OPTICALLY ACTIVE POLYMERS OF 3-ALKYLMALIC ACIDS : CONTRIBUTION OF THE BIOCONVERSION FOR DIVERSIFYING THE CHIRAL PRECURSORS

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Abstract : The need for new optically active monomers and polymers is conducive to the setting up of stereospecific synthesis routes starting from chiral precursors. The biomass can be considered as a major source for extracting such biomolecules aimed at chemoenzymatic transformation and further polymerization. Due to its versatility, β-methylaspartate ammonia-lyase, from cell-free extracts of *Clostridium tetanomorphum*, has been used in the bioconversion of alkylfumarates into optically active pure 3-alkylaspartic acids with alkyl=methyl, ethyl, isopropyl. These amino acids have been transformed in several steps into optically active benzyl 3-alkylmalolactonates leading to semi-crystalline polyesters. 3-Methylaspartic acid includes two chiral centers and the racemic compound containing the four stereoisomers can be prepared by a multiple step synthesis. The ability of β-methylaspartase to catalyse both syn- and anti-elimination of ammonia from natural 3-methylaspartic acid has been expressed to retain one stereoisomer and this bioconversion is a preparative method for obtaining unnatural stereoisomers. Moreover, the catalytic hydrogenolysis of the benzyl α,β-substituted β-lactone yields stable 3-alkylmalolactonic acid which can be coupled with functional alcohols and copolymerized. At last the introduction of (2S)-3,3-dimethyl-2-butanol, using *Rhodotorula glutinis* as microorganism in a biological synthesis step, as chiral ester pendant group, has conducted to optically active polyesters with very high melting transition temperatures. The combination of bioconversion and chemical synthesis is a very useful tool for building hydrolyzable functionalized polyesters required for temporary applications.

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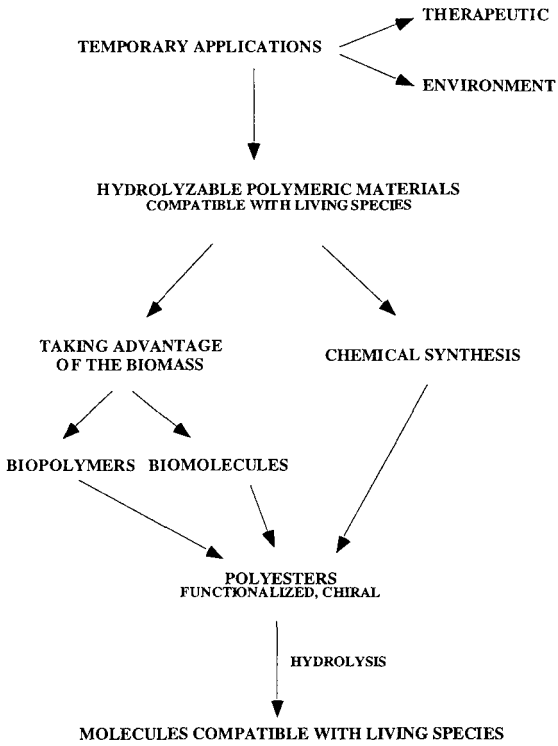
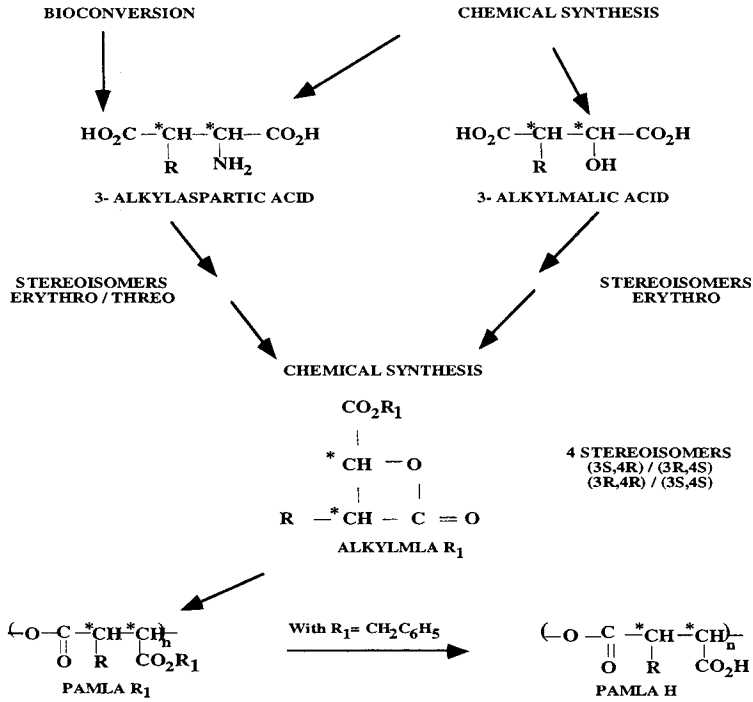
INTRODUCTION

