Title: PROCESS FOR THE ENZYMATIC RESOLUTION OF 1,3-DIOXOLANE-4-CARBOXYLATES

Abstract: The present invention relates to enzymatic resolution of enantiomeric mixtures of 1,3-dioxolane-4-carboxylate esters of Formula I. More particularly, the present invention relates to a process for preparing R and S enantiomers of methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate in high optical purity.
PROCESS FOR THE ENZYMATIC RESOLUTION OF
1,3-DIOXOLANE-4-CARBOXYLATES

TECHNICAL FIELD

The present invention relates to enzymatic
resolution of enantiomeric mixtures of 1,3-dioxolane-4-
carboxylate esters and more particularly to a process for
preparing R and S enantiomers of methyl 2,2-dimethyl-1,3-
dioxolane-4-carboxylate in high optical purity.

BACKGROUND OF THE INVENTION

Optically active 1,3-dioxolane-4-carboxylate
esters are important intermediates in the synthesis of
various pharmaceutically active compounds. Such
compounds include, for example, dioxolane nucleoside
analogs for treating HIV and HBV infections (see e.g.,
PCT patent applications WO 01/32153, WO 00/47759, and WO
00/39143). Methyl 2,2-dimethyl-1,3-dioxolane-4-
carboxylate is a particularly important C-3 chiral
building block for the preparation of pharmaceuticals,
such as dioxolane nucleoside analogs, as well as other
natural products. Conventional processes exist for
synthesizing methyl 2,2-dimethyl-1,3-dioxolane-4-
carboxylate from D- and L-serine or sugars, such as
mannitol. However, such processes involve several steps
and produce generally low yields of the desired product. Peptidase enzymes of subclass EC 3.4 and esterase enzymes of subclass EC 3.1 have been used to prepare optically active 1,3-dioxolane-4-carboxylates, but the resulting yields and optical purity are low (see e.g., German Patent Nos. DD 277700 and DD 277698; S. Hans et al., Liebigs Ann. Chem. (1993), 1, 103-04; M. Pottie et al., Tetrahedron Lett. (1989), '30, 5319-22). Therefore, there remains a need for the development of processes for the economical, large-scale stereoselective synthesis of optically active 1,3-dioxolane-4-carboxylate esters, such as methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate.

Amidohydrolase enzymes of subclass EC 3.5 have been employed in the resolution of amino acids via hydrolysis of the N-acetamide (see R. A. Sheldon, Chirotechnology, 1993, Marcel Dekker, New York, NY). However, there are relatively few instances of the use of amidohydrolases, such as acylase I (EC 3.5.1.14), for the hydrolytic resolution of carboxylate esters (see e.g., L. T. Kanerva et al., Tetrahedron: Asymmetry (2001), 12, 2059-2066; L. T. Kanerva et al., Tetrahedron: Asymmetry (2000), 11, 3957-3966; L. T. Kanerva et al., Tetrahedron: Asymmetry (1999), 10, 4405-4415).

In another example, Penicillin acylase (EC 3.5.1.11) has been used to hydrolyze the phenacetyl amide bond in penicillin G (J. Bryjak et al., Enzyme and Microbial Technology, 1996, 19, 196). Penicillin acylase has also been used to prepare alpha, beta, and gamma amino acids via enantioselective hydrolysis or acylation of amides (H. K. Chenault, J. Am. Chem. Soc. (1989), 111, 6354; Soloshonok, Tetrahedron: Asymmetry, (1994), 5, 1225; PCT application WO 94/02628; United States patent 6,214,609). There have been relatively few instances of
the use of penicillin acylase for the hydrolysis of carboxylic acid esters. One example is the penicillin acylase-catalyzed hydrolysis of phenacetyl esters of carbinol substrates (C. Fuganti et al., Tetrahedron (1988), 44, 2575-2582).

SUMMARY OF THE INVENTION

The present invention provides processes for preparing the R and S enantiomers of 1,3-dioxolane-4-carboxylate esters of Formula I, as defined herein. The processes comprise the step of contacting or reacting a solution comprising an enantiomeric mixture of 1,3-dioxolane-4-carboxylate of Formula I with an amidohydrolase enzyme, under conditions which permit the stereoselective hydrolysis of one enantiomer to its corresponding carboxylate of Formula II. The opposite enantiomer of the ester is maintained unreacted in enantiomerically enriched form.

