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PATENTS ACT 1990

NOTICE OF ENTITLEMENT

WE, LABORATORIOS MENARINI S.A. of Alfonso XII, 587, E-08912 Badalona, Spain, being the applicant in respect of the annexed application state the following:-

The inventors of the invention are as follows:

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RICHARD ANTHONY WISDOM, of 79 Station Road, Impington, Cambs CB4 4NP, England, United Kingdom.

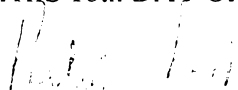
The person nominated for the grant of the patent LABORATORIOS MENARINI S.A.:

has entitlement from the inventors by virtue of the inventors assigning their rights in the invention to Chiros Limited. Chiros Limited subsequently assigned the rights in the invention to Laboratorios Menarini S.A.

The person nominated for the grant of the patent LABORATORIOS MENARINI S.A. has the consent of the basic applicant CHIROS LIMITED to claim convention priority from the basic application.

The basic application listed in the declaration made under Article 8 of the PCT is the first application made in a convention country in respect of the invention.

DATED THIS 10th DAY OF July 1995

  
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By their Patent Attorneys  
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A number of 2-arylpropionic acids are well known as anti-inflammatory agents. Examples include ibuprofen, naproxen and ketoprofen. These compounds are chiral, and it is now well established that their major therapeutic activity resides in the (S)-enantiomer.

#### Claim

1. A process for preparing predominantly (S)-2-(3-benzoylphenyl)propionic acid from a mixture of enantiomers of an alkyl ester of 2-(3-benzoylphenyl)propionic acid, which comprises using as a biocatalyst a microorganism of the genus Ophiostoma or Ceratocystis, or a material having enzymatic activity derived therefrom.
2. A process according to claim 1, which gives (S)-2-(3-benzoylphenyl)propionic acid in at least 93% enantiomeric excess, at 15-25% conversion.
3. A process according to claim 1, <sup>OR CLAIM 2</sup> wherein the biocatalyst is the microorganism Ophiostoma novo-ulmi, IMI 356050.



INTEK

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<p>(21) International Application Number: PCT/EP94/00630 (22) International Filing Date: 3 March 1994 (03.03.94) (30) Priority Data: 9304256.2 3 March 1993 (03.03.93) GB (71) Applicant (for all designated States except US): LABORATORIOS MENARINI S.A. [ES/ES]; Alfonso XII, 587, E-08912 Badalona (ES). (72) Inventors; and (75) Inventors/Applicants (for US only): WARNECK, Julie, Belinda, Hazel [GB/GB]; 6 Manor Drive, Sawtree, Huntingdon PE17 5UU (GB). WISDOM, Richard, Anthony [GB/GB]; 79 Station Road, Impington, Cambs CB4 4NP (GB). (74) Agent: MINOJA, Fabrizio; Studio Consulenza Brevettuale, Via Rossini, 8 I-20122 Milano (IT)</p>	<p>(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p><b>675700</b></p>	
<p>(54) Title: ARYLALKANOIC ACID RESOLUTION</p> <p>(57) Abstract</p> <p>An organism of the genus <i>Ophiostoma</i> or <i>Ceratocystis</i>, or a material having enzymatic activity derived therefrom, is used as a stereospecific agent, in a biotransformation reaction, for the production of (S)-ketoprofen from a mixture of enantiomers of an ester thereof.</p>		

ARYLALKANOIC ACID RESOLUTIONField of the Invention

This invention relates to arylalkanoic acid resolution, particularly to 2-(3-benzoylphenyl)propionic acid (Ketoprofen) resolution.

5 Background of the Invention

A number of 2-arylpropionic acids are well known as anti-inflammatory agents. Examples include ibuprofen, naproxen and ketoprofen. These compounds are chiral, and it is now well established that their major therapeutic  
10 activity resides in the (S)-enantiomer.

