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Resolution of (*R*,*S*)-flurbiprofen catalysed by dry mycelia in organic solvent

Patrizia Spizzo,[a] Alessandra Basso,[a] Cynthia Ebert,[a] Lucia Gardossi,[a,*] Valerio Ferrario,[a,b] Diego Romano[b] and Francesco Molinari[b]

[a]*Laboratory of Applied and Computational Biocatalysis, Dipartimento di Scienze Farmaceutiche, Università degli Studi di Trieste, Piazzale Europa 1, 34127 Trieste, Italy*
[b]*Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Sezione Microbiologia Industriale, Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy*

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Abstract—Mycelia of *Aspergillus oryzae* display high enantioselectivity towards (*R*)-flurbiprofen and can be efficiently used in pure organic solvent for the resolution of (*R*,*S*)-flurbiprofen through esterification. The use of the lyophilized mycelia facilitates the separation process so that in one step the two enantiomers of flurbiprofen, which are both valuable for pharmaceutical applications, can be easily separated. The biotransformation can be carried out in different apolar solvents using different primary alcohols as nucleophiles under very mild conditions.

1. Introduction

Flurbiprofen is a cyclooxygenase inhibiting non-steroidal anti-inflammatory drug (NSAID). Cyclooxygenase inhibiting activity resides primarily in the (*S*)-enantiomer, whereas the (*R*)-enantiomer has scarce anti-cyclooxygenase activity, but it has been found to inhibit tumour growth in various animal models.[1]

Industrially, (*R*)-flurbiprofen is obtained via the following steps: (i) synthesis of a suitable activated derivative of flurbiprofen, (ii) preparation of the corresponding amide of (*R*,*R*)-thiomicamine to obtain a diastereomeric mixture, (iii) second order asymmetric resolution and (iv) hydrolysis of the optically pure amide. The process leads to a 73% yield, although the preliminary production of (*R*,*R*)-thiomicamine has to be considered.[2]

Several studies concerning the biocatalyzed resolution of racemic flurbiprofen have been also reported in the recent years. The hydrolytic route has been pursued by starting from a suitable flurbiprofen ester and, in some cases, with the aid of chiral cyclodextrins that form selective inclusion complexes.[3,4] Novel lipases and esterases have been isolated and applied to the asymmetric hydrolysis of racemic flurbiprofen esters.[5,6]

Immobilized lipase B from *Candida antarctica* (Novozym 435®) has been reported to exhibit a relatively high enantiopreference towards the (*R*)-flurbiprofen and it has been applied to the flurbiprofen resolution via either esterification or transesterification carried out in organic media.[7–9]

In the past few years, the synthesis of ester bound has been reported by different microorganism used as dry mycelia.[10,11] Dry mycelia can show high enantioselectivity, stability to temperature and organic solvent. The cell structure may act as natural matrix that is able to protect the enzymes from the possible negative action of external agents.

In the present study we describe the use of dry mycelia in pure organic solvent in the resolution of (*R*,*S*)-flurbiprofen. Dry mycelia of moulds are a precious source of enantioselective enzymes with interesting economic and technological benefits, such as improved stability while avoiding costly and time consuming purifications.[10,12,13]

2. Results and discussion

A series of lyophilized mycelia of moulds have been screened in the enantioselective esterification of 10 mM (*R*,*S*)-flurbiprofen **1** with equimolar ethanol **2** in *n*-heptane at 50 °C (see Scheme 1 and Table 1).

Keywords: Mycelia; Flurbiprofen; Organic solvent; Enzymatic esterification.
* Corresponding author. Tel.: +39 0405583103; fax: +39 04052572; e-mail: gardossi@units.it

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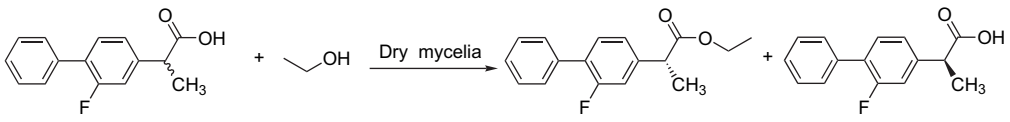


Scheme 1. Enantioselective esterification of (*R*,*S*)-flurbiprofen **1** with ethanol **2** catalysed by dry mycelia in toluene.