In the field of polymers for temporary therapeutic or environmental applications, the necessary adjustment of the material properties conducts to the tailor making of synthetic polymers with different but complementary chemical structures and reproducible characteristics. The building of such polyvalent polymers may be achieved by copolymerization, cross-linking, and chemical modification starting from a parent compound. These polymeric materials must be hydrolyzable, compatible with living species and the presence of stereogenic centers in the macromolecular chain is a major structural factor for modifying physical and mechanical properties. Polyesters contain a degradable backbone and their configurational structures can be modulated by the presence of enantiomeric or diastereoisomeric repeating units. At last, the presence of functionalized pendant groups leads to a strict adjustment of the polymer characteristics. These polyesters can be obtained according to different synthesis routes : chemical, chemoenzymatic or biological (scheme1).



Scheme 1 : Access to hydrolyzable polymeric materials

The biomass constitutes an important wealth of chiral biomolecules as precursors of optically active monomers and biopolymers. In the field of microbial polyesters, attention has been primarily paid to poly[(R)-3-hydroxybutyrate] and related materials generally referred as poly[(R)-hydroxyalkanoates][1,2]. Recently, we have displayed, *Pseudomonas oleovorans*, a versatile bacterium, can grow on 10-undecenoic acid, sole or in mixture with sodium octanoate for producing polyesters containing unsaturated side chains in different proportions up to 100%, which can be epoxidized for further chemical modifications. Moreover, it has been possible to produce new functional bacterial polyesters containing terminal epoxy groups in the side chains up to 37%[3]. Natural poly(L-malic acid) can be obtained from different microorganisms as *Penicillium cyclopium* [4], *Physarum polycephalum*[5] and *Aureobasidium SP-A* [6].



Scheme 2 : Poly(β-3-alkylmalic acid) derivatives

Synthetic poly(β-malic acid) has been known for more than a decade[7] and high molecular weight polymers are prepared by anionic ring opening polymerization of β-substituted-β-lactones from aspartic acid[8] and malic acid[9] enantiomers as chiral precursors. A wide spectrum of optically active or racemic, functional or reactive poly(β-malic acid) derivatives have been prepared by changing the chemical structure of the pendant ester group for obtaining suitable

properties such as hydrophilic/hydrophobic balance[10], morphology[11], degradation rate[12], bioactive or targeting molecules attachment[13], specific interactions with natural macromolecules[14], associating degradable hydrogels[15](scheme 2). The limitation for obtaining such functional macromolecules hangs on the possibility of synthesizing the corresponding monomers, i.e. β-substituted-β-lactones.