Several methods for obtaining the (S)-enantiomer, free of contaminating (R)-enantiomer, are known. These include asymmetric chemical syntheses and chemical resolution such as the stoichiometric crystallisation of diastereomeric  
15 salts formed with various chiral amines.

An alternative approach is biocatalytic, using a biocatalyst to selectively hydrolyse an ester of the 2-arylpropionic acid. Providing a biocatalyst with the correct stereospecificity can be identified, a reaction  
20 mixture containing the unreacted ester of one enantiomer plus the acid product of the other enantiomer can be obtained. Separation and recovery of the product are then relatively facile. Unreacted ester can be racemised and reused in a further reaction, thereby ensuring almost  
25 complete conversion of the racemic substrate to the required single enantiomer product.

EP-A-0227078 describes the use of several extracellular, commercially-available microbial lipases in such biocatalytic resolutions. In general, however, large  
30 amounts of enzyme were required and the reaction took 2-6 days. Such reactions would therefore be expensive to operate. The best enzyme powder identified was that from Candida cylindracea (also known as Candida rugosa); however, in EP-A-0407033, it was shown that this  
35 preparation, as well as having low activity, contained more than one enzyme with esterase activity. Further, in order

to obtain ketoprofen with a high ee, it was necessary to purify the preparation.

EP-A-0233656 describes the isolation and cloning of an esterase gene from Bacillus thal. This enzyme is shown to selectively hydrolyse the ethyl and methyl esters of both naproxen and ibuprofen to give the (S)-acid of respective compounds. It is also shown that cloning of the enzyme resulted in a preparation giving a higher ee product, as a result of minimising other enzyme side-activities.

WO-A-9323547 describes a number of strains which also produce an esterase which selectively hydrolyses naproxen esters. The best strain found was Zopfiella latipes, the esterase from which had been cloned.

WO-A-9304189 describes an organism, Trichosporon sp, that is able to selectively hydrolyse the (S)-enantiomer of ethyl ketoprofen to give an (S)-acid product having an ee of >90%. The enzyme that carries out this biotransformation is intracellular. As stable cell-free preparations have proved difficult to obtain, it is difficult to increase biocatalytic activities by gene cloning or classical mutagenesis.

An object behind the present invention has been to obtain a biocatalyst which has good activity against different ketoprofen esters, which gives good ee ketoprofen acid product, and which would be suitable for cloning and hyper-expression, e.g. in E. coli, to enable biocatalyst costs to be kept to a minimum.

#### Summary of the Invention

Surprisingly, a screen of microorganisms from a range of different sources showed that the ascomycete Ophiostoma novo-ulmi (also known as Ceratocystis ulmi) produced an intracellular hydrolytic enzyme which could carry out the desired reaction. Further tests demonstrated that, unlike the strain disclosed in WO-A-9304189, stable cell-free activity could be obtained and that therefore it was suitable for cloning and potential use in cell-free biotransformations. Ophiostoma novo-ulmi is a plant

pathogen known to be the virulent causative agent of Dutch Elm disease. It is known to be closely related to less virulent strains of Ophiostoma ulmi and also to O. piceae.

5 Following the primary discovery underlying the present invention, it has been found that stereospecific esterase activity is present in alternative strains of Ophiostoma novo-ulmi in Ophiostoma ulmi (also sometimes known as Ceratocystis ulmi) and Ophiostoma piceae (also sometimes known as Ceratocystis piceae) and from isolates collected  
10 from various locations in the USA, Europe, the Middle East and Uzbekistan. In addition, alternative strains of Ceratocystis sp. obtained from the IMI, Egham, Surrey, UK have been obtained and screened for activity. It has been discovered that the activity is also present in strains C.  
15 coronata (e.g. IMI 176533), C. ips (e.g. IMI 212114), C. tetropii (e.g. IMI 212117), C. cainii (e.g. IMI 176523), C. arborea (e.g. IMI 176529) and C. stenoceras (e.g. IMI 268494). The activity appears therefore to be widespread amongst a range of alternative Ceratocystis strains  
20 collected from different locations in the world.