Table 1. Screening of enantioselective esterification of (*R*,*S*)-flurbiprofen **1** with ethanol **2** catalysed by dry mycelia in *n*-heptane[a]

Biocatalyst	Time (h)	Molar conversion (%)	ee (*R*)-flurbiprofen ethyl ester **3** (%)	*E*[b]
Aspergillus oryzae MIM	24	40	85	21
Aspergillus oryzae CBS 102.07	24	40	82	17
Rhizopus oryzae CBS 112.07	168	50	20	1.8
Rhizopus oryzae CBS 260.28	168	49	42	3.6
Rhizopus oryzae CBS 328.47	168	48	46	4.0
Rhizopus oryzae CBS 391.34	168	71	25	[c]

[a] Reaction conditions: 10 mM (*R*,*S*)-flurbiprofen **1**, 10 mM ethanol **2**, 30 g l^{-1} of dry mycelia, 50 °C, *n*-heptane.
[b] $E=\ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$, c=molar conversion, ee_p=enantiomeric excess of product.[14]
[c] Not determined, the above mentioned equation give reliable results except for low and high extents of conversion.[14]

The two strains of *Aspergillus oryzae* gave the best results in terms of rate and enantioselectivity. *A. oryzae* MIM was tested in various organic solvents differing in their polarity. Reactions were performed at 50 °C (Table 2).

Solvents leading to significant molar conversions were used to perform the esterification at 30 °C (Table 3) since it is known that enantioselectivity is inversely related to temperature.[15]

The most promising results in terms of enantioselectivity and also in terms of molar conversion were obtained in *n*-heptane, benzene and toluene solvents with values of log *P* comprised between 2 and 4 (Figs. 1 and 2).

Table 2. Screening of different solvents in the enantioselective esterification of (*R*,*S*)-flurbiprofen **1** with ethanol **2** catalysed by dry mycelium of *Aspergillus oryzae* MIM at 50 °C[a]

Solvent	Log *P*	Time (h)	Molar conversion (%)	ee (*R*)-flurbiprofen ethyl ester **3** (%)
Acetonitrile	−0.3	168	<5	—[b]
Ethanol	−0.2	168	<5	—[b]
Tetrahydrofuran	0.5	168	<5	—[b]
Diisopropyl ether	1.9	168	9	—[b]
Benzene	2.0	96	32	88
Toluene	2.5	96	28	84
n-Heptane	4.0	24	40	85
Isooctane	4.5	48	27	62
Pentadecane	7.5	48	20	65

[a] Reaction conditions: 10 mM (*R*,*S*)-flurbiprofen **1**, 10 mM ethanol **2**, 30 g l^{-1} of dry mycelia, 50 °C.
[b] Not determined.

Table 3. Screening of different solvents in the enantioselective esterification of (*R*,*S*)-flurbiprofen **1** with ethanol **2** catalysed by dry mycelium of *Aspergillus oryzae* MIM at 30 °C[a]

Solvent	Log *P*	Time (h)	Molar conversion (%)	ee (*R*)-flurbiprofen ethyl ester **3** (%)
Benzene	2.0	168	32	90
Toluene	2.5	168	36	92
Isooctane	4.5	48	26	90
n-Heptane	4.0	48	28	92
Pentadecane	7.5	168	15	62

[a] Reaction conditions: 10 mM (*R*,*S*)-flurbiprofen **1**, 10 mM ethanol **2**, 30 g l^{-1} of dry mycelia, 30 °C.