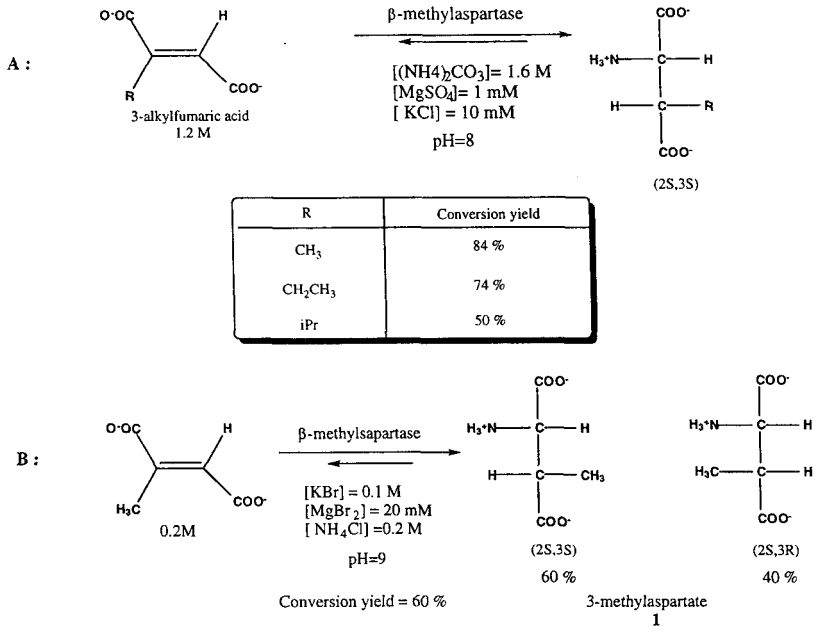
The need for novel complex chiral polymeric structures in the family of malic acid polymers has conducted to consider particular biomolecules of the metabolism as chiral precursors. 3-Methylaspartic acid has been choosen, at first, for its analogy with aspartic acid and for the presence of two chiral centers in the molecule, leading to stereoisomers wich can be prepared by chemical and chemoenzymatic syntheses. Preliminary studies on racemic 3-methylaspartic acid prepared by chemical synthesis have shown racemic benzyl 3-methylmalolactonate and corresponding poly(β-benzyl-3-methylmalate) can be prepared[16]. Another chemical synthesis route has been successfully explored from 3-alkylmalic acid[17]. Catalytic hydrogenolysis of benzyl protecting groups has conducted to water soluble racemic poly(β-3-methylmalic acid).

DISCUSSION

β-Methylaspartase as a versatile enzyme

β-Methylaspartase (EC 4.3.1.2) catalyses the second step in the catabolism of (2S)-2-glutamic acid in some clostridia and some other bacteria[18]. The *C. tetanomorphum* enzyme, which is the most studied, is known for catalysing the elimination of ammonia from (2S,3S)-3-methylaspartic acid to give mesaconic acid. It has been shown, that β-methylaspartase from *C. tetanomorphum* could be efficiently used in the reverse reaction. Moreover, the enzyme from cell-free extracts is useful in providing access to various 3-substituted aspartic acids via the direct amination of corresponding substituted fumaric acids[19]. The bioconversion of ammonium mesaconate , ammonium 3-ethyl- and 3-isopropylfumarates have been carried out in batch, yielding large amounts of the corresponding 3-alkylaspartic acids. ^{1}H NMR spectra of the extracted and purified parent 3-alkylaspartic acids displayed, in our experimental conditions, signals corresponding to the sole (2S,3S)-stereoisomer[20]. Yields are depending on the size of the alkyl groups (scheme 3).

Early reports claimed that β-methylaspartase could also catalyse the syn-deamination of L-erythro-(2S,3R)-3-methylaspartic acid, at 1% of the rate of the natural substrate to give mesaconic acid[21]. The presence of a L-erythro-3-methylaspartase activity has been confirmed by incubation of the enzyme with diammonium mesaconate at pH 9.0 in the presence of K^+ and Mg^{2+} ions. Exclusion of chemical epimerisation has been shown by chemical and biological experiments. Salts and substrate concentrations (scheme 3) were quite different compared to those used in the preparation of the sole L-threo-(2S,3S)-stereoisomer (medium A).