A strain of the original isolate screened, herein described as AJ3, was deposited at the IMI on 15th February 1993, with the accession number IMI 356050, under the terms of the Budapest Treaty. This strain provides a good  
25 example of the activity; however, given the widespread nature of the activity in a wide variety of related strains, the scope of the invention is not intended to be limited to this particular organism. The invention also extends to the use of enzymatic activity isolated from such  
30 organisms, by any suitable technique.

#### Description of the Invention

In accordance with the present invention, microorganisms of the genus Ophiostoma and their enzymatic activity can be used to hydrolyse the racemic alkyl ester  
35 of ketoprofen stereo-selectively, to yield the acid substantially enriched in the (S)-enantiomer, e.g. to 93-96% enantiomeric excess, or more, ~~and leave residual ester~~ <sup>AT 15-25% CONVERSION</sup>



enriched with the (R)-enantiomer. The reaction can be conducted on any mixture of enantiomers of the ester, although that mixture will usually be the racemate. The alkyl group of the ester is preferably of 1 to 3 carbon atoms. The reaction is preferably conducted at pH 8-11, more preferably 9.5-10.5, and most preferably about 10. An organic solvent such as cyclohexane is usually employed.

Standard chemical procedures may then be used to separate the acid product from the ester. Where necessary, the optical purity of the acid can be improved by the use of a chiral amine such as (R)- $\alpha$ -methylbenzylamine. The ester that is left, unconverted, can be racemised, for further use.

The following Examples illustrate the invention.

Example 1 Biotransformation of racemic ethyl Ketoprofen by AJ3

The following medium was used for the growth of the organism:

	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (g/l)	0.5
20	MgSO <sub>4</sub> .7H <sub>2</sub> O (g/l)	0.25
	CaCl <sub>2</sub> .2H <sub>2</sub> O (g/l)	0.1
	KH <sub>2</sub> PO <sub>4</sub> (g/l)	8.0
	Yeast Extract (g/l)	10.0
	Glucose (g/l)	5.0
25	Trace element solution ( $\mu$ l/l)	100
	pH (with NaOH)	6.5

The trace element solution used was:

	CaCl <sub>2</sub> .2H <sub>2</sub> O (g/l)	3.57
	ZnO (g/l)	2.0
30	CuCl <sub>2</sub> .2H <sub>2</sub> O (g/l)	0.85
	Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O (g/l)	4.8
	MnCl <sub>2</sub> .4H <sub>2</sub> O (g/l)	2.0
	FeCl <sub>3</sub> .6H <sub>2</sub> O (g/l)	5.4
	H <sub>3</sub> BO <sub>3</sub> (g/l)	0.3
35	CoCl <sub>2</sub> .6H <sub>2</sub> O (g/l)	2.4
	HCl (ml/l)	250

Cells of AJ3 were inoculated from a malt extract agar plate into 30 ml growth medium in a 250 ml baffled flask.

This was shaken for 30 hours at 23°C and then 2.5 ml transferred into a second 250 ml baffled flask containing 30 ml of medium. Following growth for 40 hours, at 23°C with shaking, racemic ethyl ketoprofen was added to the  
5 broth to a concentration of 20 g/l. The biotransformation was then allowed to proceed for 120 hours, after which HPLC analysis showed there to have been 18.2% hydrolysis of the added ester. The enantiomeric excess of the ketoprofen acid formed was found to be 96% in favour of the (S)-  
10 enantiomer.

Example 2 Effect of organic solvent

A crude enzyme solution was prepared, using the same growth medium as in Example 1, minus glucose and at a pH of 6.0. Cells of AJ3 were inoculated into 100 ml medium in a  
15 1 litre shake flask. This culture was grown with shaking at 30°C for 48 hours. 10 ml was then transferred into 90 ml fresh medium in a 1 litre shake flask and grown for a further 8 hours at 30°C with shaking. This flask was used to inoculate a 2.8 l laboratory fermenter with 1.5 l medium  
20 plus 5 g/l glucose and 0.5 ml/l of a silicone/PPG-based antifoam (XFO371, Ivanhoe Chemicals, Ill., USA). This fermenter was operated for 48 hours with agitation and aeration, controlled to maintain a DOT >50% air saturation and temperature and pH at 30°C and 6.0 respectively.