DETAILED DESCRIPTION

In the following description, terms are defined as follows:

Alkyl -- represents a substituted (by halogen, hydroxyl or C6-C20 aryl) or unsubstituted straight chain, branched chain, or cyclic hydrocarbon moiety having 1 to 30 carbon atoms and preferably, from 1-8 carbon atoms.

Aryl -- represents a carbocyclic moiety which may be substituted by at least one heteroatom (e.g., N, O, or S) and containing at least one benzenoid-type ring and preferably containing from 6-15 carbon atoms (e.g. phenyl and naphthyl).

Chiral compound -- a compound that is not superimposable on its mirror image (e.g., an asymmetric
carbon atom, where four different substituents are attached to the same carbon).

Enantiomers -- pairs of stereoisomers that are nonsuperimposable mirror images of each other. Separate enantiomers rotate plane of polarized light in equal but opposite directions. Enantiomers are distinguished by absolute configuration (using the R and S designations) and by the direction of rotation of plane polarized light that has passed through the sample (dextrorotatory (+) right or levorotatory (-) left).

Enantiomeric mixture -- a mixture of two enantiomers.

Enantiomeric excess -- in a mixture of two enantiomers where one enantiomer is present to a greater extent, the percentage of the enantiomer found in excess over that of the racemic mixture is the enantiomeric excess. The enantiomeric excess is calculated as follows:

\[
\frac{([R]-[S]) + ([R]+[S])}{([R]-[S]) + ([R]+[S])} \times 100 = \% \text{ enantiomer excess.}
\]

Racemic mixture -- an equimolar mixture of two enantiomers.

Resolution -- the process of separating pairs of enantiomers from an enantiomeric mixture.

According to the present invention, the resolution of an enantiomeric mixture of a 1,3-dioxolane-4-carboxylate ester of Formula I:
wherein:

R₁ and R₂ are each independently selected from hydrogen, -(C₁-C₉)-straight or branched alkyl, -(C₅-C₁₂)-cycloalkyl, -(C₆-C₁₅)-aryl, -(C₁-C₄)-straight or branched alkyl-O-C(0)Ph, -(C₁-C₉)-straight or branched alkyl-O-(C₆-C₁₅)-aryl, or -(C₁-C₄)-straight or branched alkyl-O-benzyl,

R₁ and R₂ may be taken together as an oxygen, or

R₁ and R₂ may be taken together with the carbon atom to which they are bound to form a 3 to 6 member
cycloalkyl group;

R₃ is -(C₁-C₉)-straight or branched alkyl or -(C₆-C₁₅)-aryl;

R₄ is hydrogen, -(C₁-C₉)-straight or branched alkyl, -(C₅-C₁₂)-cycloalkyl, or -(C₆-C₁₅)-aryl,

is performed by a process comprising the steps of:

(a) contacting a solution comprising said
enantiomeric mixture of ester of Formula I with an
amidohydrolase enzyme, under conditions which permit
selective hydrolysis of one of the enantiomers to produce
a carboxylate of Formula II:

\[
\begin{array}{c}
\text{II} \\
\text{COOH}
\end{array}
\]

wherein:

R₁, R₂, and R₄ are each defined as in Formula I;

and (b) separating said carboxylate of Formula
II from the unhydrolyzed ester of Formula I.
The processes of present invention achieve high yields and enantioselectivity by employing amidohydrolases of subclass EC 3.5 which act on carbon-nitrogen bonds other than peptide bonds.

5 Amidohydrolases, known for hydrolysis of the carbon-nitrogen bond in amides, are unexpectedly efficient in the hydrolysis of the 1,3-dioxolane-4-carboxylates in the processes of this invention.

