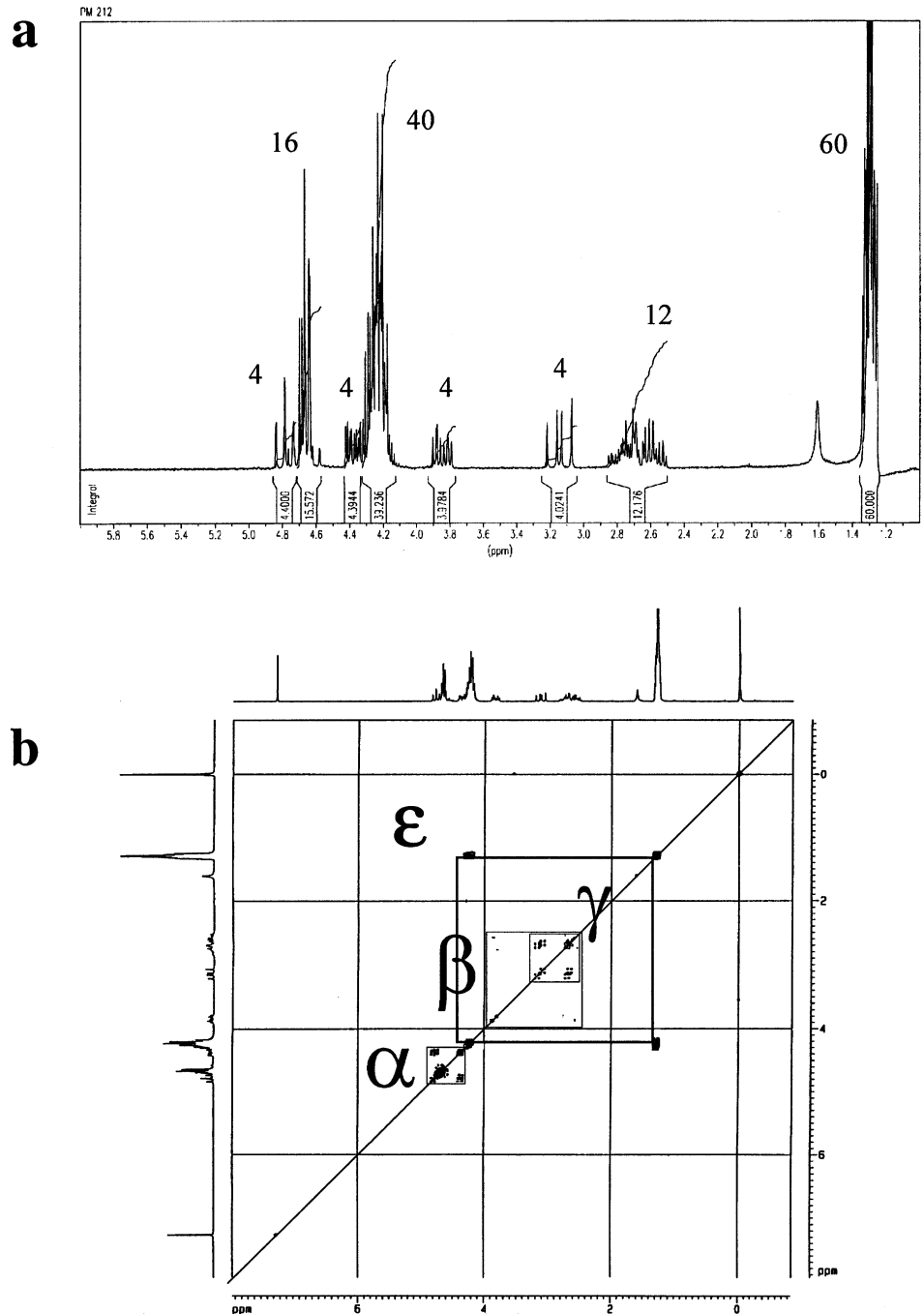
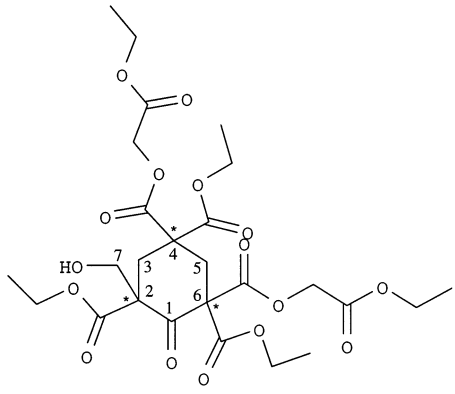


Fig. 4. (a) ^{1}H NMR spectra and (b) correlation ^{1}H-^{1}H (cosy 90) of the major oligomer (X_3).

quently, the unexpected series could result from ion fragmentation of the X series. To confirm this hypothesis LC-MS experiments were undertaken.