25 At the completion of the fermentation the cells were harvested by centrifugation and the pellet resuspended at 10% w/v (based on wet cell weight) in lysis buffer, i.e. 0.1M sodium carbonate in 5% Triton X-100 aqueous solution. Lysis was carried out overnight at 8°C with shaking after  
30 which the enzyme activity-containing lysate was separated from the cell debris by centrifugation.

Racemic ethyl ketoprofen was dissolved in each of four organic solvents: toluene, cyclohexane, methanol and MTBE (methyl t-butyl ether) to a concentration of 20 g/l. 2 ml of each of these solutions was added to 5 ml aliquots of crude enzyme solution in glass vials. A control aqueous biotransformation containing 5 ml crude enzyme solution, 400  $\mu$ l 50% racemic ethyl ketoprofen, 0.5% w/v Tween 80 stock solution and 2.5% w/v Triton x-100 was also prepared in a glass vial. All samples were reacted with shaking for 48 hours at 25°C. Analysis of the aqueous phase of each reaction mixture gave the results shown in Table 1.

Table 1 HYDROLYSIS OF RACEMIC ETHYL KETOPROFEN IN 2-PHASE BIOTRANSFORMATIONS

SAMPLE SOLVENT	ACID PRODUCED (mg.ml <sup>-1</sup> )	CONVERSION (%)
AQUEOUS	2.3	6.2
TOLUENE	0.1	1.3
CYCLOHEXANE	1.1	14.1
METHANOL	0.4	4.6
MTBE	0.7	8.3

It is observed that the biotransformation works to a certain extent in the presence of cyclohexane or MTBE as solvents, but is quite strongly inhibited by toluene under these conditions.

Example 3 Effect of the alkyl group

Various esters of racemic ketoprofen were dissolved in cyclohexane to a concentration of 20 g/l. The esters used were methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl. In the case of methyl ester, a suspension was prepared due to low solubility of ester in cyclohexane.

To 5 ml aliquots of crude enzyme solution in glass vials was added 2 ml of each of the six racemic ketoprofen ester solutions above. An aqueous control biotransformation was also prepared in a glass vial containing 5 ml crude enzyme solution, 200  $\mu$ l 50% racemic ethyl ketoprofen, 0.5% Tween 80 stock solution and 2.5% w/v Triton x-100. Reactions were conducted with shaking for 24 hours at 25°C. HPLC analysis of the aqueous phase of each biotransformation gave the results shown in Table 2.

Table 2 HYDROLYSIS OF RACEMIC KETOPROFEN ESTERS IN 2 PHASE BIOTRANSFORMATIONS.

5	KETOPROFEN ESTER	ACID PRODUCED (mg.ml <sup>-1</sup> )	CONVERSION (%)	EE (%)(S)-enantiomer
	AQUEOUS CONTROL	4.6	22.9	90
	METHYL	1.7	42.3	90
	ETHYL	1.1	27.5	100
10	n-PROPYL	1.0	25.6	86
	n-BUTYL	0.3	8.0	N/D
	n-PENTYL	0.18	4.4	N/D
	n-HEXYL	0.09	2.3	N/D

N/D = not determined owing to the low acid accumulated.

Example 4 Preparation of AJ3 lysate

The AJ3 isolate was grown to the 500 litre scale using the following methodology.

AJ3 was inoculated into 100 ml medium (as Example 1) in a 1 litre point baffled flask. Following growth for 48 hours at 25°C with orbital shaking (300 rpm, 25 mm throw), 1 ml was transferred to each of three 1 litre baffled flasks each containing 100 ml medium. The cultures were then grown for a further 48 hours under similar conditions before being combined and used to inoculate a 750 l fermenter containing 500 l medium.