Preferred enzymes for the processes of the present invention include those in the EC class of enzymes known as hydrolases acting on linear amides (EC 3.5.1.*; class, hydrolase; subclass, acting on carbon-nitrogen bonds, other than peptide bonds; sub-subclass linear amides). Most preferred enzymes are amidohydrolase enzymes of EC 3.5.1.11 and EC 3.5.1.14. Those most preferred enzymes and their common names are provided in Table I below.

Table I

<table>
<thead>
<tr>
<th>EC Number</th>
<th>Common Name</th>
<th>Other Names</th>
</tr>
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<tbody>
<tr>
<td>EC 3.5.1.11 Penicillin amidase</td>
<td>penicillin acylase; benzylpenicillin acylase; novozym 217; semacylase; α-acylamino-β-lactam acylhydrolase; ampicillin acylase</td>
<td></td>
</tr>
<tr>
<td>EC 3.5.1.14 Aminoacylase</td>
<td>Amano acylase; acylase I; dehydropeptidase II; histozyme; hippuricase; benzamidase; hippurate; amido acid deacylase; L-aminoacylase; acylase; aminoacylase I; L-amino-acid acylase; α-N-acylaminoacid hydrolase; long acyl amidoacylase; short acyl amidoacylase</td>
<td></td>
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</tbody>
</table>
Many of the enzymes useful in the processes of the present invention are commercially available. Alternatively, they may be derived from a variety of sources, such as plants, animals, microbes, or fungi. The enzymes may be used as a part of a whole cell culture, an enzyme extract, an isolated enzyme, an isolated enzyme attached to a solid support, or a cross-linked enzyme crystal. The reaction processes may be performed in any conventional reaction vessel, such as a batch reactor, column, hollow-fiber membrane or membrane reactor.

The hydrolysis is generally carried out in buffer or buffer-organic solvent mixtures. Any organic solvent may be used. These include, for example, alcohols, aromatics, ketone ethers, alkanes, haloalkanes, acetone, and dimethyl sulfide. Additionally, multiple organic solvents may be used in combination. Either a homogenous or multiphasic solution may be employed.

The reaction can be carried out at any pH, ranging from about 5 to about 9. Typically, the pH of the reaction is about 7. A suitable temperature for the reaction may be in the range of about 0°C to about 60°C. Typically, the temperature is lower than about 50°C.

The concentration of the enantiomeric mixture of dioxolane ester of Formula I (weight/volume) is between about 1% and about 70%, the preferred concentration being between about 5% and about 50%, and the most preferred concentration being between about 10% and about 20%.

Conventional techniques for separating an ester from an acid may be used to separate the unreacted ester of Formula I from the acid of Formula II. Typically, the
reaction mixture is adjusted to a basic pH (e.g., pH >7.0) and then extracted with an appropriate organic solvent. As a result, the unreacted ester is dissolved in the organic solvent, while the carboxylic acid remains in the aqueous layer. Drying and evaporation of the organic solvent provides the desired chiral ester. Similarly, adjustment of the pH of the aqueous layer, followed by extraction with an organic solvent, leads to isolation of the acid. Alternatively, the separation process may be carried out without the addition of solvent, in cases in which the ester is sparingly soluble in water. Other standard separation techniques, such as the use of ion exchange resins or distillation, may be also used to separate the ester from the acid.

The products prepared by the processes of this invention are substantially enriched in one enantiomer. The high selectivity of the amidohydrolase for the hydrolysis of one enantiomer advantageously provides a high yield of the enantiomerically enriched acid of Formula II and the chiral, non-racemic unreacted ester of Formula I.

The processes of the present invention may be readily carried out on a large scale. One advantage of the processes of this invention is that, depending on the enzyme used, they allow stereoselective hydrolysis of the R vs. S enantiomer, and vice versa.

In one embodiment of the present invention, the enzyme penicillin acylase from *E. coli* is used to stereoselectively hydrolyze predominantly the S-enantiomer of Formula I, leaving the R-enantiomer unreacted. This embodiment is illustrated in Scheme A below:
In another embodiment of this invention, either Amano acylase from *Aspergillus melleus* or acylase I from porcine kidney is used to stereoselectively hydrolyze predominantly the R-enantiomer of Formula I, leaving the S-enantiomer unreacted. This embodiment is illustrated in Scheme B:

It will be apparent to those of skill in the art that the general processes outlined in Schemes A and B may also be used to prepare optically active acids of Formula II. Accordingly, the isolated optically active ester resulting from the processes of Schemes A or B may be hydrolyzed using organic acids to provide access to both enantiomers of Formula II, by means of a single stereoselective reaction. The isolated, optically active esters of Formula I may also be hydrolyzed enzymatically.