A second set of experiments was performed in an attempt to obtain the two enantiomers with higher volumetric yields since they both are of pharmacological relevance. Toluene was selected due to the higher solubility of (*R*,*S*)-flurbiprofen **1** in such solvent, as compared to *n*-heptane whereas benzene was discarded because of its toxicity. This allowed us to perform further experiments at 50 mM concentration of (*R*,*S*)-flurbiprofen **1**. A molar ratio of acid/alcohol of 1:0.55 was used to prevent the esterification proceeding much further than 50% conversion.

1-Octanol **4** (Scheme 2) was selected in order to increase the hydrophobicity of the produced ester, thus favouring the synthetic route of the reaction as compared to the hydrolytic one.[16,17]

Reactions were performed at 30 and 50 °C (Figs. 3 and 4) and data show how at 30 °C reaction kinetics are unacceptably low. This causes a low ee of the unreacted acid, although no significant effect of the temperature on the enantioselectivity of the biocatalyst was observed. As a matter of fact, at 40% conversion the ee of the (*S*)-flurbiprofen **1**-**S** was about 60% in both cases.

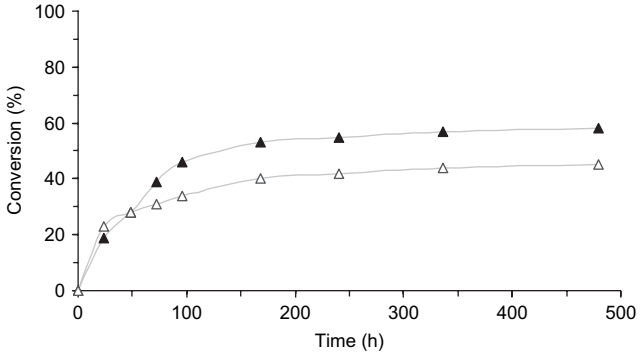


Figure 1. Molar conversion of the esterification of (*R*,*S*)-flurbiprofen **1** with ethanol **2** catalysed by dry mycelium (30 g l^{-1}) of *Aspergillus oryzae* MIM at 30 °C in *n*-heptane (▲) and in toluene (△).

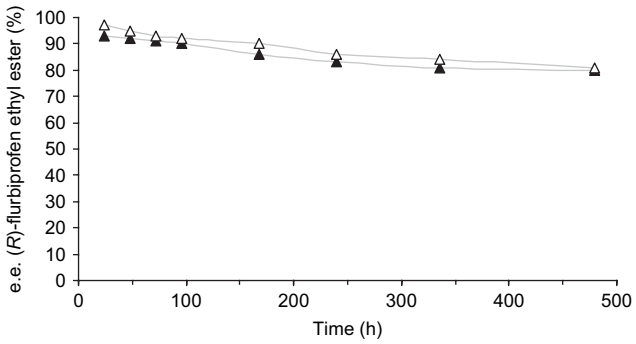


Figure 2. Enantioselectivity (ee of the product (*R*)-flurbiprofen ethyl ester **3**) in the esterification of (*R*,*S*)-flurbiprofen **1** with ethanol **2** catalysed by dry mycelium (30 g l^{-1}) of *Aspergillus oryzae* MIM at 30 °C in *n*-heptane (▲) and in toluene (△).

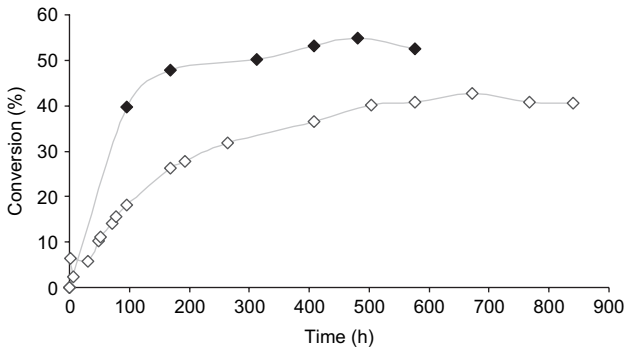


Figure 3. Molar conversion of the esterification of (*R*,*S*)-flurbiprofen **1** with 1-octanol **4** catalysed by dry mycelium (10 g l^{-1}) of *Aspergillus oryzae* MIM in toluene at 30 °C (◇) and 50 °C (◆).