The following medium was used:

	MgSO <sub>4</sub> .7H <sub>2</sub> O	0.4 g/l
	CaCl <sub>2</sub> .2H <sub>2</sub> O	0.1 g/l
15	KH <sub>2</sub> PO <sub>4</sub>	8.0 g/l
	Yeast Extract	30.0 g/l
	Trace Element Solution	200 µl/l (as Example 1)
	Antifoam (XFO-371)	0.5 ml/l
	pH (with NaOH)	6.0

This medium was sterilised at 121°C for 50 minutes prior to the addition of 15 l of 50% w/v sterile glucose solution.

Control conditions were: temperature 25°C, pH at 6.0 phosphoric acid or sodium hydroxide addition and DOT ≥50% of air saturation by agitation and aeration control.

After 64 hours growth, cells were treated in situ to kill them and to aid lysis. This was done by dropping the temperature to 15°C and adding sodium hydroxide to bring the pH to 10. After 50 minutes' treatment, the broth was readjusted to pH 7 with phosphoric acid and the cells harvested in a continuous centrifuge. The collected cells were distributed into different containers and stored at -20°C until required.

Frozen cells were thawed and resuspended at 25% w/v (based on wet cell weight) in 50 mM KH<sub>2</sub>PO<sub>4</sub>, pH 6.5. After stirring for 30 minutes, the cells were collected by centrifugation. Cells were then resuspended at 25% w/v

(based upon wet cell weight) in lysis buffer, i.e. 0.3 M  $\text{Na}_2\text{CO}_3$ , pH 10.5. Lysis was conducted overnight at 4°C with stirring, after which the lysate was clarified by centrifugation to separate cell debris from the supernatant. The enzyme solution (i.e. lysate supernatant) was assayed for activity using standard biotransformation conditions, i.e. 1 ml enzyme solution suitably diluted in 0.1 M  $\text{Na}_2\text{CO}_3$ , pH 10.0, 40  $\mu$  50% racemic ethyl ketoprofen, 0-5% Tween 80 stock solution, 2.5% w/v Triton x-100. Biotransformations were carried out in a sealed 20 ml volume glass vial with shaking for 1 hour at 25°C. Activity is expressed as U/ml activity where 1 unit (U) = 1 mg ketoprofen acid produced per hour at 25°C. Enzyme solutions were considered suitably diluted if the assayed activity was in the range of 1 to 5 U/ml.

A 50% racemic ethyl ketoprofen stock solution was prepared as follows: 25 g racemic ethyl ketoprofen and 0.25 g Tween 80 were added to 10 ml distilled water and this mixture sonicated for 5 mins (15 amplitude  $\mu\text{m}$ , 10 seconds on/10 seconds off cycle). The volume was then made up to 50 ml with distilled water, and the stock solution autoclaved to sterilise and allow for longer term storage.

A typical lysate activity was 1.5 U/ml.

Example 5 Isolation and purification of hydrolytic activity

Clarified lysate (Example 4) was diafiltered against distilled water (Amicon DC2 ultra-filtration unit using 30,000 molecular weight cut off hollow fibre cartridges) until the conductivity of the lysate reached that of 20 mM  $\text{Na}_2\text{CO}_3$ , pH 9.2 buffer (Buffer A). The solution was then concentrated 4-5 fold and typically retained >90% initial activity. Enzyme activity was loaded batchwise onto pre-equilibrated QA52 anion exchange resin (Pharmacia) for 1 hour at ambient temperature, at a loading of 7-10 U of activity per ml of wet QA52 gel. The loaded gel was packed into a column at a linear flow rate of 200 mm/h. Chromatography was subsequently conducted at 4°C.

The column was washed to baseline with Buffer A. Elution was performed using a 10 column volume gradient of 0-05 M NaCl in Buffer A and fractions corresponding to 1/35 gradient volume were collected. The elution profile typically obtained is shown in Table 3.