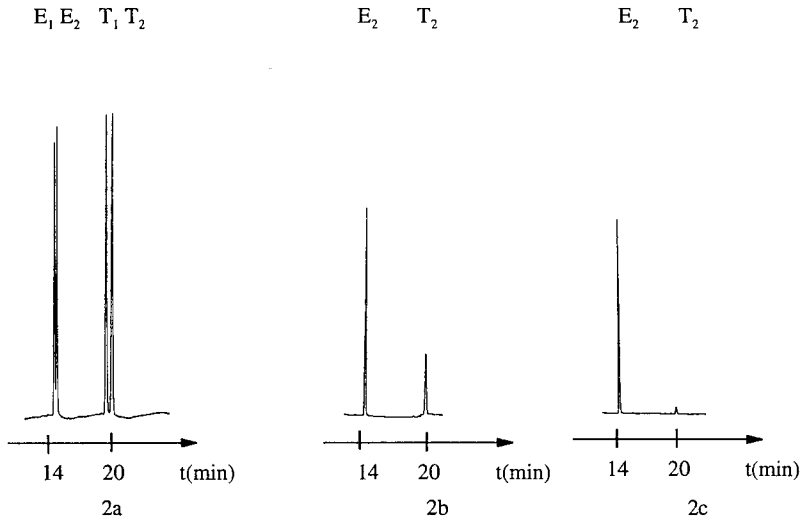
R	Conversion yield
CH_3	84 %
CH_2CH_3	74 %
iPr	50 %

Scheme 3 : Bioconversion using β-methylaspartase

Under these particular conditions, it was possible to obtain 52 g (for 1.6 l of bioconversion medium) of a 60/40 mol/mol mixture of (2S,3R)/(2S,3S)-3-methylaspartic acid **1** with a yield of 60% starting from 60g of mesaconic acid (scheme 3). The extraction of **1** from the bioconversion medium was complicated by the presence of salts in high concentration and the high solubility of the (2S,3R)-stereoisomer. Two methods were carried out for the recovery of (2S,3RS)-**1** : by using an ion exchange resin (DOWEX AG1-X8) and by chemical modification of **1** and further organic solvent extraction and deprotection. The principal interest lies in the possibility of obtaining pure compound **1** for its study (conformational analysis, stereoisomer characterization and further biotransformations).

According to the experimental conditions for recovering 3-methylaspartic acid during the chemical racemic amino acid synthesis, it is possible to obtain a mixture of the four stereoisomers or to separate threo and erythro racemic isomers. β-Methylaspartase can be therefore used for isolating non-natural stereoisomers by the α,β elimination of ammonia from natural isomers. This possibility has been, at first, exemplified on a 70/30 (2S,3S)/(2S,3R) mixture of extracted stereoisomers. The substrate concentration was lower than in the case of amination reaction (0.04M) and pH was corresponding to the optimum condition for the enzyme

(pH= 9.7). After 30 minutes of reaction, most of the L-threo-(2S,3S)-stereoisomer has been transformed in mesaconic acid as shown by chiral GC (figure 1). If the reaction is continued, the yield of L-erythro-(2S,3R)-stereoisomer is reduced, due its biotransformation. This result is important in regard to the obtaining of the non-natural stereoisomer at a preparation scale.