The present invention is illustrated in the following examples. These are for illustrative purposes only and are not to be construed as limiting the invention in any manner.
EXAMPLE 1

Penicillin acylase-catalyzed resolution of methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate at 20°C.

Racemic methyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate (0.55 g) was dissolved in 15 ml of 0.3 M, pH 7.5 phosphate buffer. A suspension of penicillin acylase from *E. coli*, obtained from Roche Diagnostics GmbH (50 μl of 1.68 U/μl suspension) was then added to the mixture and the resulting reaction mixture was stirred for 2 h with gentle agitation at room temperature (20°C). The pH was maintained at 7.5 by the addition of 1 M aqueous sodium hydroxide as necessary. The optical purity of the unreacted (R)-ester and (S)-acid product were monitored by HPLC using a chiral stationary phase column. The optical purity of the unreacted (R)-ester was also monitored by gas chromatography using a chiral stationary phase capillary column. The chemical conversion was monitored by reverse phase HPLC. After 2 hours, the enantiomeric excess of (R)-ester reached >99% and the conversion was 60%. Extraction of the unreacted ester from the reaction mixture with tert-butyl methyl ether and evaporation of the organic solvent yielded the desired (R)-ester.

Chiral HPLC Conditions: CHIRALCEL® OJ, 0.46 cm x 25 cm HPLC column (Daicel Chemical Inc.), mobile phase = Hexane:Acetonitrile:TFA (95:5:0.1), flow rate = 1 ml/min., UV detection at 220 nm. Retention times: (S)-ester, 6.3 min.; (R)-ester, 7.5 min.; (S)-acid, 9.3 min.; (R)-acid, 13.5 min.

Chiral gas chromatography conditions: J&W Scientific Cyclodex B column 0.25 micron, 30 M X 0.25 mm column. Temperature program: after 4 minutes at 130°C,
the temperature of the oven was increased at a rate of 10°C/min. to 140°C (column head pressure 20 psi). Retention times: S-methyl ester 2.84 min, R-methyl ester 2.92 min.

Reverse Phase HPLC Conditions: Varian Microsorb-MV™ 100 Å, C18, 0.46 cm x 15 cm HPLC column, mobile phase = Water:Acetonitrile:TFA (90:10:0.1), flow rate = 1 ml/min., UV detection at 220 nm. Retention times: methyl ester; 13.31 min.; acid, 5.75 min.

EXAMPLE 2

Penicillin acylase-catalyzed resolution of methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate at 15°C.

Racemic methyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate (1.1 g) was dissolved in 30 ml of 0.3 M pH 7.5 phosphate buffer. A 50 µl aliquot of 1.68 U/µl of penicillin acylase (from E. coli, obtained from Roche Diagnostics GmbH) was then added to the mixture and the resulting reaction mixture was stirred with gentle agitation at 15°C for 3 h. The temperature was maintained at 15°C. The pH was maintained at 7.5 by the addition of 1 M aqueous sodium hydroxide as necessary. After 3 hours, the enantiomeric excess of (R)-ester reached 98.8%. The conversion was 54.7%. Extraction of the unreacted ester from the reaction mixture with tert-buty1 methyl ether and evaporation of the organic solvent yielded the desired (R)-ester.

EXAMPLE 3

Penicillin acylase-catalyzed resolution of methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate at 10°C.

Racemic methyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate (1.1 g) was dissolved in 30 ml of 0.3 M pH 7.5 phosphate buffer. A 100 µl aliquot of 1.68 U/µl of
penicillin acylase (from \textit{E. coli}, obtained from Roche Diagnostics GmbH) was then added to the mixture and the resulting reaction mixture was stirred with gentle agitation at 10°C for 3.5 h. The temperature was maintained at 10°C. The pH was maintained at 7.5 by the addition of 1 M aqueous sodium hydroxide as necessary. After 3.5 hours, the enantiomeric excess of (R)-ester reached 98.5%. The conversion was 50.9%. Extraction of the unreacted ester from the reaction mixture with tert-butyl methyl ether and evaporation of the organic solvent yielded the desired (R)-ester.