Table 3 ELUTION PROFILE FOR 1ST PASS QA52 CHROMATOGRAPHY

	FRACTION	TOTAL ACTIVITY (U.)	RECOVERY (%)
5	LOAD	99	-
	FLOW THROUGH	0	0
	WASH	0	0
	1	0	0
	5	0.3	0.3
10	6	2.0	2.0
	7	6.6	6.7
	8	19.5	19.7
	9	22.2	22.4
	10	10.0	10.1
15	11	2.5	2.5
	13	1.0	1.0
	17	0.3	0.3

Activity was eluted in the NaCl concentration range of 0.12-0.18 M. Fractions 7-10 were pooled inclusively to give:

20

pooled fraction activity recovery = 63.5%

pooled fraction protein recovery = 15.8%

25 The pooled activity was concentrated using a YM 30 membrane in a stirred cell ultrafiltration unit (AMICON) with no loss of activity, and dialysed against Buffer A until a conductivity of 3.2-3.6 mS was attained. This was then re-chromatographed on QA52 resin with the results shown in Table 4.

Table 4 ELUTION PROFILE FOR 2ND PASS QA52 CHROMATOGRAPHY

	FRACTION	TOTAL ACTIVITY (U)	RECOVERY (%)
5	LOAD	3111	-
	FLOW THROUGH	0	0
	WASH	0	0
	13	60	1.9
	14	193	6.2
10	15	407	13.1
	16	828	26.6
	17	890	28.6
	18	445	14.3
	19	152	4.9
15	20	81	2.6

In this second pass, activity was eluted in the NaCl concentration range of 0.2-0.25 M NaCl. Fractions 15-17 were pooled inclusively. The pooled sample was assayed for activity and protein content.

20 Activity = 27.8 U.ml<sup>-1</sup>  
 Total activity = 2599 U  
 Total activity recovery = 83.5%

25 Protein (biuret method) = 1.34 mg.ml<sup>-1</sup>  
 Total protein = 125 mg  
 Total protein recovery = 5.5 %

30 The pooled fractions were further purified using a Pharmacia MONO P HR 5/5 packed anion exchange FPLC. The chromatographic conditions were as follows:

Low salt buffer = 20 mM Ethanolamine-HCl pH 9.0  
 High salt buffer = 20 mM Ethanolamine-HCL + 0.3M NaCl pH 9.0  
 Flow rate = 1 ml/min .

Gradient profile	Time	NaCl concentration
	0-5 mins	0 M
	5-7 mins	0-0.1 M
5	7-20 mins	0.1-0.3 M
	20-22 mins	0.3 M
	22-23 mins	0.3-0 M
	23-25 mins	0 M

10 Sample volume = 1 ml  
 Fraction volume = 1 ml

15 The pooled fractions from the 2nd pass QA52 chromatography were concentrated 10 fold through a 30,000 molecular weight cut off ultrafiltration membrane and the resulting concentrate desalted into low salt buffer for HPLC, using a Pharmacia G-25 PD10 gel filtration column. Approximately 5 mg total protein were loaded onto the HPLC column per run. The following elution profile was obtained (Table 5):

20 Table 5 ELUTION PROFILE FROM MONO P ANION EXCHANGE HPLC

FRACTION (mins)	ACTIVITY (U.ml <sup>-1</sup> )	RECOVERY (%)
LOAD	104.0	-
8	5.7	5.5
25 9	63.8	61.3
10	28.3	27.2
11	7.5	7.2
12	1.9	1.8
13	0.0	0.0

30 Fractions 9 and 10 were pooled to give a total activity recovery of 88.5%. This pooled activity was then further purified by size exclusion HPLC.

