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Microbiological Transformations 34: Enantioselective Hydrolysis of a Key-Lactone Involved in the Synthesis of the Antidepressant Milnacipran®

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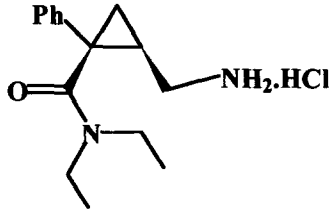
Abstract: The enantioselective hydrolysis of a key-lactone allowing for the synthesis of the antidepressant milnacipran® is described. Several biocatalysts were screened in order to achieve this biotransformation, the best results being obtained using whole-cells cultures of the fungi *Beauveria sulfurescens* and *Cunninghamella blakesleeana*.

Molecules endowed with antidepressant activity are of utmost importance on today drug market as illustrated by the recent success of Prozac® (fluoxetine).[1] Compound **1** ((Z)-1-phenyl-1-N,N-diethylamino carbonyl-2-aminomethyl cyclopropane, hydrochloride), a new molecule whose INN is milnacipran®, has been discovered to possess similar biological activities,[2] and is currently in phase III trials under racemic form.[3] However, it has been shown - as is very often the case for chiral biologically active compounds - that each enantiomer of this molecule showed different pharmacokinetic properties, and that its dextrorotatory (1*S*,2*R*) enantiomer (the eutomer) was two times more active than the racemate, the antipode (distomer) being less active. Thus, it was of high interest to devise an efficient procedure allowing for the synthesis of the most active enantiomer of **1** in high enantiomeric purity. We describe here the results we have obtained studying the enantioselective biohydrolysis of the racemic lactone **2**, the key building block used in the synthesis of milnacipran®.



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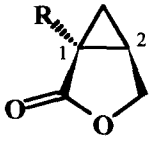
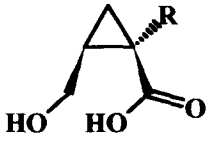


2 R = Ph

3 R = H



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If countless publications[4] concern hydrolysis of various esters using lipases or esterases, only few deal with hydrolysis of lactones[5-8] in spite of the fact that such chiral building blocks are of considerable interest for the synthesis of enantiomerically pure compounds.[9,10] In this context, Rousseau *et al.*[7] have previously described the highly enantioselective hydrolysis of **3**, a lactone structurally close to **2**, using Horse Liver Esterase. Thus, our first try was to test this enzyme on **2**. Unfortunately, no enantioselective hydrolysis of **2** occurred at all using the standard experimental conditions. One obvious explanation to this failure could have been that **2** is a white solid which appears to be very insoluble in water. Thus, we explored the possibility to achieve this biohydrolysis using different experimental conditions i.e. (i) to use this substrate under micronized form (ii) to achieve this reaction under biphasic conditions (iPr_2O or Toluene/water) or (iii) in solution in an organic solvent (iPr_2O or THF). Neither of these experimental conditions appeared to be operative using HLE.

Screening of 38 other commercially available hydrolases (9 esterases, 13 proteases and 16 lipases) was therefore performed using these diverse experimental conditions, and led to the conclusion that only two of them (i.e. *Bacillus* sp. esterase (Fluka) and cholesterol esterase from *B. sulfurescens* (Sigma)) were able to achieve - although quite slowly - the enantioselective hydrolysis of **2**. The results obtained are shown in Table 1 and indicate that the best - but rather disappointing - ee value obtained for the residual (-)-(*1S,2R*)-lactone was 52%.

Table 1: Hydrolysis of **2** by an esterase from *Bacillus* sp. or a cholesterol esterase

Enzyme	Medium	residual lactone ee (%)				
		5h	21h	48h	72h	96h
Esterase from *Bacillus* sp.	H_2O (pH 8)	8	13	**52**	28	26*
	iPr_2O/H_2O (pH8)	nd	15	**16**	nd	8
	Toluene/H_2O (pH8)	nd	7	nd	nd	6
Cholesterol esterase	H_2O (pH8)	0	0	4	30	36

* The hydroxyacid **4** ee was 21% ((+)-enantiomer)

Another strategy was to screen for microorganisms able to achieve this hydrolysis. Only some lactone hydrolases from bacterial origin were reported,[11,12] but most of them were shown not to hydrolyse γ-lactones. Thus, although, to the best of our knowledge, no fungal lactone hydrolase has been described up to now, we chose to explore the possibilities offered by using various fungi, since these microorganisms are known to contain a large variety of enzymes and to secrete surfactants able to partly solubilize lipophilic substrates. A total of 41 strains were assayed, and we were pleased to observe that 27 out of them did metabolize enantioselectively the key lactone **2**. Interestingly, 16 fungi hydrolyzed the dextrorotatory (*1R,2S*) antipode and