At 50 °C, the esterification proceeds up to the maximum conversion (about 55%) with 84% ee of the unreacted (*S*)-flurbiprofen **1-S**. It is noteworthy that molar conversion was complete, although during the direct esterification water is produced. Moreover Figure 5 shows the production and ee of the (*R*)-flurbiprofen octyl ester **5** performed at 50 °C.

Recovery of the unreacted (*S*)-flurbiprofen **1-S** and its separation from the ester were easily accomplished by removing the biomass by centrifugation and extraction of the acid from the organic solvent.

Results obtained with the dry mycelia were finally compared with the data obtained using two commercial preparations of lipase. Figures 6 and 7 report conversions and enantiomeric excess obtained by using the lipase B from *C. antarctica* (CalB) in its native form (Chirazyme L-2) and as immobilized enzyme (Novozym 435®), at 50 °C.

Maximum conversion was achieved in all cases, but enantiomeric excess of the (*S*)-flurbiprofen **1-S** was lower (about 50%) than what observed with *A. oryzae* MIM (>80%). In the case of immobilized CalB, the reaction was reversible, with enantiomeric excess (ee) of the substrate reaching a maximum after 2 days, and progressively decreasing as the reaction progressed further. A possible effect of the polymeric support on the equilibrium and, therefore, on the overall enantioselectivity of the transformation can be ascribed to substrate adsorption as previously pointed out in the case of 4-methyloctanoic acid esterification with ethanol catalysed by Novozym 435®.[18] As a matter of fact, we have verified that, after the first day of reaction, about 5% of racemic (*R*,*S*)-flurbiprofen **1** is adsorbed by the support in the experimental conditions used for the enzymatic resolution.

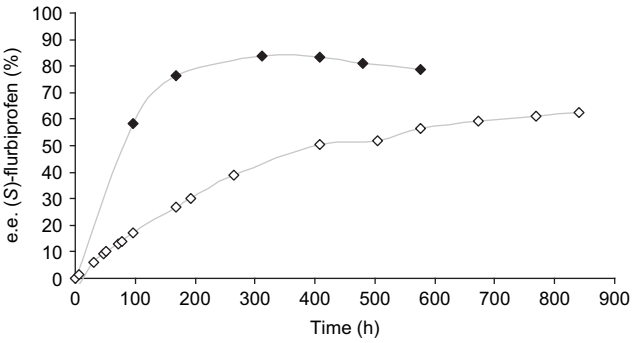


Figure 4. Enantioselectivity (ee of the residual (*S*)-flurbiprofen **1-S**) in the esterification of (*R*,*S*)-flurbiprofen **1** with 1-octanol **4** catalysed by dry mycelium (10 g l^{-1}) of *Aspergillus oryzae* MIM in toluene at 30 °C (◇) and 50 °C (◆).