E_1 : (2R,3S)-3-methylaspartic acid, t= 14.3 min

E_2 : (2S,3R)-3-methylaspartic acid, t= 14.6 min

T_1 : (2R,3R)-3-methylaspartic acid, t=19.7 min

T_2 : (2S,3S)-3-methylaspartic acid, t=20.3 min

2a : chromatogram of the four stereoisomers of 3-methylaspartic acid

2b : chromatogram of the 70/30 (2S,3R)/(2S,3S) mixture of 3-methylaspartic acid

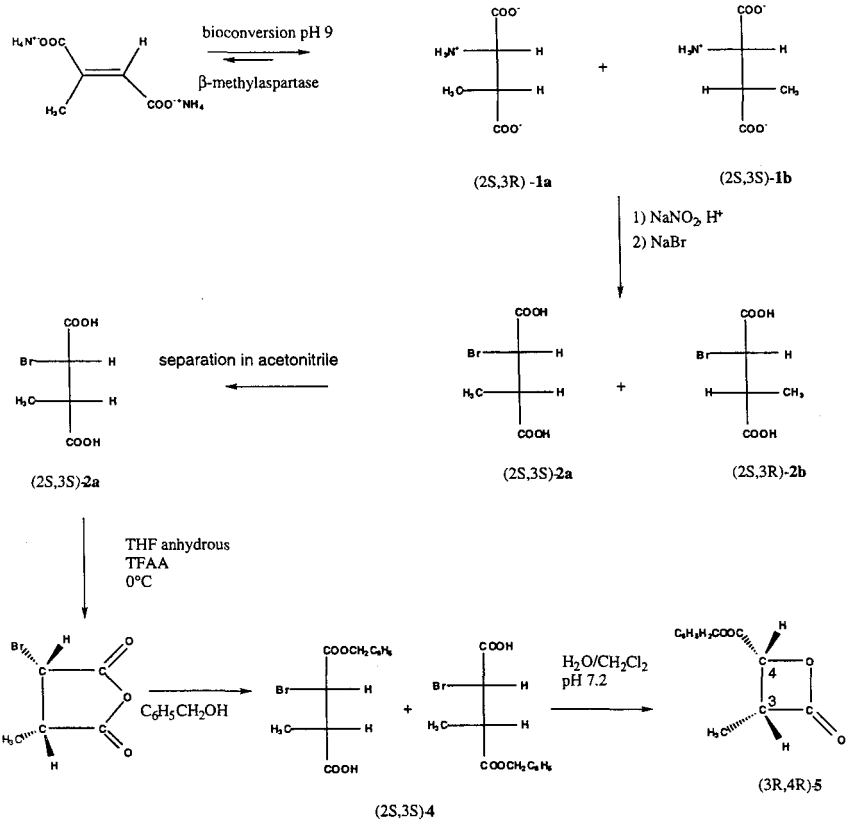
2c : chromatogram of the 70/30 (2S,3R)/(2S,3S) mixture of 3-methylaspartic acid after 30 min of exposure with β-methylaspartase

Figure 1 : Action of the β-methylaspartase on a mixture of (2S,3RS)-3-methylaspartic acid

Preparation of the optically active 3-methylalkylmalolactonate esters

The different β-substituted-β-lactones were synthesized starting from 3-alkylaspartic acids by using a now well established four-steps process. In the case of benzyl (3S,4R)-3-alkylmalolactonate, starting amino acid was pure (2S,3S)-stereoisomer. Scheme 4 concerns the preparation of benzyl (3R,4R)-3-methylmalolactonate. The separation of both diastereoisomers (**2a** and **2b**) was done at the bromation step thanks to the very low solubility of (2S,3S)-**2a** in

acetonitrile contrary to (2S,3R)-**2b**. During the synthesis of **2**, the substitution reaction does not affect the relative configuration, due to a double inversion. Both following steps do not affect the configuration of both asymmetric carbons, and only the lactonization reaction could lead to the formation of a couple of diastereoisomers by racemization of C_4 in the lactone **5** (scheme 3). The cyclization reaction, which takes place on the sole 2-alkyl-3-bromosuccinic acid monoester isomer **4**, proceeds according to an *intra-bimolecular mechanism*, with inversion of the configuration of the carbon bearing the bromine atom[22].



Scheme 4 : Synthesis route to benzyl (3R,4R)-3-methylmalolactonate

Diastereoisomeric excess of the lactones was determined by ^{1}H NMR and chiral GC. In the case of benzyl 3-methylmalolactonate, erythro-(3R,4R) and threo-(3S,4R) diastereoisomers present two different ^{1}H NMR spectra and the proton coupling constants are not identical . The methyl group chemical shift of benzyl (3S,4R)-3-alkylmalolactonate is at 1.5 ppm and the

coupling constant ^3J=4.3Hz is corresponding to a "threo" isomer compared to δ=1.15 ppm and ^3J=7Hz (isomer cis) in the case of the "erythro"-(3R,4R)-stereoisomer. ^1H NMR spectra displayed, for the optically active monomers, resonances corresponding to only one pure stereoisomer . This result was confirmed by chiral GC ; each compound contained only one peak, contrary to a diastereoisomer mixture which displays two peaks. It can be concluded, contrary to some malolactonic acid esters, benzyl 3-alkylmalolactonates are optically pure.

