ELSEVIER

Polymer 40 (1999) 6521–6528

polymer

Biosynthetic stereocopolymer of 3-methylmalic acid as hydrolyzable and biocompatible polyester for temporary therapeutic applications

Marie Maud Bear[a], Karine Lozac'h[b], Solo Randriamahefa[a], Valérie Langlois[a], Richard Bourbouze[b], Philippe Guerin[a,*]

[a]*Laboratoire de Recherche sur les Polymères, UMR CNRS C7581, Université Paris XII, 2 à 8, rue Henri Dunant, 94320 Thiais, France*
[b]*Laboratoire de Biochimie et de Glycobiologie, Faculté des Sciences Pharmaceutiques et Biologiques, 4, Avenue de l'Observatoire, 75270 Paris, Cedex 06 France*

Received 20 October 1998; received in revised form 3 December 1998; accepted 3 December 1998

Abstract

A mixture of (2*S*,3*S*) and (2*S*,3*R*)-3-methylaspartic acid, obtained by bioconversion of mesaconic acid in the presence of 3-methylaspartase as enzymatic catalyst, was transformed into the corresponding benzyl 3-methylmalolactonate stereoisomers using a multiple-step synthesis. A mixture of (3*R*,4*R*) and (3*S*,4*R*) (80/20 (mol/mol) ratio) benzyl-3-methylmalolactonate was transformed by anionic ring-opening polymerization to an optically active and stereoregular stereocopolymer constituted by 80 mol% of benzyl (3*R*,4*S*) 3-methylmalate repeating units and 20 mol% of benzyl (3*S*,4*S*) 3-methylmalate units, as determined by ^{1}H NMR. The corresponding optically active poly(β-3-methylmalic acid) was obtained by catalytic hydrogenolysis of the protecting benzyl ester groups, in *N*-methylpyrrolidone as solvent. This functionalized hydrosoluble polyester was degraded by simple hydrolysis in a phosphate buffer at pH 7, as shown by SEC measurements. It is worth noting that the kinetic profile was equivalent to the one of poly(β-malic acid). Moreover, at 37°C, the hydrolysis was complete within six weeks, yielding to the corresponding optically active 3-methylmalic acid. In order to use this polymeric material for temporary therapeutic applications, polymers containing both diastereoisomers as repeating units as well as their ultimate products of degradation were evaluated based on harmlessness towards their environment. No toxicity was detected against a human cell line, HepG2.

Keywords: (2*S*,3*S*) and (2*S*3*R*)-3-methylaspartic acids; Poly(β-3-methylmalic acid); Hydrolytic degradation

1. Introduction

The need for biocompatible and degradable polymers is increasing in the field of bioactive molecules loading and controlled delivery. These systems are used in pharmacy, agriculture, navigation and food. It is, therefore, very important to take into account their interactions with living species from the utilization period, until the ultimate degradation stage. Further, in order to adjust the macromolecular architecture to the required properties, the polymer configurational structure can be modulated by taking advantage of the presence, in the repeating units, of one or two stereogenic centers. The chirality can be introduced in the polymer, starting from optically active biomolecules as precursors. Natural or artificial biopolyesters constitute a large qualified family for specific bioapplications. The access to multiple functionalized derivatives can be obtained by chemical or biological synthesis routes as examplified by poly(α or β-hydroxyacids) such as polylactides [1], polyhydroxyalkanoates [2], poly(malic acid esters) [3].

Biomass constitutes an unfailing wealth of chiral precursors and the observation of the metabolic pathway in the bacterium *Clostridium tetanomorphum* has conducted to select 3-methylaspartic acid for its analogy with aspartic acid and for the presence of two chiral centers in the molecule. The (2*S*,3*S*)-stereoisomer is an intermediate in the catabolism of (2*S*)-glutamate in mesaconate, and also in citramalate. 3-methylaspartase from *Clostridium tetanomorphum*, which catalyses the bioconversion of 3-methylaspartate to mesaconate, can be efficiently used in the retrophysiological reaction [4]. We have used the enzyme from cell extracts in the enantiospecific synthesis of (2*S*,3*S*)-3-alkylaspartic acids (alkyl = methyl, ethyl, isopropyl) [5,6] using a modified Barker's original procedure [5]. The optically active benzyl 3-alkylmalolactonates were prepared according to the synthesis route described for optically active malolactonates [7] and polymerized by anionic ring

* Corresponding author. Tel.: 33 1 49 78 12 86; fax: 33 1 49 78 12 08.

0032-3861/99/$ - see front matter
PII: S0032-3861(98)00847-7

opening polymerization yielding optically active semi-crystalline polyesters [6,8]. Moreover, all biotransformations can be carried out in very large quantities.

Under particular experimental conditions of bioconversion [9], the same enzyme presents a (2*S*,3*R*)-3-methylaspartase activity. A 60/40 mol/mol mixture of (2*S*,3*R*)/(2*S*,3*S*)-3-methylaspartic acid was therefore prepared and extracted, after chemical modification, under its 2-bromo-3-methyl succinic acid form. After diastereoisomers resolution and further monomer synthesis, the sole benzyl (3*R*,4*R*)-3-methylmalolactonate was obtained as confirmed by chiral GC. It is worth noting that no racemization occurred during the multiple step synthesis. Anionic polymerization of the lactone yielded a semi-crystalline polymer poly[benzyl β-(3*R*,4*S*)-3-methylmalate] ($T_m = 120°C$) with only one configurational structure type as shown by ^{13}C NMR.