LC-MS investigations with APCI enabled the separation of products identified as X series oligomers. Elution gradient up to 100% ACN shows an oligomerization distribution from 3 to 8 monomeric units (Fig. 3). The ions extracted from total ion current (TIC) showed that under each chromatographic peak, there is a mass, which corresponds to a sequential loss of 105. We may conclude that the unforeseen series observed by APCI resulted from ion fragmentation of the X series.

In conclusion, MS and LC-MS allowed the possibility of termination (iii) to be eliminated, but did not permit conclusions to be drawn about termination (i) or (ii). Termination (iv) probably occurred but is minor, so studies focused on the major *X* series oligomers and especially on the trimer ($n = 0$).



$\underline{CH_3}CH_2$	5	15 protons
$\underline{CH_2}CH_3$	5	10 protons
$CO\underline{CH_2}OCO$	2	4 protons
$\underline{CH_2}OH$	1	2 protons
$CH_2O\underline{H}$	1	1 proton
$\underline{CH_2}$ cyclohexanone ring	2	4 protons
		36 protons
	x	4
		144 protons

Fig. 5. Structure of the diastereoisomers of the major oligomer: a 2,4,6-ethoxycarbonyl-4,6-ethoxycarbonylmethoxy-carbonyl-2-hydroxymethyl cyclohexanone.

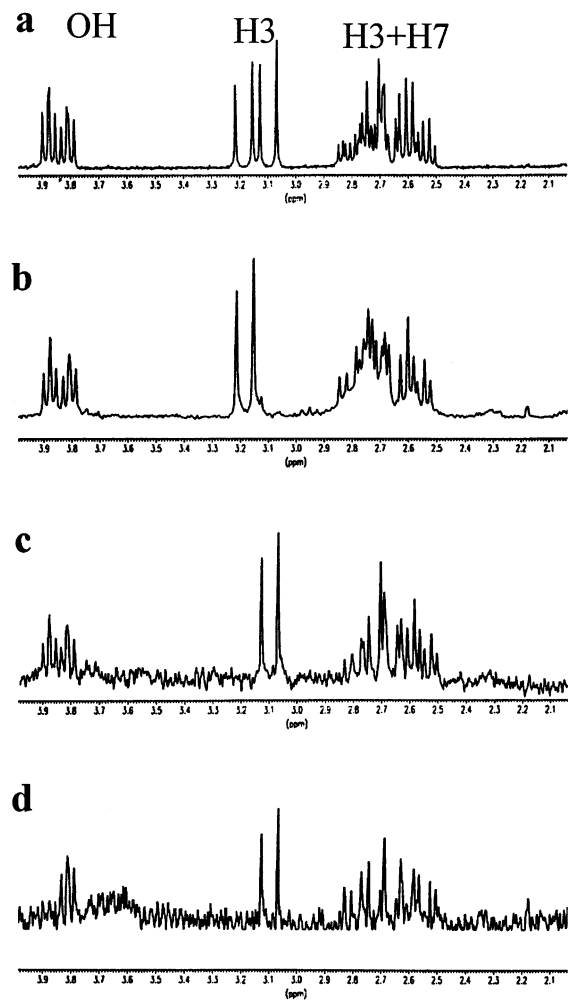


Fig. 6. ^{1}H NMR spectra (2.0–4.0 ppm) of a mixture of the four diastereoisomer couples of X_3 (a), of peak 1 collected on Hypercarb S column or peak A collected on amino stationary phase (mixture of two diastereoisomer couples) (b), of peak B collected on an amino stationary phase (mixture of two diastereoisomer couples) (c) and of peak 2 collected on Hypercarb S column corresponding to a pure diastereoisomer couple (d).

NMR easily differentiates between (i) or (ii) due to the specificity of ethylenic proton resonance. Preparative LC fraction of X_3 representing the major oligomer (Mw = 604.6) [7] was therefore

collected in order to perform detailed NMR investigations and to elucidate the structure.