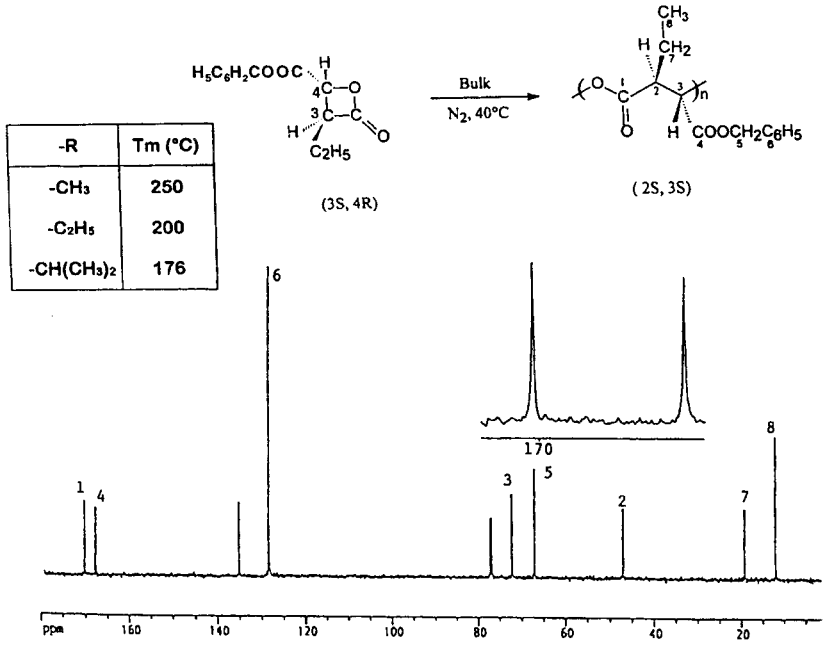
Anionic polymerization of the lactones

The chiral lactones were anionically polymerized in bulk in the range of 40 to 70°C using the potassium acetate/dibenzo-18-crown-6-complex (molar ratio 1/1) and tetramethylammonium benzoate as initiators (monomer/initiator molar ratio =1000). A quantitative yield (determined by IR) in polymer was obtained after 3 to 7 days of reaction according to the alkyl group size. All optically active poly(benzyl β-3-alkylmalates) present a melting temperature. This characteristic temperature varies with the size of the alkyl group as shown on figure 2.

Moreover, the configurational structure of repeating units is an important factor (figure 2). For example in the case of poly(benzyl β-3-methylmalate), it is worth noting the difference of behaviour between both (2S,3S) and (2R,3S) polystereoisomers. The polyester containing benzyl (2S,3S)-3-methylmalate repeating units is completely insoluble in organic solvents and presents a melting transition temperature of 250°C contrary to (2R,3S) stereoisomer which is soluble in common organic solvents and whose melting transition temperature is 130°C. Besides, organosoluble (2RS,3RS) and (2S,3RS) stereocopolymers are amorphous[16,17].

In order to confirm the stereoregularity of polymers, solid state ^{13}C NMR have been done on the highly cristalline poly[(2S,3S) benzyl β-3-methylmalate][23]. The spectrum exhibits single resonances. The relative narrowness of the resonance peaks is in favour of the presence of only one stereosequence of monomer units. This result agrees with the configurational structure of a closely related compound : poly[(2S,3S)-benzyl β-3-ethylmalate] which presents a ^{13}C NMR spectrum in $CDCl_3$ with only one fine peak for each carbon, due to the exclusive iso-sequence (figure 2). It has been demonstrated on poly[(R) or (S)-benzyl β-malate] that ring opening polymerization proceeds with configuration inversion of the carbon 4 of the lactone without any racemization of this carbon and with the formation of a carboxylate growing chain end[24].