In this article, we wish to report that by mixing stereoisomers of benzyl-3-methylmalolactonate, it is possible to prepare the corresponding copolystereoisomer, which can be transformed in a water soluble and hydrolyzable poly(3-methylmalic acid).

The limiting step in the use of degradable polymeric materials is the interactions of both the polymer and the degradation products in living organisms. Consequently, poly(β-3-alkylmalic acids) and degradation by-products were evaluated in an in vitro cytotoxic test designed to check rapidly their biocompatibility. The liver is responsible for the metabolism of most xenobiotics, and is often the site of damage induced by them or their metabolites. Cultured hepatocytes have been widely used to evaluate xenobiotics hepatotoxicity. Consequently, we have used the human hepatoma cell line Hep G2 for hepatotoxicological evaluation [10]. Therefore, the spectrum of biocompatible and degradable polymers for the biomedical domain is enlarged, as shown by the report of a preliminary in vitro toxicity study.

2. Experimental

2.1. Preparation of optically active poly(3-methylmalic acid)

2.1.1. Synthesis of (3R,4R)/(3S,4R)-3-methyl-4-(benzyloxycarbonyl)-2-oxetanone (benzyl 3-methylmalolactonate) ***5***

3-methylaspartase was obtained from cell-free extracts of *C. tetanomorphum* DSM 528 (strain ^1H of Barker), as previously described [5]. The bioconversion was carried out by introducing the dialysis tube containing the enzyme into a reaction mixture, in a conventional stirred tank. Incubation of the enzyme was carried out at pH 9 in the presence of KBr (0.1 M), $MgBr_2$ (0.01 M), NH_4Br (0.2 M) and ammonium mesaconate (0.2 M).

Under these conditions we could obtain from 1.6 l of bioconversion medium, 52 g of a 60/40 mol/mol mixture of (2*S*,3*S*) and (2*S*,3*R*)-3-methylaspartic acid **1** with a yield of 70% starting from 60 g of mesaconate ammonium.

To avoid the extraction of (2*S*,3*RS*)-3-methylaspartic acid **1** from the bioconversion medium, the well established first step of the lactone synthesis consisting in a deamination–bromation reaction for obtaining (2*S*,3*RS*)-2-bromo-3-methylsuccinic acid **2** was carried out directly on the bioconversion medium. The separation of both diastereoisomers (**2a** and **2b**) was made at this step as a result of the very low solubility of (2*S*,3*S*)-**2a** in acetonitrile contrary to (2*S*,3*R*)-**2b**. At first, pure **2a** was removed by filtration and after concentration of the resulting solution, a 80/20 mixture of **2a** and **2b** was recovered for the lactonization reaction.

(2S,3S)/(2S/3R) 80/20 ***2a/b***: $[\alpha]_D^5$ (c = 1.4, THF) = −43.7; RMN ^1H (200 MHz, $CDCl_3$, δ ppm): 1.36 (d, J = 7 Hz, 0.8 × 3H, CH_3), 1.43 (d, J = 7 Hz, 0.2 × 3H), 3.14 (m, 1H), 4.52 (d, J = 9 Hz, 0.8 × 2H), 4.52 (d, J = 9 Hz, 0.2 × 2H).

The following steps carried out on a mixture of **2a** and **2b** (80/20) lead to **5a**/**b** mixture in a ratio 80/20 (3*R*,4*R*)/(3*S*/4*R*) mixture, as determined by chiral GC and mass spectroscopy.

5: m = 4.3 g (yield 70%).$[\alpha]_D^{25}$ (c = 2.0, dioxane) = +30; ^1H NMR (200 MHz), $CDCl_3$, δ ppm): 1.15 (d, J = 7.8 Hz, 0.85 × 3H, CH_3 (3*R*,4*R*), 1.47 (d, J = 7.5 Hz, 0.15 × 3H, CH_3 (3*S*,4*R*), 4.04 (m, 0.15 × 1H, C*H*CH_3), 4.23 (m, 0.85 × 1H, C*H*CH_3 (3*R*,4*R*), 4.87 (d, J = 7 Hz 0.15 × 1H, C*H*COO (3*S*,4*R*), 5.20 (d, J = 7 Hz, 0.85 × 1H, C*H*COO (3*R*,4*R*), 5.25 (d, J = 1.7 Hz, 2H, CH_2), 5.30 (d, J = 1.7 Hz, 2H, CH_2), 7.42 (m, 5H, C_6H_5); $^1{}^3C$ NMR (50 MHz, CD_3COCD_3, δ ppm): 9.6 (CH_3 (3*R*,4*R*)), 12.4 (CH_3 (3*S*,4*R*), 50.8 (*C*HCH_3 (3*R*,4*R*)), 52.6 (*C*HCH_3 (3*S*,4*R*)), 67.8 (CH_2), 71.3 (CH (3*R*,4*R*)),73.2 (CH (3*S*,4*R*)), 129.0, 129.3, 136.2 (C_6H_5 (3*S*,4*R*)), 129.2, 129.5, 136.3 (C_6H_5 (3*R*,4*R*)), 168.2 (lateral CO, (3*R*,4*R*)), 168.7 (lateral CO (3*S*,4*R*)), 170.8 (cycle CO (3*R*,4*R*)), 171 (cycle CO (3*S*,4*R*)).