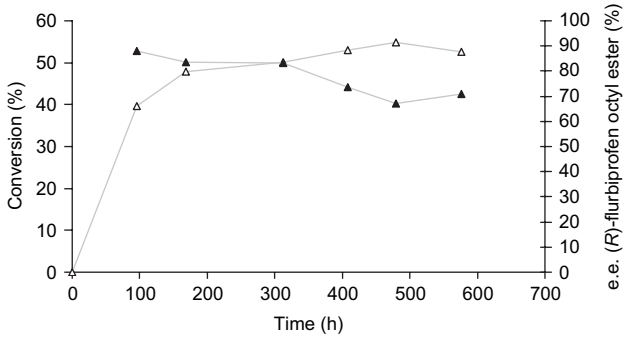
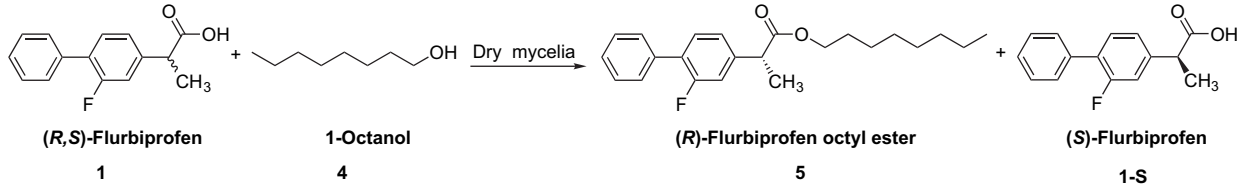


Figure 5. Conversion (△) and ee (▲) of the (*R*)-flurbiprofen octyl ester **5** in the esterification of (*R*,*S*)-flurbiprofen **1** with 1-octanol **4** catalysed by dry mycelium (10 g l^{-1}) of *Aspergillus oryzae* MIM in toluene at 50 °C.



Scheme 2. Enantioselective esterification of (*R*,*S*)-flurbiprofen **1** with 1-octanol **4** catalysed by dry mycelia in toluene.

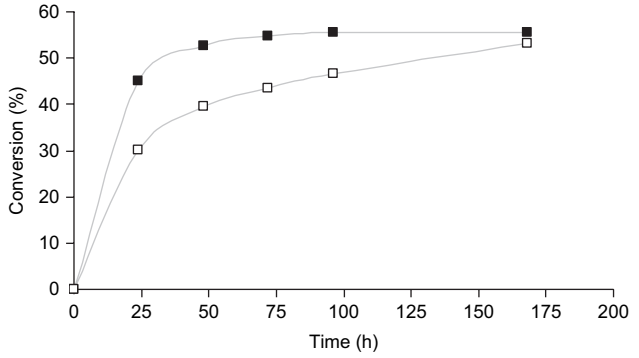


Figure 6. Molar conversion of the esterification of (*R*,*S*)-flurbiprofen **1** with 1-octanol **4** catalysed by immobilized CalB (Novozym 435®) (■) and native CalB (Chirazyme L-2) (□) in toluene at 50 °C.

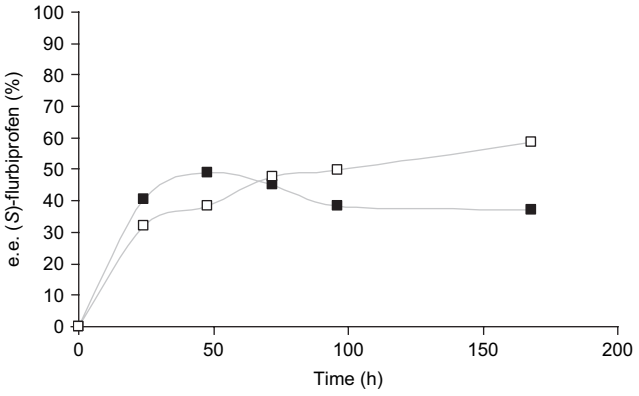


Figure 7. Enantioselectivity (ee of the residual (*S*)-flurbiprofen **1-S**) of the esterification of (*R*,*S*)-flurbiprofen **1** with 1-octanol **4** catalysed by immobilized CalB (Novozym 435®) (■) and native CalB (Chirazyme L-2) (□) in toluene at 50 °C.

3. Conclusions

Mycelia of *A. oryzae* are a valuable source of carboxylesterases that display high enantioselectivity towards (*R*)-flurbiprofen. The use of the lyophilized mycelium allows to separate in one step the two enantiomers, which are valuable for pharmaceutical application; recovery and separation of the products are easy and fast. The biotransformation can be carried out in different solvents (i.e., *n*-heptane or toluene) and with different primary alcohols (i.e., ethanol or 1-octanol) under very mild conditions.