11 preferred to metabolize the levorotatory (1*S*,2*R*) enantiomer. Among these, *Beauveria sulfurescens* ATCC 7159 afforded the best results for the obtention of the residual (+)-lactone **2** (37 % analytical yield, 95 % ee), whereas *Cunninghamella blakesleeana* DSM 1906 led, after two days, to a 29 % analytical yield of the (-)-lactone **2** showing a 95 % ee. It is to emphasize that this levorotatory lactone is the precursor of the dextrorotatory - and biologically more active - enantiomer of milnacipran®. These ees were determined by chiral G.C. analysis of either the residual lactone or the lactone obtained after acid catalyzed cyclisation (TsOH, AcOEt) of the corresponding hydroxyacid **4**. The respective E values, calculated following Sih's equations[13] using the ee of the residual substrate **2** and of the formed hydroxyacid **4**, were respectively of 12 and 7, indicating a moderate enantioselectivity.

In order to achieve preparative-scale biotransformations using *Cunninghamella blakesleeana* DSM 1906, several parameters were determined. First, we checked the localization of the enzyme which proved to be intracellular, an interesting result which allowed us to achieve these reactions using a resting-cells methodology. Analytical studies carried out at different pH values indicated that the best enzymatic activity was observed at pH 8, that an optimum 2 g/L concentration of the substrate could be used with a cell suspension containing 32 g/L (dry weight) and that this biotransformation could be conducted in the presence of 1 % DMF as a cosolvent, in order to improve the solubility - and thus the rate - of the reaction.

Using these optimized conditions, 2 g (11.5 mM) of micronized lactone **2** were subjected to biohydrolysis. This led, after 41 hours, to a 32 % isolated yield (640 mg) of the remaining (-)-(1*S*,2*R*)-lactone **2** which showed an ee of 99 % ($[\alpha]_D^{20}$ = -85,3 (c = 2, EtOH)). Thus, this opens the way to the synthesis of the most potent enantiomer of milnacipran®. We also isolated 860 mg (48 % yield) of (+)-(1*R*,2*S*)-lactone **2** in 73 % ee obtained after cyclisation of the formed hydroxyacid **4**.

These results interestingly show that lactone hydrolases appear to be widely spread among fungi and that using a judicious choice of strains it is possible to prepare both enantiomers of the lactone **2** in high enantiomeric purity.[14] Nevertheless there still remains some work to be carried out in order to optimize this biohydrolysis and make it possible at an industrial scale.

Experimental

Chiral GC analysis - These were performed using a Lipodex® E chiral column (25 m × 0.25 mm, Macherey-Nagel - He, 1 bar, 160 °C); (-)-enantiomer t_R: 21 min; (+)-enantiomer t_R: 23 min.

Enzyme screening - In 5 mL vials (horizontal agitation, 120 strokes/min, 30 °C) - *Aqueous media*: 20 mg lactone **2** (29 mM), 4 mL phosphate buffer pH 7 or 8, 3-15 mg enzymatic preparation. (sampling: 100 µL reaction medium, 100 µL buffer pH 8, 500 µL AcOEt). *Biphasic media*: 3-15 mg enzymatic preparation, 1 mL phosphate buffer pH 8/ 1 mL iPr_2O or toluene, 10 mg lactone **2** (58 mM). *Organic media*: 3-15 mg enzymatic preparation, 15 mg lactone **2** (57 mM), 1.5 mL iPr_2O or THF. Organic layers were directly analyzed by chiral GC.

Fungi screening - 500 mL shake-flasks, 150 spm, 27 °C. *Medium*: Corn Steep Liquor 20 g/L, glucose 10 g/L, KH_2PO4 10 g/L, K_2HPO4 5 g/L. *Cultures*: 48 to 64 h. *Biotransformations*: 48 to 120 h. *Sampling*: 1 mL medium, 1 mL buffer pH8, 1 mL AcOEt containing octadecane (2 mM) as internal standard.

Preparative biotransformations - *Culture*: 7 L fermentor filled with 5 L of medium (100 g Corn Steep Liquor, 50 g glucose, 1 mL Pluronic PE 8100 (BASF), 0,25 mL Antifoam Silicon 426R (Prolabo)), 27 °C, 48 h. *Biotransformation*: After growing, cells were washed and suspended in 1 L of phosphate buffer (pH 8) in a 2 L fermentor. Lactone **2** (2 g in 10 mL DMF) is added directly. Residual lactone was extracted with toluene then, after acidification of the medium, the hydroxyacid was extracted continuously with CH_2Cl_2. Under these conditions, lactonisation took place spontaneously and the resulting lactone was analyzed by GC.

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