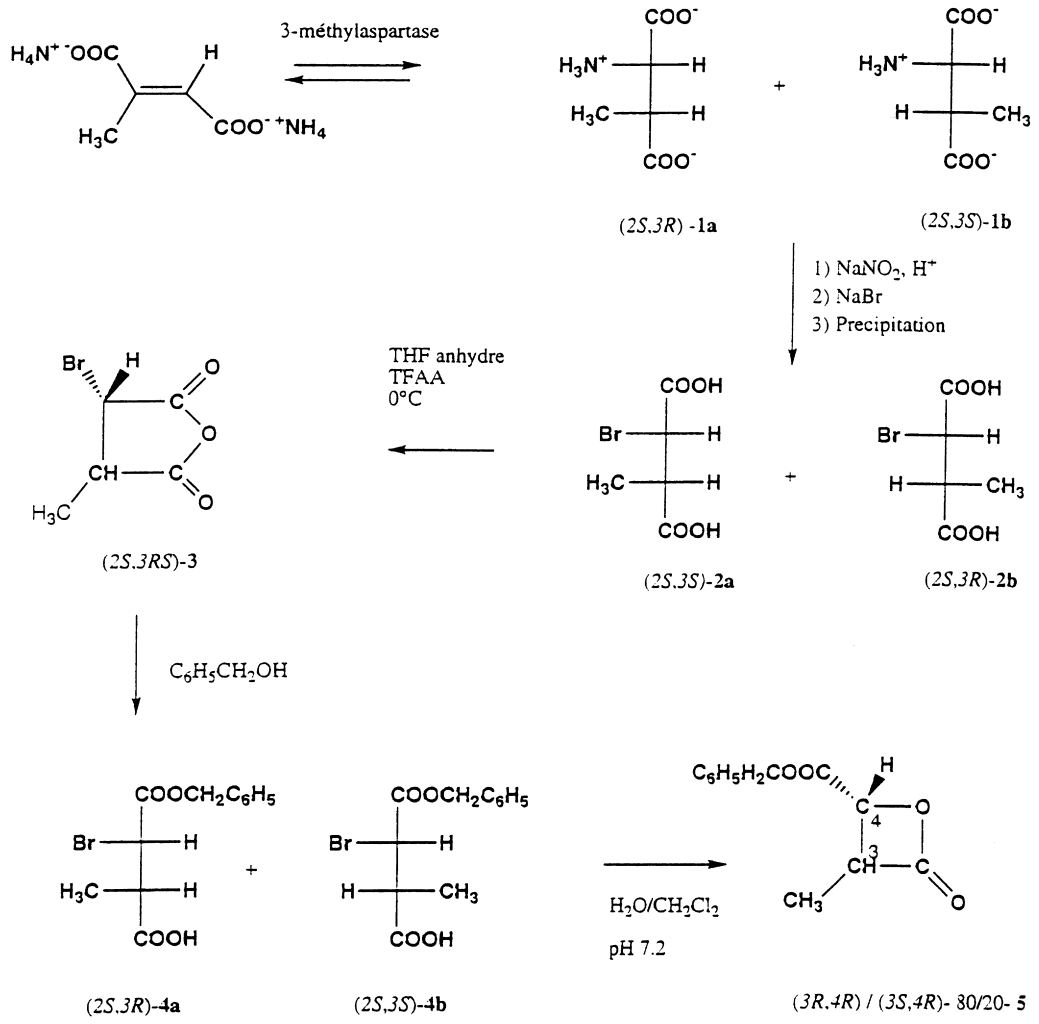
2.1.2. Polymerization of benzyl-3-methylmalolactonate ***5***

5 was polymerized at 40°C using tetramethylammonium benzoate as initiator (initiator/monomer in a 1/1000 molar ratio). The reaction was completed in four days (FTIR determination on the lactone band at 1850 cm^{-1}). **6** was dissolved in $CDCl_3$ and precipitated in ethanol.

6: $[\alpha]_D^{25}$ (c = 1, $CHCl_3$) = −1.43; T_g = 31°C and T_m = 41°C (ΔH_f = 22.5 J/g); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): 1.09 (d, J = 7 Hz, 3H, CH_3), 3.11 (m, 1H, $CHCH_3$), 5.09 (s, 2H, CH_2), 5.26 (d, J = 5 Hz, 1H, C*H*COO), 7.27 (m, 5H, C_6H_5); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 12.4 (CH_3); 40.5 (*C*HCH_3), 67.4 (CH_2), 73.0 (*C*HCOO), 128.42, 128.47, 128.48, 134.95 (C_6H_5), 167.4 (CO), 170.6 (CO).

2.1.3. Hydrogenolysis of poly[benzyl (3R,4S)/(3S,4S) (80/20)-β-3-methylmalate (PMeMLABe) **6**

Hydrogenolysis of PMeMLABe **6** was carried out in



Scheme 1. Synthesis of (3*R*,4*R*)/(3*S*,4*R*)-3-methyl-4-(benzyloxycarbonyl)-2-oxetanone (benzyl 3-methylmalolactonate).