35 An Analgel-TSK G3000 SWXL 30 cm x 7.8 mm size exclusion HPLC column was used for the following separation. The following chromatographic conditions were employed:

Buffer - 0.1 M K<sub>2</sub>HPO<sub>4</sub> + 20 mM Na<sub>2</sub>EDTA pH 7.0

Flow rate = 0.4 ml.min<sup>-1</sup>

Sample volume = 200 µl

40 Fraction volume = 400 µl

The pooled fractions from the MONO P ion exchange HPLC were concentrated 1.5 fold

through a 10,000 molecular weight cut off ultrafiltration membrane . The concentrated sample was desalted into size exclusion HPLC buffer using a Pharmacia G25 pd10 column. The following elution profile was obtained (Table 6).

5 Table 6 ELUTION PROFILE FROM SIZE EXCLUSION HPLC

FRACTION (mins)	ACTIVITY (U.ml <sup>-1</sup> )	RECOVERY (%)
LOAD	131.0	-
10 17.5	0.0	0
18.5	0.0	0
19.5	1.5	2.3
20.5	13.3	20.3
21.5	7.5	11.5
15 22.5	2.6	4.0
23.5	0.8	1.2

20 An estimation of the molecular weight of our AJ3 racemic ethyl ketoprofen esterase was made by SDS-PAGE (sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis). A pre-cast 12% homogeneous SDS-PAGE mini-gel (BDH) was run using prestained molecular weight protein standard markers (BISC) for molecular weight comparison. The running Buffer was Electrograd Buffer TTS (BDH).

25 Electrophoresis was carried out at a constant voltage (200 V) and limiting current (40 mA per gel) until the blue dye front had passed completely through the gel. Protein was visualised by Coomassie Blue staining.

30 The band of protein corresponding to the esterase was identified by comparison of protein banding in active and non-active samples. Comparison of the relative migration distances of the active protein and the protein standards indicated the novel purified denatured protein had a molecular weight slightly greater than the 32,500 Da marker.

Example 6 Effect of pH on the hydrolytic activity

The pH profile of the biotransformation was tested using the pooled material described above, following 4 months storage at -20°C. An aliquot was thawed at ambient temperature, and diluted 10-fold with distilled water (diluted enzyme solution). 1.1 M buffer stock solutions were prepared at the following pH, using the salts indicated (pH was adjusted as necessary using sodium hydroxide or hydrochloric acid):

10	pH 6.0	:	NaH <sub>2</sub> PO <sub>4</sub>
	pH 7.0	:	NaH <sub>2</sub> PO <sub>4</sub>
	pH 8.0	:	NaH <sub>2</sub> PO <sub>4</sub>
	pH 9.0	:	Glycine-HCl
	pH 10.0	:	Glycine-HCl
15	pH 11.0	:	Na <sub>2</sub> HPO <sub>4</sub>

The biotransformation reactions were set up in sealed glass vials as follows:

	1 ml diluted enzyme solution
	100 µl relevant 1.1 M pH buffer stock solution
20	40 µl 50% racemic ethyl stock solution
	2.5% w/v Triton X-100

For a biotransformation at pH 12.0, 1M NaOH solution was added directly to a pH 11.0 reaction mixture until the correct pH was reached. All biotransformations were set up in duplicate and reactions were carried out for 1 hour with shaking at 25°C. The main results of the reactions are shown in Table 7. Maximum activity was found at pH 10.

TABLE 7 AJ3 BIOTRANSFORMATION pH PROFILE

BIOTRANSFORMATION pH	ACTIVITY (U.ml <sup>-1</sup> )	NORMALISED RECOVERY (%)
5 6	1.32	27.6
7	1.97	41.2
8	2.47	51.7
9	2.95	61.7
10 10	4.78	100.0
11	2.35	49.2
12	0.36	7.5