\textbf{EXAMPLE 4}

Penicillin acylase-catalyzed resolution of methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate at room temperature.

Racemic methyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate (0.55 g) was dissolved in 15 ml of 0.3 M pH 7.5 phosphate buffer. A 50 \mu l aliquot of 1.68 U/\mu l of penicillin acylase (from \textit{E. coli}, obtained from Roche Diagnostics GmbH) was then added to the mixture and the resulting reaction mixture was stirred with gentle agitation at room temperature for 20 h. The pH was maintained at 7.5 by the addition of 1 M aqueous sodium hydroxide as necessary.

After 1 hour 35 min., the enantiomeric excess of the (R)-ester reached 95.8% and a further 0.50 g of racemic methyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate was added to the reaction mixture.

After 2.5 hours, the enantiomeric excess of the (R)-ester reached 63.1% and a further 1.1 g of racemic methyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate was added to the reaction mixture.
After 5 hours 20 min., the enantiomeric excess of (R)-ester reached 69% and a further 2.2 g of racemic methyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate was added to the reaction mixture.

After 20 hours, the enantiomeric excess of (R)-ester reached 81.7%. The conversion was 58.2%. Extraction of the unreacted ester from the reaction mixture with tert-butyl methyl ether and evaporation of the organic solvent yielded the desired (R)-ester.

**EXAMPLE 5**

Penicillin acylase-catalyzed resolution of methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate at 10°C.

Racemic methyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate (6 g) was dissolved in 24 ml of 0.3 M pH 7.5 phosphate buffer. A 500 µl aliquot of 1.68 U/µl of penicillin acylase (from *E. coli*, obtained from Roche Diagnostics GmbH) was then added to the mixture and the resulting reaction mixture was stirred with gentle agitation at 10°C for 4 h. The pH was maintained at 7.5 by the addition of 1 M aqueous sodium hydroxide as necessary. After 4 hours, the enantiomeric excess of (R)-ester reached 98.3%. The conversion was 57.4%. Extraction of the unreacted ester from the reaction mixture with tert-butyl methyl ether and evaporation of the organic solvent yielded 2.5 g (83% yield based on 50% yield) of the desired (R)-ester.

**EXAMPLE 6**

Penicillin acylase-catalyzed resolution of methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate at 20°C.

Racemic methyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate (1.1 g) was dissolved in 30 ml of 0.3 M pH 7.5 phosphate buffer. A 5 µl aliquot of 1.68 U/µl of
penicillin acylase (from *E. coli*, obtained from Roche Diagnostics GmbH) was then added to the mixture and the resulting reaction mixture was stirred with gentle agitation at 20°C for 20 h. The pH was maintained at 7.5 by the addition of 1 M aqueous sodium hydroxide as necessary. After 20 hours, the enantiomeric excess of (R)-ester reached 80%. The conversion was 60%. Extraction of the unreacted ester from the reaction mixture with tert-butyl methyl ether and evaporation of the organic solvent yielded the desired (R)-ester.

**EXAMPLE 7**

Amano acylase-catalyzed resolution of methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate at room temperature.

Racemic methyl-2,2-dimethyl-1,3-dioxolane-4-carboxylate (0.55 g) was dissolved in 15 ml of 0.3 M pH 7.5 phosphate buffer. A 66 mg aliquot of Amano acylase (*Aspergillus melleus* from Amano Pharma Corp. Ltd. as a lyophilized powder; 30,000 U/g) was then added to the mixture and the resulting reaction mixture was stirred with gentle agitation at room temperature for 10 h. The pH was maintained at 7.5 by the addition of 1 M aqueous sodium hydroxide as necessary. After 10 hours, the enantiomeric excess of (S)-ester reached 99.1%. The conversion was 51.5%. Extraction of the unreacted ester from the reaction mixture with tert-butyl methyl ether and evaporation of the organic solvent yielded the desired (S)-ester.