N-methylpyrrolidone as a solvent, using a 10% charcoal/Pd catalyst, according to the process previously described [12].

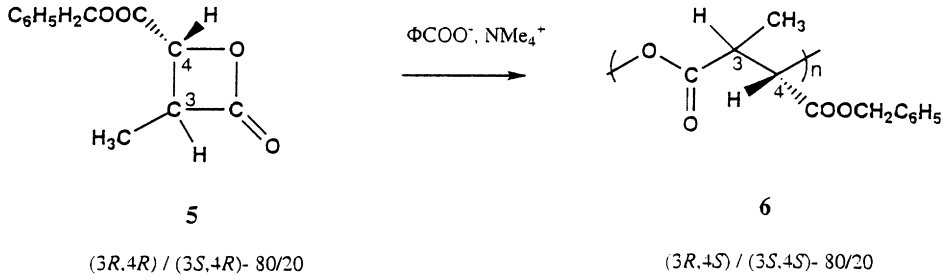
Poly[(3*R*,4*S*)/(3*S*,4*S*) 80/20-β-3-methylmalate] **7**: ^{1}H NMR (500 MHz, D_2O, δ ppm): 1.03 (broad peak, 3H, CH_3), 3.13 (broad peak, 1H, C*H*CH_3), 5.11 (broad peak, 0.85 × 1H, (3*R*,4*S*) CHCOO), 5.34 (broad peak, 0.15 × 1H, (3*S*,4*S*) C*H*COO). ^{13}C NMR (125 MHz, D_2O, δ ppm): 12.8 (CH_3 (3*S*,4*S*)), 14.7 (CH_3 (3*R*,4*S*)), 43.4 (*C*HCH_3), 67.4 (CH_2), 76.9 (*C*HCOO), 174.1 and 175.3 (2CO).

2.2. Degradation

Samples were dissolved (4 mg ml^{-1}) in aqueous sodium phosphate buffer solution (0.05 M, pH 7.0), containing sodium chloride (0.75 M) and allowed to age in a thermostated bath (37°C). Degradation was monitored by aqueous SEC with the same phosphate buffer solution as eluent with a flow rate of 1 ml min^{-1} (BECKMAN 110B pump), using two TSK GEL G3000PW type columns (30 cm × 7.5 mm internal diameter). Chromatograms were recorded using a UV detection at 214 nm (SPECTRA PHYSICS 100) and a differential refractometer detector (WATER R401) for calibration with poly(ethylene glycol) standards.

2.3. In vitro cytotoxic assays

Hep G2 cells were grown in Earle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum and antibiotics (100 μg/ml penicillin; 100 μg/ml streptomycin). All treatments were performed on cells in exponential phase growth disrtributed in six-well plates at a cellular density of 4×10^5 cells/well and in a humidified atmosphere (5% CO_2). Typically, cells were incubated for 24 h with various concentrations of products. At the end of the experiment, cell viability was determined by Trypan blue assay. The survival rate of the treated cells was calculated in regard to untreated cultures.



Scheme 2. Ring opening polymerization of benzyl 3-methylmalolactonate.

3. Results and discussion

3.1. Chemoenzymatic synthesis to benzyl 3-methylmalolactonate (3R,4RS)

The four steps process for the preparation of optically active lactone (3*R*,4*RS*)-3-methyl-4-benzyloxycarbonyl-2-oxetanone **5** is reported in scheme 1, starting from mesaconate **1**.

Previous studies [11] have shown that the different steps before the lactonization reaction do not affect the configuration of both asymmetric carbon atoms. The cyclization reaction, which takes place on the sole 2-bromo-3-methylsuccinic acid monoester isomers **4a** and **4b**, proceeds according to an intra-bimolecular mechanism, with inversion of the configuration at the C_2 bearing the bromine atom [12].

For the synthesis of the lactone, the mixture of 3-methylaspartic acid diastereoisomers **1** was transformed into 2-bromo-3-methylsuccinic acid **2** without preliminary extraction. For this purpose, the bioconversion medium contained potassium bromide (0.1 N), ammonium bromide

