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Screening of Liver Acetone Powders in the Enantioselective Hydrolysis of Naproxen Esters

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Abstract. LAPs from different sources were used to hydrolyze the methyl and butyl esters of naproxen. The results revealed that no generalization can be done about the reaction conditions, since they greatly depend on the LAP source and the substrate. For example, in the hydrolysis of methyl naproxenate by rabbit LAP the conversion was 20% at pH 8, but the ee for the acid produced was 44%, in contrast to 80% ee at pH 7.5 with chicken LAP at the same conversion. In other hand cat LAP gave a conversion of 80% at all pHs and 90% ee at pH 7.5. The results for the butyl naproxenate hydrolysis were different; with pig LAP the conversion was 40% but only 34% ee, turkey LAP gave 12% conversion and 90% ee, and cat provided 17% conversion and 90% ee, all these results at pH 7.5. All LAP tested, except sheep, gave enantiopreference for the hydrolysis of the *R*-enantiomer
Key words: Naproxen, enzymatic resolution, liver acetone powder.

Resumen. Polvos acetónicos de hígado de diferentes fuentes fueron aplicados a la hidrólisis de los ésteres metílico y butílico del naproxén. Los resultados muestran que es difícil hacer generalizaciones respecto a las condiciones de reacción, ya que estas dependen significativamente de la fuente del LAP y del sustrato. Por ejemplo, en la hidrólisis del naproxenato de metilo por el LAP de conejo la conversión fue del 20% con un ee en el ácido producido del 44% a pH 8, en contraste a un 80% de ee a pH 7.5 con el LAP de pollo a la misma conversión. El LAP de gato dio una conversión del 80% en todos los pHs con un 90% de ee a pH 7.5. Los resultados para la hidrólisis del naproxenato de butílo fueron diferentes; con el LAP de puerco la conversión fue del 40%, pero solo dio 43% ee, el LAP de guajolote dio solo 12% de conversión y un 90% de ee y el LAP de gato dio 17% de conversión y 90% de ee, todos estos resultados a un pH de 7.5. Todos los LAPs probados, a excepción del de borrego, mostraron una preferencia hacia la hidrólisis del enantiómero *R*.
Palabras clave: Naproxen, resolución enzimática, polvo acetónico de hígado.

Introduction

Hydrolytic enzymes are the most employed biocatalysts in organic chemistry, they have become a common reagent in the organic laboratories, and without a doubt esterases and lipases are the most used hydrolases of all; they have been used to catalyze synthetically useful reactions such as ester hydrolysis, esterification, transesterification, intraesterification and transfer of acyl groups from esters to other nucleophiles such as amines, thiols and hydroperoxides [1]. Since the biological activity can differ for each enantiomer, enantioselective resolution using lipases has been highly applied to the preparation of very valuable chiral intermediates, for the synthesis of pharmaceuticals and agrochemicals [2]. For example (*S*)-Naproxen [2-(6-methoxy-2-naphtyl)-propionic acid] is 28-fold more active than the corresponding (*R*)-enantiomer, for this reason and because most regulations ask for warranting elimination of the unwanted enantiomer from the pharmaceutical formulations, there is an increasing need for efficient methods for the synthesis of optically pure products. Hence, (*R,S*)-naproxen is a suitable commercial candidate for the study of the application of crude hydrolytic enzymatic preparations, as liver acetone powders, for the resolution of chiral compounds.

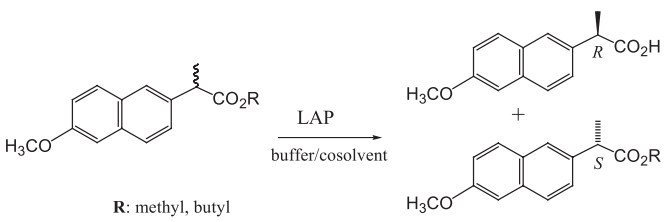
Since commercially available enzymes are expensive, liver acetone powders (LAPs) have been recommended as an easy to handle and low cost crude esterase source, for example LAPs can be used to carry out biotransformations involving carboxylic acids, esters and alcohols [3]. The use of LAPs has been extended to some industrial applications as demonstrated by the use of this methodology in patented procedures [4].

In this paper we describe the use of several LAPs for the resolution of naproxen esters, under different reaction conditions, in order to determine the influence of factors such as cosolvent, pH, temperature and chain length of the alkyl group, on their biocatalytic activity.