3.2. NMR analysis

The ^{1}H-NMR spectra of the major oligomer X_3 recorded in $CDCl_3$ are shown in Fig. 4(a, b). These spectra corresponded to a mixture of several optical isomer couples (enantiomers and diastereoisomers) and required additional NMR techniques (i.e. Correlation Spectroscopy (COSY), Distortionals Enhancement by Polarization Transfer (DEPT), Insensitive Nuclei Enhancement by Polarization Transfer (INEPT)) in order to be fully assigned. However, from Fig. 4, it clearly appeared that there were no ethylenic protons (chemical shift for ethylenic protons of MM 2.1.2 was 6.7 ppm). Thus, it could be concluded that the X series did not correspond to an intermolecular termination (type (ii) — Fig. 1), but certainly to a back-biting reaction (type (i) — Fig. 1). In such a situation, the 2,4,6-trisubstituted cyclohexanone (Fig. 5) yielded bore three asymmetric carbons accounting for four diastereoisomer couples. NMR spectra did not provide data concerning diastereoisomer distribution.

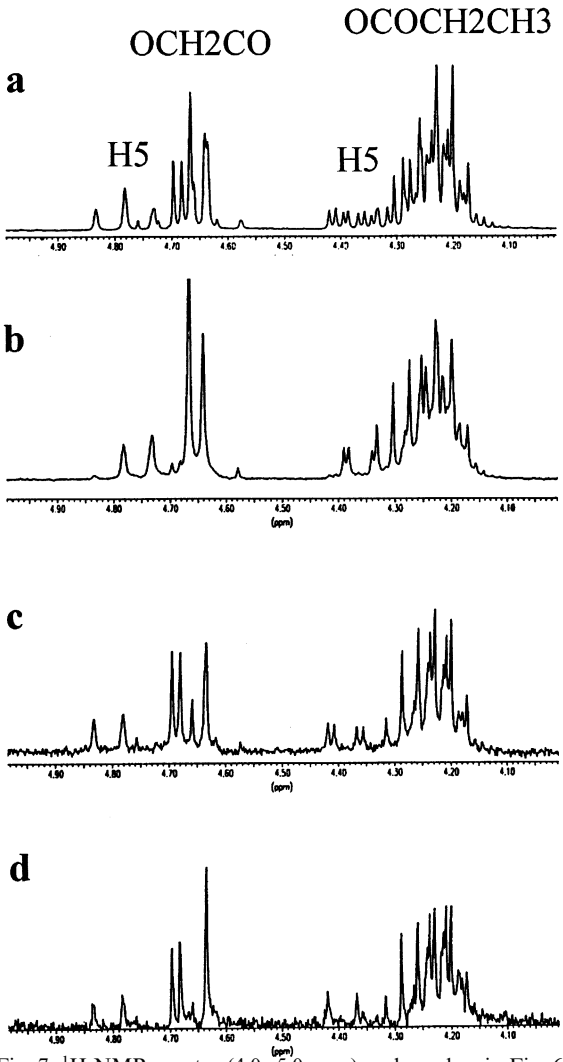


Fig. 7. ^{1}H NMR spectra (4.0–5.0 ppm). a, b, c, d as in Fig. 6.

The signal integration ratio calculated from methyl group chemical shift at 1.3 ppm and the proton summary shown on Fig. 5 allowed the attribution of heterocyclic methylene groups –$\underline{CH_2}$–CH_3 and –CO–$\underline{CH_2}$–CO–O– at about 4.2–4.3 and 4.6–4.7 ppm, respectively. Coherently, this was supported by H-H COSY spectra depicted on Fig. 4(b) where proton correlation ε wasperfectly brought to the fore. Hydroxyl protons were assigned at $\approx$3.8–3.9 ppm through proton exchanges performed in D_2O (data not shown). Signals corresponding to methylene protons from the hydroxy methyl group –$\underline{CH_2}$–OH were easily localized between 2.5 and 2.8 ppm from the proton correlation β (Fig. 4(b)) involving hydroxyl protons. In addition, H-H COSY (Fig. 4(b)) showed two other correlations (α and γ) which involved cyclic protons responsible for approximate chemical shifts at 2.5–2.8, 3.1–3.2, 4.3–4.4 and 4.7–4.9 ppm, without providing us with enough clues to come to a full proton indexation. In fact, in order to reach this objective and completely elucidate NMR data, purification of diastereoisomer couples (DCs) was required.