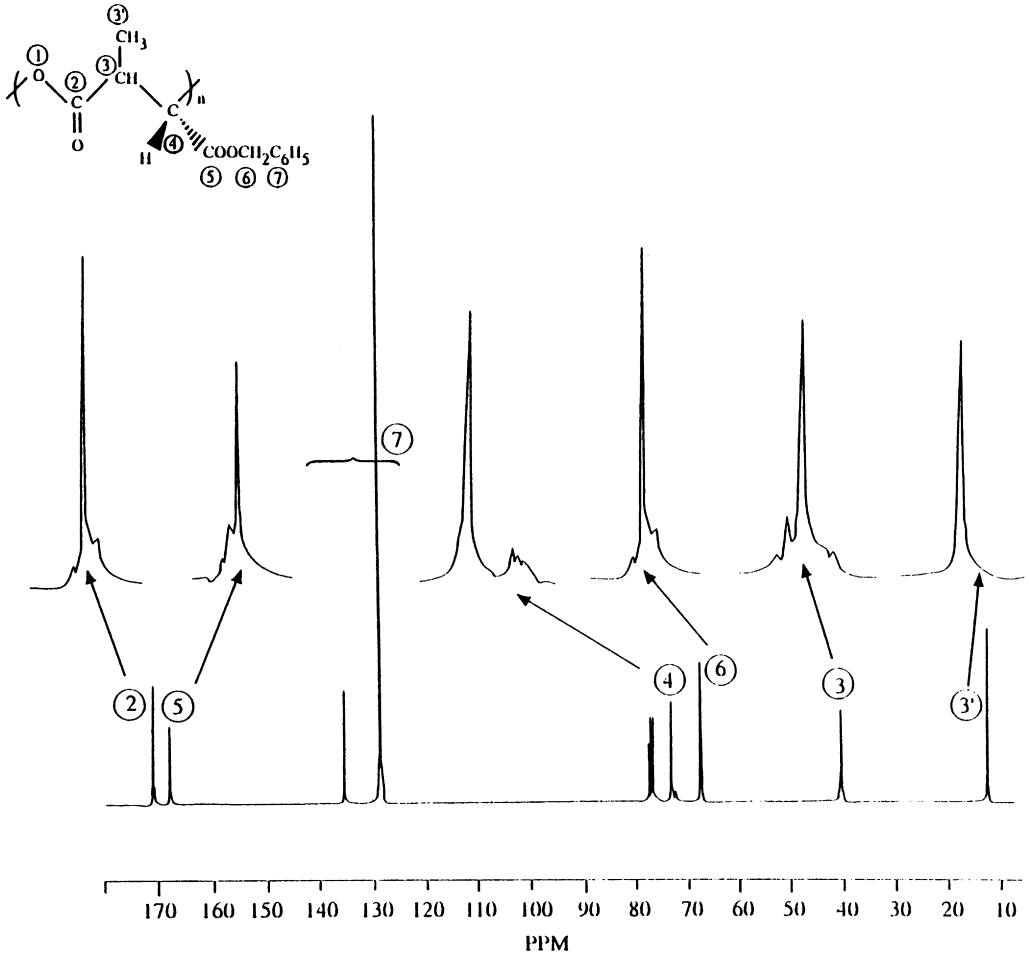
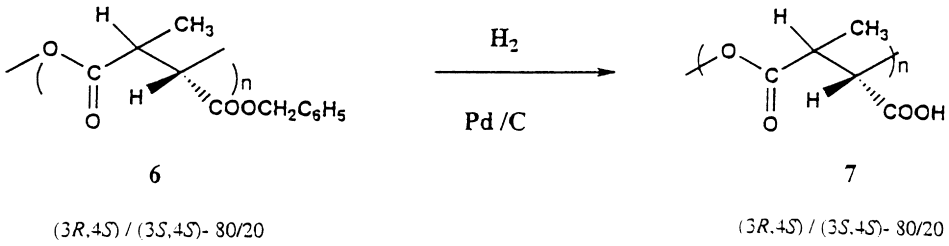


Fig. 1. ^{13}C NMR (75 MHz, $CDCl_3$) spectra of poly[benzyl (3*R*,4*S*)/(3*S*,4*S*) 80/20-β-3-methylmalate].



6 7

(3*R*,4*S*) / (3*S*,4*S*)- 80/20 (3*R*,4*S*) / (3*S*,4*S*)- 80/20

Scheme 3. Hydrogenolysis of poly[benzyl (3*R*,4*S*)/(3*S*,4*S*) 80/20 β-3-methylmalate].

(0.1 N) and magnesium bromide (0.01 N). pH of the reaction solution was adjusted at 9 with ethanolamine. The bioconversion yield (65%) and the proportion of (2*S*,3*R*)-**1** were comparable to those obtained with usual bioconversion conditions. In order to prepare a lactone containing a mixture of both (2*S*,3*S*)/(2*S*,3*R*) diastereoisomers in a ratio 80/20 mol/mol, **2** was treated in actonitrile, taking into account the difference of solubility in this solvent between **2a** and **2b**; in contrast to **2b**, **2a** has a very low solubility in acetonitrile. The following steps lead to (3*R*,4*R*)/(3*S*,4*R*)-5 in a ratio 80/20 mol/mol as determined by chiral chromatography and mass spectrometry.

3.2. Stereocopolymer of 3-methylmalic acid

The optically active lactone was anionically polymerized in bulk at 40°C using tetraethylammonium benzoate as initiator (initiator/monomer in a 1/1000 molar ratio). The reaction was complete after four days (disappearance of the lactonic band (ν C=O) at 1850 cm^{-1}) (Scheme 2).