The obtaining of poly(β-3-alkylmalic acid) (PAMA) has been exemplified on organosoluble poly[(2S,3S) benzyl β-3-isopropylmalate] and poly[(2R,3S) benzyl β-3-methylmalate]. After catalytic hydrogenolysis of benzyl protecting groups, these polymers are pH-sensitive water soluble and are under study as pH-sensitive hydrogels. Moreover, the ultimate degradation product of poly[(2S,3S)-β-3-isopropylmalic acid] is also a biomolecule ; this compound is a metabolite in the leucine biosynthesis.



-R	Tm (°C)
$-CH_3$	250
$-C_2H_5$	200
$-CH(CH_3)_2$	176

Figure 2 : Melting transition temperatures of poly[(2S,3S) benzyl β-3-alkylmalate] and ^{13}C NMR spectrum in $CDCl_3$ of poly[(2S,3S)-benzyl β-3-ethylmalate]

At last, it has been possible to introduce a third stereogenic center in the repeating unit for planing the tailor making of chiral enantioselective supported catalysts.

3,3-Dimethyl-butan-2-ol has been used under racemic or optically active form. (2S)-3,3-dimethyl-butan-2-ol has been obtained, by bioconversion, from 3,3-dimethyl-2-butanone, using *Rhodotorula glutinis* as microorganism. The corresponding lactone is optically pure as determined by chiral GC and the homopolymer obtained is insoluble in organic solvents and present a melting temperature at 320°C.

Starting from the racemic alcohol, the lactone displayed two peaks by chiral GC and the stereoregular polymer was also very crystalline and present a melting temperature at 313°C (figure 3). These experiments show, it is possible to obtained very organized optically active polymeric structures.

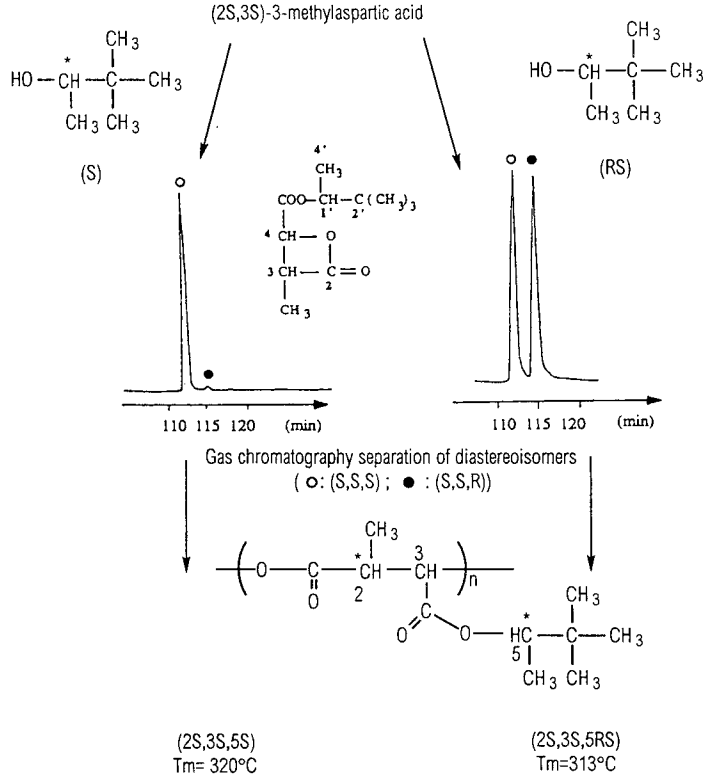


Figure 3 : Preparation of highly stereoregular polyesters

In conclusion, β-methylaspartate ammonia-lyase is a very versatile tool in the preparation of stereoisomers of 3-methylaspartic acids by reversible amination and deamination reactions. This chemoenzymatic route is under study to obtain both separated non-natural stereoisomers in order to prepare the corresponding optically active lactones. The combination of bioconversion and chemistry is a convenient method to yield all the separated stereoisomers of a chiral functional monomer aimed at the tailor making of polyvalent macromolecular drug carriers.

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