While we have hereinbefore presented a number of embodiments of this invention, it is apparent that our basic construction can be altered to provide other embodiments which utilize the process of this invention.
Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than the specific embodiments which have been presented hereinbefore by way of example.
We claim:

1. A process for the resolution of an enantiomeric mixture of a 1,3-dioxolane-4-carboxylate ester of Formula I:

\[
\begin{align*}
\text{R}_1 & \quad \text{R}_2 \\
\text{O} & \quad \text{O} \\
\text{R}_4 & \quad \text{COOR}_3
\end{align*}
\]

wherein:

- \( \text{R}_1 \) and \( \text{R}_2 \) are each independently selected from hydrogen, \(-(C_1-C_8)\)-straight or branched alkyl, \(-(C_5-C_{12})\)-cycloalkyl, \(-(C_6-C_{15})\)-aryl, \(-(C_1-C_4)\)-straight or branched alkyl-O-C(O)Ph, \-(C_1-C_4)\)-straight or branched alkyl-O-(C_6-C_{15})-aryl, or \-(C_1-C_4)\)-straight or branched alkyl-O-benzyl,

- \( \text{R}_1 \) and \( \text{R}_2 \) may be taken together as an oxygen, or

- \( \text{R}_1 \) and \( \text{R}_2 \) may be taken together with the carbon atom to which they are bound to form a 3 to 6 member cycloalkyl group;

- \( \text{R}_3 \) is \-(C_1-C_8)\)-straight or branched alkyl or \-(C_6-C_{15})\)-aryl;

- \( \text{R}_4 \) is hydrogen, \-(C_1-C_8)\)-straight or branched alkyl, \-(C_5-C_{12})\)-cycloalkyl, or \-(C_6-C_{15})\)-aryl,

the process comprising the steps of:
(a) contacting a solution comprising said enantiomeric mixture of ester of Formula I with an amidohydrolase enzyme, under conditions which permit selective hydrolysis of one of the enantiomers to produce a carboxylate of Formula II:

wherein:

R₁, R₂, and R₄ are each defined as in Formula I; and

(b) separating said carboxylate of Formula II from the unhydrolyzed ester of Formula I.

2. The process according to claim 1, wherein said solution comprises an aqueous buffer.

3. The process according to claim 2, wherein said aqueous buffer has a pH between about 5 and about 9.

4. The process according to claim 3, wherein said aqueous buffer has a pH of about 7.

5. The process according to claim 1, wherein said solution comprises an aqueous buffer and an organic solvent.

6. The process according to claim 5, wherein said aqueous buffer has a pH between about 5 and about 9.

7. The process according to claim 6, wherein said aqueous buffer has a pH of about 7.
8. The process according to any one of claims 1 to 7, wherein said amidohydrolase enzyme is penicillin acylase.

9. The process according to claim 8, wherein said penicillin acylase is *E. coli* penicillin acylase.

10. The process according to claim 8, wherein said process is carried out at a temperature between about 0°C and about 60°C.

11. The process according to claim 10, wherein said unhydrolyzed ester of Formula I is the R-enantiomer.

12. The process according to claim 10, wherein \( R_1, R_2 \) and \( R_3 \) are each methyl and \( R_4 \) is hydrogen.

13. The process according to claim 8, wherein said amidohydrolase enzyme is a soluble enzyme.

14. The process according to claim 8, wherein said amidohydrolase enzyme is an immobilized enzyme.

15. The process according to claim 8, wherein said amidohydrolase enzyme is a crosslinked enzyme crystal.

16. The process according to claim 8, wherein said enantiomeric mixture of ester of Formula I is a racemic mixture.

17. The process according to claim 8, wherein the concentration (weight/volume) of said enantiomeric mixture of ester of Formula I is between about 1% and about 70%.

18. The process according to any one of claims 1 to 7, wherein said enzyme is acylase I.
19. The process according to claim 18, wherein said acylase I is porcine kidney acylase I.