The low crystalline structure of the polymer, as expected by mixing two diastereoisomers was confirmed by DSC, with a low melting temperature at 40°C. The ^{13}C NMR spectrum in $CDCl_3$ at 100 MHz displayed a fine high peak corresponding to iso-stereosequences and small broad peaks

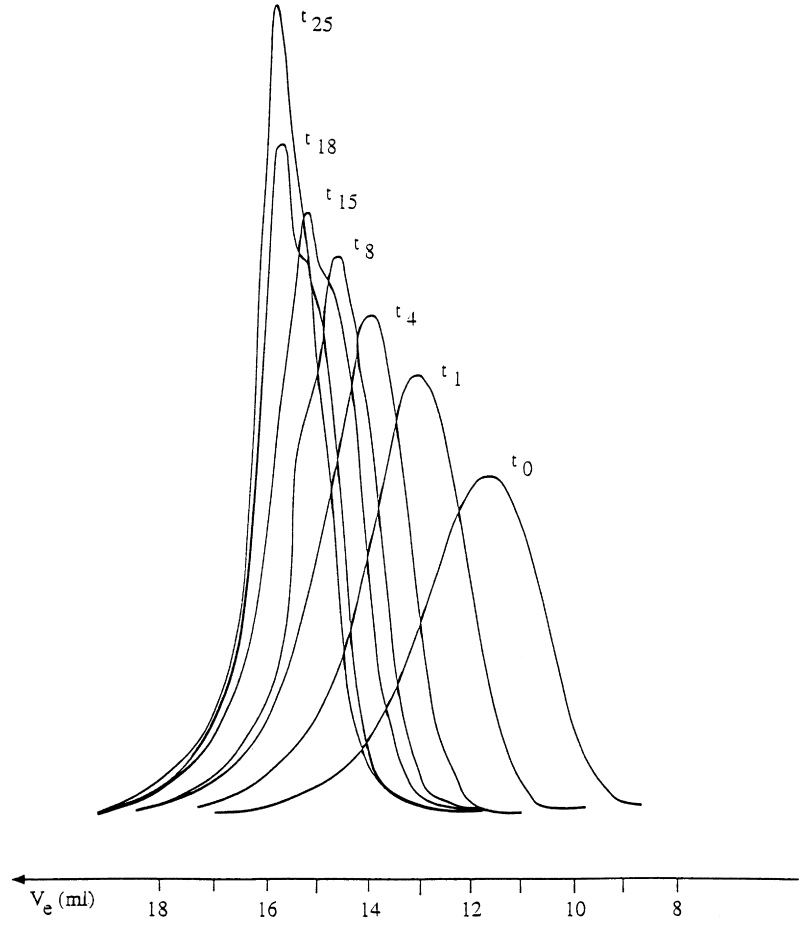


Fig. 2. UV detected SEC chromatograms versus aging time (*t* days) (phosphate buffer, NaCl 0.75 M, pH 7), during hydrolysis of poly[(3*R*,4*S*)/(3*S*,4*S*) (80/20)-β-3-methylmalate].

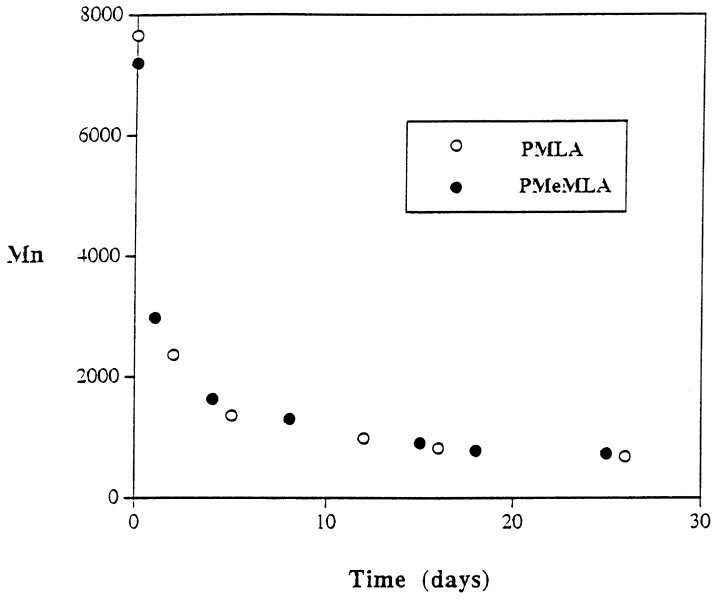


Fig. 3. Molecular weight (M_n) evolutions of poly(β-malic acid) (PMLA) and poly(β-3-methylmalic acid) (PMeMLA) during their hydrolysis in phosphate buffer at pH 7.

corresponding to hetero-stereosequences for each carbon (Fig. 1). It was demonstrated on poly[(*R*) or (*S*)-benzyl β-malate] that ring-opening polymerization proceeds with configuration inversion of the β-carbon of the lactone and without any racemization of this carbon, with the formation of carboxylate growing chain end [13].

It is worthy noting the different behavior between stereopolymers of benzyl-3-methylmalolactonate. The polymer containing benzyl (3*S*,4*S*)-3-methylmalate repeating units is completely insoluble in organic solvents and presents a melting transition temperature at 250°C [7]. On the contrary, poly[benzyl (3*R*,4*S*)-β-3-methylmalate] is soluble in organic solvents and melts at 130°C. However, catalytic hydrogenolysis of this polymer to remove the benzyl protecting groups had not been complete in *N*-methylpyrrolidone. In the case of poly[benzyl (3*R*,4*S*)/(3*S*,4*S*) 80/20-β-3-methylmalate] **6** and using identical experimental conditions of hydrogenolysis, optically active poly(3-methylmalic acid), PMeMLAH **7** was obtained. The ^{1}H and ^{13}C NMR spectra do not display any more peak corresponding to the benzyl group (Scheme 3).