4. Materials

4.1. Microorganisms

A. oryzae MIM (Microbiologia Industriale Milano), *A. oryzae* CBS 102.07 (Centraalbureau voor Schimmelcultures, Baarn, Holland), *Rhizopus oryzae* CBS 112.07, *R. oryzae* CBS 260.28, *R. oryzae* CBS 328.47, *R. oryzae* CBS 391.34 were used throughout this study and routinely maintained on malt extract (8 g l^{-1}, agar 15 g l^{-1}, pH 5.5). The microorganisms were cultured in 500 ml Erlenmeyer flasks containing 100 ml of medium and incubated for 48 h at 28 °C on a reciprocal shaker (100 rpm). The liquid media contained a basal medium (BM: Difco yeast extract 1 g l^{-1}, $(NH_4)_2SO_4$ 5 g l^{-1}, K_2HPO_4 1 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 g l^{-1} pH 5.8) added with Tween 80 (0.5%). Suspensions of spores (1.6×10^4) were used as inoculum. The microorganisms were also cultured in 10 l stirred tank reactor containing 2 l of medium at 28 °C, 200 rpm and aeration 1 vvm. Cells grown for 48 h in submerged cultures were harvested by filtration at 4 °C, washed with phosphate buffer (Kpi 0.1 M, pH 7.0) and lyophilized.

4.2. Enzymes and chemicals

Lipase B from *C. antarctica* (Chirazyme L-2) was obtained from Roche, while Novozym 435® was kindly donated by Novozymes.

All chemicals were purchased from Sigma–Aldrich and Fluka and were used without any further purification. Log *P* of solvents was from the literature.[19]

4.2.1. Resolution via esterification with ethanol 2. The esterifications of (*R*,*S*)-flurbiprofen **1** with absolute ethanol **2** were carried out by suspending the dried cells (30 mg ml^{-1}) in 2.5 ml of different organic solvents (acetonitrile, ethanol, tetrahydrofuran, diisopropyl ether, benzene, toluene, isooctane, *n*-heptane and pentadecane). Equimolar concentrations (10 mM) of acid (6.1 mg, 0.025 mmol) and alcohol (1.2 mg, 0.025 mmol) were added to the suspension and the reaction mixture was maintained in a thermostatted bath at 30 or 50 °C under constant magnetic stirring. Withdrawals of 100 μl were taken from the reaction mixture and analysed by gas chromatography. To each sample 100 μl of internal standard ((*R*,*S*)-flurbiprofen methyl ester, 2 mg ml^{-1}) was added and, after centrifugation, samples were injected in the gas chromatography.

The work-up of the microbial-catalysed esterification with ethanol is reported as an example: mycelium was removed by centrifugation and the organic solution was extracted twice with 4 ml of an aqueous NaOH solution (1%). The organic extracts were evaporated to give the crude ester, which was purified by flash chromatography (hexane/ethyl acetate, 7:3). The aqueous extracts were brought to pH 1.0 with an aqueous HCl solution (5%) and re-extracted with AcOEt and the organic extracts were dried over Na_2SO_4. The solvent was evaporated and the unreacted (*S*)-flurbiprofen **1-S** was purified by crystallisation.

4.2.2. Resolution via esterification with 1-octanol 4. Resolution of (*R*,*S*)-flurbiprofen **1** was carried out in anhydrous toluene (6 ml) at 30 and 50 °C by direct esterification of (*R*,*S*)-flurbiprofen **1** (73.3 mg, 50 mM, 0.3 mmol) with 1-octanol **4** (26 μl, 27.5 mM, 0.165 mmol) catalysed by whole cells (10 mg ml^{-1}, 0.043 U/ml, *p*-nitrophenyl palmitate units) of the dry mycelia of *A. oryzae* MIM under constant stirring (orbital shaker, 150 rpm).