20. The process according to claim 18, wherein said process is carried out at a temperature between about 0°C and about 60°C.

21. The process according to claim 20, wherein said unhydrolyzed ester of Formula I is the S-enantiomer.

22. The process according to claim 20, wherein R₁, R₂ and R₃ are each methyl and R₄ is hydrogen.

23. The process according to claim 18, wherein said amidohydrolase enzyme is a soluble enzyme.

24. The process according to claim 18, wherein said amidohydrolase enzyme is an immobilized enzyme.

25. The process according to claim 18, wherein said amidohydrolase enzyme is a crosslinked enzyme crystal.

26. The process according to claim 18, wherein said enantiomeric mixture of ester of Formula I is a racemic mixture.

27. The process according to claim 18, wherein the concentration (weight/volume) of said enantiomeric mixture of ester of Formula I is between about 1% and about 70%.

28. The process according to any one of claims 1 to 7, wherein said amidohydrolase enzyme is an Amano acylase.

29. The process according to claim 28, wherein said Amano acylase is *Aspergillus* *melleus* Amano acylase.
30. The process according to claim 28, wherein said process is carried out at a temperature between about 0°C and about 60°C.

31. The process according to claim 30, wherein said unhydrolyzed ester of Formula I is the S-enantiomer.

32. The process according to claim 30, wherein R₁, R₂ and R₃ are each methyl and R₄ is hydrogen.

33. The process according to claim 28, wherein said amidohydrolase enzyme is a soluble enzyme.

34. The process according to claim 28, wherein said amidohydrolase enzyme is an immobilized enzyme.

35. The process according to claim 28, wherein said amidohydrolase enzyme is a crosslinked enzyme crystal.

36. The process according to claim 28, wherein said enantiomeric mixture of ester of Formula I is a racemic mixture.

37. The process according to claim 28, wherein the concentration (weight/volume) of said enantiomeric mixture of ester of Formula I is between about 1% and about 70%.

38. The process according to claim 1, wherein R₁, R₂ are each methyl.

39. The process according to claim 38, wherein R₃ is methyl and R₄ is hydrogen.

40. The process according to claim 1, wherein said process is carried out at a temperature between about 0°C and about 60°C.
41. The process according to claim 1, wherein said enantiomeric mixture of ester of Formula I is a racemic mixture.

42. The process according to claim 1, wherein the concentration (weight/volume) of said enantiomeric mixture of ester of Formula I is between about 1% and about 70%.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C07H 19/00; C12P 1/00, 41/00
US CL. : 536/27.14, 22.1; 435/41, 87, 88, 280
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 536/27.14, 22.1; 435/41, 87, 88, 280

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Chemical Abstracts electronic search; US PTO East electronic Database search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>A, P</td>
<td>US 6,541,625 B2 (CIMPOIA et al.) 01 April 2003, see abstract.</td>
<td>1-42</td>
</tr>
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</table>

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
  A - document defining the general state of the art which is not considered to be of particular relevance
  E - earlier application or patent published on or after the international filing date
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Date of the actual completion of the international search
11 November 2003 (11.11.2003)

Date of mailing of the international search report
12 Dec 2003

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Form PCT/ISA/210 (second sheet) (July 1998)
Box No. VIII (ii)  DECLARATION: ENTITLEMENT TO APPLY FOR AND BE GRANTED A PATENT

The declaration must conform to the standardized wording provided for in Section 212; see Notes to Boxes Nos. VIII, VIII (i) to (v) (in general) and the specific Notes to Box No. VIII (ii). If this Box is not used, this sheet should not be included in the request.

Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent (Rules 4.17(ii) and 51bis.1(a)(ii)), in a case where the declaration under Rule 4.17(iv) is not appropriate:

In relation to this international application,

ALTUS BIOLOGICS INC. is entitled to apply for and be granted a patent by virtue of the following:

an assignment from LALONDE, James J. and YAO, Yiming to ALTUS BIOLOGICS INC., dated 19 June 2003.

This declaration is made for the purposes of:

all designations except the designation of the United States of America.

☐ This declaration is continued on the following sheet, "Continuation of Box No. VIII (ii)".