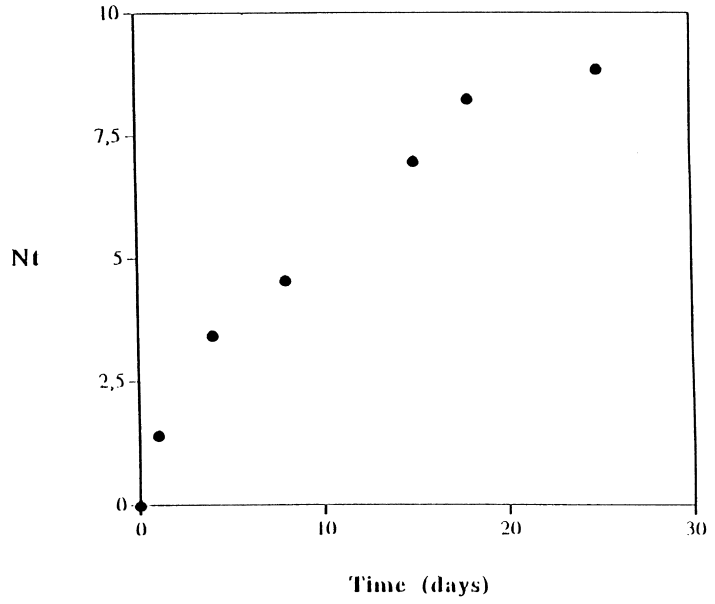


Fig. 4. Average number of ester bond cleavages per original macromolecule versus aging time.

Table 1
Half life time versus initial number average molecular weight during degradation of poly (β-3-methylmalate)

Polymer	M_n[a]	DP_n	$t_{1/2}$
PMLA[b]	7700	74	17
PmeMLA 80/20[c]	7200	61	19
PmeMLA 80/20[c]	5100	44	48
PmeMLA 50/50[d]	3500	30	120

[a] (SEC, equiv. PEG).
[b] Determined by SLS = 16 000.
[c] (3*R*,4*S*)/(3*S*,4*S*) 80/20.
[d] (3*RS*,4*RS*).

3.3. Degradation of poly[(3R,4S)/(3S,4S) (80/20)-β-3-methylmalate] 7

Degradation was monitored by aqueous SEC. Polymer **7** (M_n = 5100) was allowed to age in ionizated form at pH 7, 0.05 M phosphate buffer. To compare its hydrolysis rate with that of a now well known polymer, a sample of poly(β-malic acid) (M_n = 7700) was studied in the same conditions. Experiments were carried out at 37°C. Fig. 2 displays the different UV detected chromatograms at different time corresponding to the PMeMLAH degradation.