Comparison between dry mycelia of *A. oryzae* MIM and native lipase B from *C. antarctica* (Chirazyme L-2) and immobilized lipase B (Novozym 435®) was carried out at 50 °C under the same experimental conditions using Chirazyme L-2 (1 mg ml^{-1}, 0.02 U/ml, *p*-nitrophenyl palmitate units) and Novozym 435® (35 mg ml^{-1}, 0.07 U/ml, *p*-nitrophenyl palmitate units), respectively, in anhydrous toluene.

4.3. Analytical methods

4.3.1. Resolution via esterification with ethanol 2. Molar conversions were determined by GLC carried out on a Carlo Erba Fractovap 2150 gas chromatograph equipped with hydrogen flame ionisation detector; the column temperature was kept at 190 °C. The column (4×1500 mm) was packed with OV-17. Retention time: (*R*,*S*)-flurbiprofen methyl ester 12.57 min, (*R*,*S*)-flurbiprofen ethyl ester **3** 15.32 min.

The absolute configuration of the ethyl ester was determined by comparison with the optical rotation of standards of the optically pure enantiomers obtained by esterification of optically pure (*S*)-flurbiprofen **1-S** with ethanol **2** using conventional esterification procedures.[20] The enantiomeric composition was determined by gas chromatography carried out on a Dani 6500 gas chromatograph equipped with hydrogen flame ionisation detector using a chiral capillary column at 160 °C (diameter 0.25 mm, length 25 m, thickness 0.25 μm, DMePeBeta-CDX-PS086, MEGA, Legnano, Italia). Retention time: (*R*)-flurbiprofen ethyl ester **3** 50.2 min, (*S*)-flurbiprofen ethyl ester 51.4 min. The stereochemical outcome of the transformations was expressed as enantiomeric excess (ee) of the major enantiomer.

4.3.2. Resolution via esterification with 1-octanol 4. Molar conversion (referred to (*R*,*S*)-flurbiprofen **1**) and enantiomeric excess (ee) of (*S*)-flurbiprofen **1-S** were evaluated by RP-HPLC (HPLC Gilson 321 system equipped with UV–vis Agilent DA and Gilson Dual length). Sample of 50 μl was diluted to 1 ml using the same mobile phase (acetonitrile or hexane) of the HPLC analysis and immediately analysed. Molar conversions were calculated by analysing the samples at 260 nm, using a Phenomenex Gemini 5μ C18 110A column (250×4.6 mm) with a gradient starting from 50% of acetonitrile for 23 min, then for 15 min with 100%. Trifluoroacetic acid (0.1% v/v) was added to the mobile phases and a flow of 1 ml min^{-1} was used. Retention times: (*R*,*S*)-flurbiprofen **1** 10.5 min, (*R*,*S*)-flurbirofen octyl ester **5** 33.6 min. Enantiomeric excess was calculated by analysing the samples at 260 nm using a Daicel Chiralpak® AD column (250×4.6 mm), with an isocratic elution with a mobile phase hexane/isopropanol=80:20 and with a 1 ml min^{-1} flow. Retention time: (*R*)-flurbiprofen 4.8 min, (*S*)-flurbiprofen **1-S** 6.3 min. The resolution factor (α) of the two enantiomers is 0.61.

4.4. Extraction of (*R*,*S*)-flurbiprofen 1 and of (*R*)-flurbiprofen octyl ester 5

Dry mycelia of *A. oryzae* MIM were removed by filtration and rinsed with toluene to recover eventually adsorbed acid and esters. After partial removing of toluene the organic pools were extracted with a saturated solution of $NaHCO_3$.