Accordingly, DCs were partially isolated by semi-preparative LC as recently described elsewhere [7]. Two (A and B) or three (1, 2 and 3) peaks, out of a maximum of four possible, and respectively corresponding to those shown on chromatograms obtained with an amino or a Hypercarb S column [7], were carefully collected and individually analyzed by NMR spectroscopy. Figs.

Table 2
Chemical shift of the different diastereoisomer couples in various LC peaks (δ TMS = 0.00 ppm)

Peak	H'_7	H''_7	H_{5eq}'	H_{5ax}''	H_{3eq}'	H_{3ax}''	OH	O–CH_2–CO
2		2.58	4.83	4.42	3.13	2.70	3.79	4.70
		2.56	4.78	4.37	3.07	2.64	3.81	4.68
		2.52					3.83	4.64
		2.50						
3	2.85	2.59	4.83	4.41	3.13	2.67	3.90	4.70
	2.82	2.58	4.78	4.36	3.07	2.73	3.88	4.68
	2.79	2.53					3.86	4.67
	2.76	2.51						
A or 1			4.78	4.39	3.21		3.90	4.67
			4.73	4.34	3.15		3.88	4.64
							3.86	
				4.38			3.83	
				4.33			3.81	
							3.79	

6 and 7 depict the NMR spectra obtained with some of them. The two unequivalent protons of each ring-borne methylene residue numbered 3 and 5 on Fig. 5 were tentatively attributed. However, considering their carbonyl group-enriched conformational surrounding, H_5 protons would probably be shifted downfield as doublets at 4.83/4.78 ppm for one proton (equatorial) and at 4.42/4.37 ppm for the other (axial) with a ^2J constant of 12.85 Hz (Fig. 7). Similarly, H_3 protons gave two doublets at 3.13/3.07 ppm (equatorial) and at 2.70/2.64 ppm (axial) with a ^2J constant value of 15.07 Hz (Fig. 6). Equatorial and axial protons were distinguished on the basis that equatorial hydrogens are usually less shielded [12]. The chemical shifts of all the protons are summarized in Table 2.

Obviously, diastereoisomer purification dramatically reduced the spectrum complexity and rendered it more easily interpretable since the split of most of the signals was overcome (Figs. 6 and 7). For instance, the hydroxyl proton led to a single triplet in the region 3.79–3.83 ppm with a ^3J constant of 5.02 Hz (Fig. 6) with adjacent protons of the methylene group numbered 7 on Fig. 5. In addition, since peaks A and B gave two triplets for hydroxyl protons, it provided evidence that Hypercarb S column provides better discrimination of diastereoisomers than amino column. Also, it was noticeable that H_5 and H_3 axial protons of both DCs shared the same chemical shifts while those of H_5 and H_3 equatorial protons were distinct. From one pure DC NMR spectrum (Fig. 6(d) and Fig. 7(d)) compared to others (Fig. 6(a–c) and Fig. 7(a–c)), it was deduced that the four DCs were equally formed during the oligomerization-cyclization process. As can be assumed from data recently published by Salvador et al. [7], LC peaks A, B and 1 corresponded to two DCs whereas peaks 2 and 3 each represented one single DC. For peak 3, no NMR experiments was performed, deduction was made from NMR data of the other peaks.

Because insufficient quantities of pure DCs were available, the determination of the absolute configuration has not yet been performed. As soon as this problem is solved Nuclear Overhauser Effect (NOE), Nuclear Overhauser Effect Spectroscopy (NOESY) NMR experiments will be carried out to differentiate the different DCs.

4. Conclusion

A combination of several spectroscopic techniques (MS, LC-MS and NMR) permit to elucidate the termination of MM 2.1.2. polymerization and to identify the major compounds produced

during oligomerization: a mixture of four cyclic diastereoisomer couples of (2R, 2S, 4R, 4S, 6R, 6S) 2,4,6-ethoxycarbonyl-4,6-ethoxycarbonylmethoxycarbonyl-2-hydroxymethyl cyclohexanone. Identification of this cyclic trimer is important since it is always produced even in a polymerization medium and probably influences the physicochemical properties of nanoparticles [8]

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