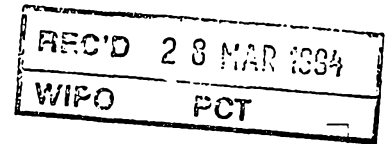
CLAIMS

1. A process for preparing predominantly (S)-2-(3-benzoylphenyl)propionic acid from a mixture of enantiomers of an alkyl ester of 2-(3-benzoylphenyl)propionic acid, which comprises using as  
5 a biocatalyst a microorganism of the genus Ophiostoma or Ceratocystis, or a material having enzymatic activity derived therefrom.
2. A process according to claim 1, which gives (S)-2-  
10 (3-benzoylphenyl)propionic acid in at least 93% enantiomeric excess, at 15-25% conversion.
3. A process according to claim 1, <sup>OR CLAIM 2</sup> wherein the biocatalyst is the microorganism Ophiostoma novo-ulmi, IMI 356050.
- 15 4. A process according to claim 3, wherein the alkyl ester has 1 to 3 carbon atoms.
5. A process according to claim 4, wherein the alkyl ester is an ethyl ester.
6. A process according to any preceding claim,  
20 wherein the reaction is conducted at pH 8 to 11.
7. A process according to any preceding claim, wherein the reaction is conducted at pH 2.5 to 10.5.



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A.I.P.P.I.

MARCO SPADARO  
Dr. Medicinal Chemistry

Milano, March 2, 1994

Your Ref.

Our Ref. SCB 215 PCT/em  
(to be indicated in the reply)

By DHL courier

Ladies and Gentlemen,

RE.: International application under the PCT  
claiming priority from British Patent  
application no.9304256.2 filed on March 3, 1993  
in the name of LABORATORIOS MENARINI S.A.

I inform you hereby that the text of the overmentioned application relates to a microorganism, which was filed at the International Mycological Institute, UK, on February 15, 1993, and was allotted accession number IMI 356050, as per the enclosed photocopy of the relative receipt.

Further, I state in advance that - according to Rule 28 (4) EPC - it is respectfully requested to restrict availability of the microorganism in question to experts only, nominated by the requester.

I would be grateful to receive a confirmation in this respect, and thank you very much in advance for your kind cooperation.

With best regards,

Yours sincerely,

*F. Minoja*

Fabrizio Minoja  
STUDIO CONSULENZA BREVETTUALE

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 94/00630

A. CLASSIFICATION OF SUBJECT MATTER		
C 12 P 41/00, C 12 P 7/40		
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)		
C 12 P		
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 practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	WO, A1, 93/04 189 (LABORATORIOS MENARINI) 04 March 1993 (04.03.93), claims 1-4.	1, 4, 5
X, P	WO, A1, 93/25 704 (LABORATORIOS MENARINI) 23 December 1993 (23.12.93), claim 1.	1
X, P	WO, A1, 93/25 703 (LABORATORIOS MENARINI) 23 December 1993 (23.12.93), claim 1.	1
A	EP, A2, 0 407 033 (RHONE-POULENC) 09 January 1991 (09.01.91), abstract.	1
<input type="checkbox"/> Further documents are listed in the continuation of box C. <input type="checkbox"/> Patent family members are listed in 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 document but published on or after the international filing date 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) 'O' document referring to an oral disclosure, use, exhibition or other means 'P' document published prior to the international filing date but later than the priority date claimed 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. '&' document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
24 June 1994		09. 08. 94
Name and mailing address of the ISA		Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 cpo nl. Fax (+ 31-70) 340-3016		WOLF e.h.

INTERNATIONAL SEARCH REPORT

-2-

International application No.  
PCT/EP 94/00630

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p style="text-align: center;">---</p> <p>EP, A1, 0 233 656 (GIST-BROCADES et al.) 26 August 1987 (26.08.87), abstract.</p> <p style="text-align: center;">----</p>	1

ANHANG

ANNEX

ANNEXE

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

to the International Search Report to the International Patent Application No.

au rapport de recherche international relatif à la demande de brevet international n°

PCT/EP 94/00630 SAE 87566

In diesem Anhang sind die Mitglieder der Patentfamilien der in obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

La présente annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visée ci-dessus. Les renseignements fournis sont donnés à titre indicatif et n'engagent pas la responsabilité de l'Office.

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
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ZA A 8700086	30-12-87		