As time increases, the peak shifts to large elution volumes

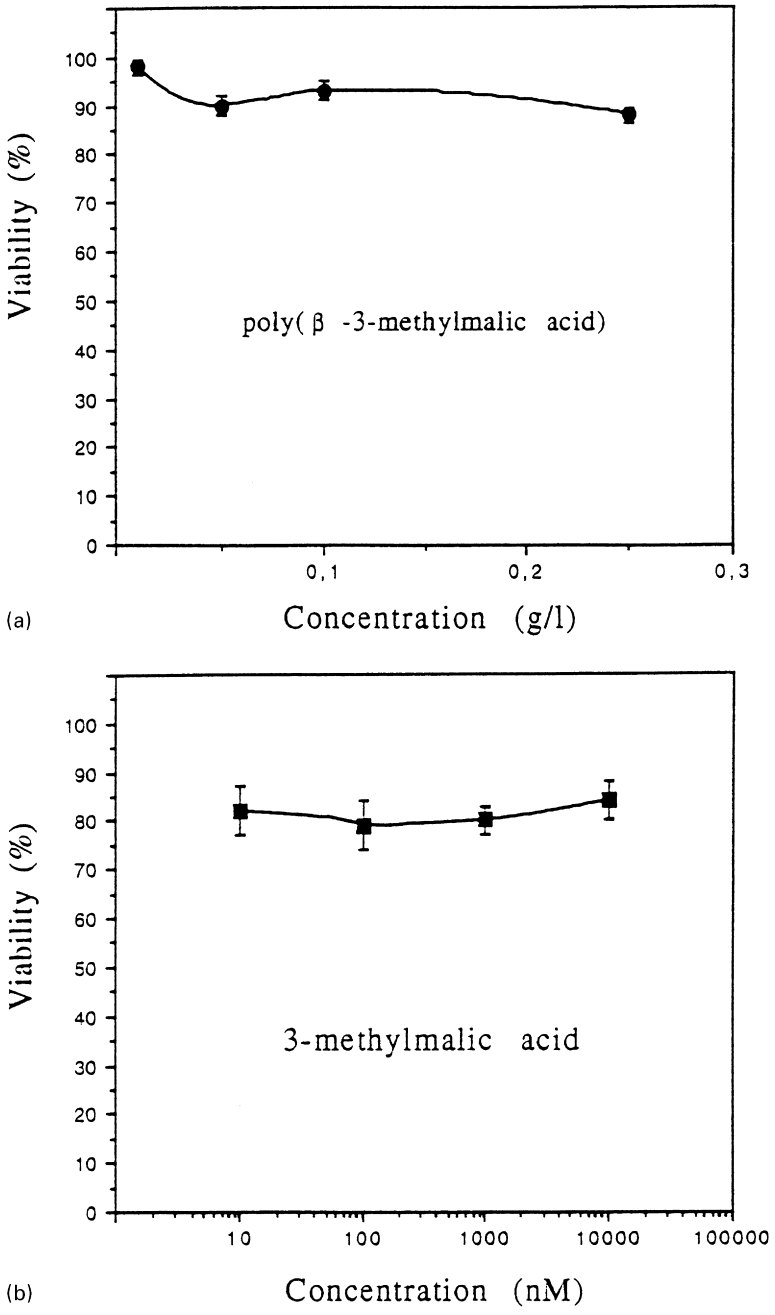


Fig. 5. (a) Toxicity of poly(β-3-methylmalic acid): dose dependance. (b) Toxicity of 3-methylmalic acid: dose dependance.

and the area broadens because of great change in UV absorption at the wavelength used for detection. As in the case of poly(β-malic acid), the cleaved main chain ester chromophores are replaced by hydroxyl and carboxylate ones as end groups, the latter absorbs in the range of 210–220 nm as ester chromophores, but with larger absorption coefficient ($\epsilon COO^- = 20 \times \epsilon COOR$). The curve corresponding to the M_n decrease is identical for both polymers (Fig. 3).

The average number of ester bond cleavages per original macromolecule, at time t, $N(t)$, was calculated using Eq. (1) [14].

$$N(t) = \frac{M_n(0)}{M_n(t)} - 1. \quad (1)$$

Fig. 4 represents the evolution of N values as a function of time. According to the literature [15], if the chain scission is a random phenomenon, the value of N should be anticipated to be a linear function of time. In the case of PMeMLAH, $N(t)$ was not a linear function of time which means that ester cleavages occurred at preferential domain in the macromolecular chain.

The rate of degradation seems to depend on the initial molecular weight of the polymer. By comparison with degradation of poly(β-3-methylmalate) presented in Table 1, half life time measured in terms of $DP_n/2$ increased when the DP_n^0 decreased. These findings suggest that cleavage of backbone ester bonds is easier inside the chain than ester bond close to chain ends.

3.4. In vitro cytotoxic assays

The dose dependence of poly(β-3-alkylmalic acids) (0.01–0.25 g/l) and final products resulting from their hydrolysis degradation (methyl-, isopropyl-, ethyl-, malic acids) (10–10 000 nM/l) against Hep G2 cells in vitro was investigated. Results of the different experiments are collected in Fig. 5. We can conclude that, in our conditions, polymer and its degradation products do not present any effect on cell viability of Hep G2 cells within a large scale of concentrations.

4. Conclusion

The choice of biomolecule which can be transformed by bioconversion in chiral precursor open the route to the chemioenzymatic synthesis of new polymers. In the case of 3-methylaspartic acid, it is possible to take advantage of the two chiral centers in the biomolecule for the tailor making of optically active polymers with a controlled architecture or to prepare polymeric materials aimed at temporary biomedical application. The evaluation of these polymers as biodegradable micelles and nanoparticles is under investigation.

Acknowledgements

This work has been supported by DGA Grant 96 057.

References

[1] Zhang X, Goosen MFA, Wyo UP, Pichona DJ. Macromol Sci Rev Macromol Chem Phys 1993;C33:81.
[2] Poirier Y, Nawrath C, Somerville C. Bio/technology 1995;13:142.
[3] Moine L, Cammas S, Amiel C, Sébille B, Guérin Ph. Polymer 1998;38:3121.
[4] Akhtar M, Botting NP, Cohen MA, Gani D. Tetrahedron 1987;24:5899.
[5] Monne C, Robic D, Campion G, Bourbouze R, Rimbault A, Masure M, Langlois V, Hemery P, Guérin Ph. Chirality 1996;8:300.
[6] Bean MM, Langlois V, Masure M, Guérin Ph. Macromol Symp 1998;132:337.
[7] Guérin Ph, Braud C, Vert M. Polymer Bull 1985;14:187.
[8] Mabille C, Masure M, Hemery P, Guérin P. Makromol Rapid Commun 1996;17:209.
[9] Bear MM, Cammas S, Guérin Ph. CR Acad Sci Paris Ser 2b 1998;315:165.
[10] Hall TJ, James PR. Res Comm Pathol Pharmacol 1993;79:249.
[11] Guérin Ph, Vert M. Polym Commun 1987;28:11.
[12] Braud C, Caron A, Francillette J, Guérin Ph, Vert M. ACS Polymer Preprints 1988;29:600.
[13] Renard I, Cammas S, Langlois V, Bourbouze R, Guérin Ph. ACS Polymer Preprints 1996;37:137.
[14] Mallardé D, Valière M, David C, Menet M, Guérin Ph. Polymer 1998;39:3387.
[15] Yoon JS, Chin IJ, Kim MN, Kim CK. Makromolecules 1996;29:3303.