The organic phase, containing the (*R*)-flurbiprofen octyl ester **5**, was dried with Na_2SO_4, filtrated and the solvent was evaporated. A yellow oil was obtained (53 mg, 90%). The aqueous phase, containing the (*S*)-flurbiprofen **1-S**, was acidified to pH 2.0 with 6 N HCl. The extraction was carried out with diethyl ether. Organic phase was then washed with distilled water in order to solubilize the salts and finally dried with Na_2SO_4. After solvent evaporation a white solid was obtained (27 mg, 82%).

The (*S*)-flurbiprofen **1-S** and the (*R*)-flurbiprofen octyl ester **5** were characterized by ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, FTIR, mass spectrometry and polarimetry. ^{1}H and ^{13}C NMR spectra were registered with a Varian Gemini 200 spectrometer (200 MHz), the ^{19}F NMR spectra were recorded with a Bruker AC 200F (188 MHz). Chemical shifts were expressed in parts per million with respect to the tetramethylsilane signal for ^{1}H and ^{13}C NMR and Ar–CF_3 for ^{19}F NMR. Signal multiplicity is expressed as follow: s (singlet), d (doublet), dd (double doublet), t (triplet), br t (broad triplet), q (quartet), m (multiplet).

Infrared spectra were recorded on a JASCO FTIR-200.

Mass spectra, carried out with electron impact method, were registered at 70 eV using a ION TRAP GCQ FINNIGAN mass spectrometer. Optical rotations were measured on a JASCO DIP-1000.

4.4.1. (*S*)-Flurbiprofen 1-S. ^{1}H NMR ($CDCl_3$): δ 1.59 (d, 3H, J=7.3 Hz, CH_3), 3.85 (q, 1H, J=7.1 Hz, C*H*), 7.14–7.24 (m, 2H, *H*-Ph), 7.38–7.60 (m, 6H, *H*-Ph). ^{13}C NMR ($CDCl_3$): δ 18.2, 45.0, 115.3, 115.8, 123.8, 123.9, 127.9, 128.2, 128.5, 128.6, 129.1, 129.2, 131.0, 131.1, 135.6, 141.0, 141.1, 157.4, 162.4, 179.6. ^{19}F NMR ($CDCl_3$): δ −117.67 (dd, J_1=8.4 Hz, J_2=11.3 Hz). FTIR (Nujol): cm^{-1} 3000 (br), 1698, 1580, 1514, 1216, 765, 724, 698. EI ($CHCl_3$): *m*/*z* 244. $[\alpha]_D^{20}$ −1.46 (*c* 2.5, CH_2Cl_2).

4.4.2. (*R*)-Flurbiprofen octyl ester 5. ^{1}H NMR ($CDCl_3$): δ 0.88 (br t, 3H, J=6.6 Hz, CH_3), 1.30 (s, 10H, CH_2), 1.55 (d, 3H, J=7.3 Hz, CH_3), 1.62 (m, 2H, CH_2), 3.77 (q, 1H, J=7.2 Hz, C*H*), 4.20 (t, 2H, J=6.8 Hz, CH_2), 7.11–7.21 (m, 2H, *H*-Ph), 7.36–7.65 (m, 6H, *H*-Ph). ^{13}C NMR ($CDCl_3$): δ 14.7, 18.9, 23.3, 26.5, 29.2, 29.8, 30.4, 32.4, 45.8, 65.7, 115.6, 116.0, 124.0, 124.1, 128.2, 128.5, 128.8, 128.9, 129.4, 129.5, 131.2, 131.3, 136.1, 142.5, 142.6, 157.7, 162.7, 174.6. ^{19}F NMR ($CDCl_3$): δ −118.06 (dd, J_1=7.9 Hz, J_2=11.2 Hz). FTIR (Nujol): cm^{-1} 1738, 1583, 1514, 1216, 1177, 766, 724, 697. EI ($CHCl_3$): *m*/*z* 356. $[\alpha]_D^{20}$ −1.96 (*c* 2.5, CH_2Cl_2). ee at maximum conversion (55% conversion of (*R*,*S*)-flurbiprofen **1**): 69%.

Acknowledgements

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