Results and discussion

Effect of the LAP source and pH of the reaction media

A great variety of liver enzymes have been used as biocatalyst, for example that from pigeon, cat, dog, eel, horse, calf, guinea pig, mouse, goat, chicken, sheep, seal, rattlesnake, trout, turtle, lungfish, salmon, and lemon shark [3, 5]. However, there is



R: methyl, butyl

Scheme 1

not a generalization about the catalytic properties of any of the biocatalyst in particular or even in groups. So, first we decided to do a screening of LAPs from different sources that could be able to carry out the naproxen ester hydrolysis. The LAPs tested were from mammals like, bovine, pig, rat, rabbit, cat, sheep; birds like chicken and turkey, and fishes like jurel, trout, croacker, caitipia mojarra and spanish mackerel. We tested the last ones despite the literature reports about their inefficiency in hydrolytic reactions [4c, 6].

As expected neither of the fish material showed any hydrolytic activity, at least in the reaction conditions used. Because we found hydrolytic activity only in the mammalian and birds livers, we decided to evaluate the effect of the pH of the reaction medium on the biocatalytic activity of those LAPs. The hydrolytic reaction was performed in 0.1M buffer solutions at the following pH values: 6.44, 7.04, 7.5 and 8.0. Since these were preliminary experiments, a 10% of dioxane was added as co-solvent to help the solution of the methyl naproxenate, and the reaction time was set at 24 h, at room temperature, regardless the extent of conversion. Regarding enantioselectivity, with the exception of the sheep material, all the LAPs have preference for the hydrolysis of the (*R*)-ester.

In graph 1 are shown the results of the evaluation of pH effect for each LAP source respect to the conversion percentage. The results indicated that the higher conversion percentage for all the LAPS were at pH 7, and the lower at pH 6.5. It is also noted that at pH 8.0, chicken, rabbit and cat LAPs gave the best conversions (close to 20%), whereas all the other LAPs and pHs gave conversions lower than 10%. In overall the best results were for the cat LAP with conversions near 20% for all pHs.

Graph 2 shows similar results to those in graph 1, but respect to the enantioselectivity of the process. Once again the best results were given for the cat LAP (84-90% ee), followed by the chicken (74-80% ee) and turkey (64-72% ee), at all pH values. It is noteworthy that, although the enantioselectivity was moderate, the sheep LAP showed preference toward the hydrolysis of the *S*-enantiomer leading to the biologically active product.

From the previous results it can be observed that there is not a general tendency to determine the pH for the best biocatalytic activity for all the LAPs, it seems that for each source the optimum conditions are specific, regarding the conversion and enantioselectivity. In summary from the above discussed is stated that the best results are for the cat LAP at pH 7.5, so we decided to continue our study with these reaction conditions.

It is worth to mention that a blank reaction for each pH and LAP source showed no conversion after 360 h, so we can conclude that the observed hydrolysis is a consequence of an enzymatic reaction.

Effect of the ester chain length

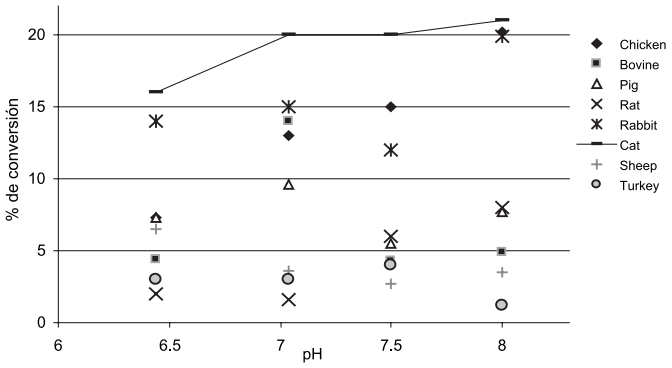
It is known that the length of the alcohol chain in an ester has an influence on the reactivity of these compounds during enzymatic transformation, so we evaluated the hydrolysis of the butyl naproxenate under the reaction conditions used to hydrolyze the corresponding methyl ester.

From Table 1 it can be observed that with pig LAP the reaction was faster using the butyl ester than with the methyl ester, also the enantioselectivity was better. In the case of cat LAP the results were very close to those for the hydrolysis of the methyl ester. For turkey there was an increase in enantioselectivity when the butyl ester was the substrate, with a moderate increase in the conversion.

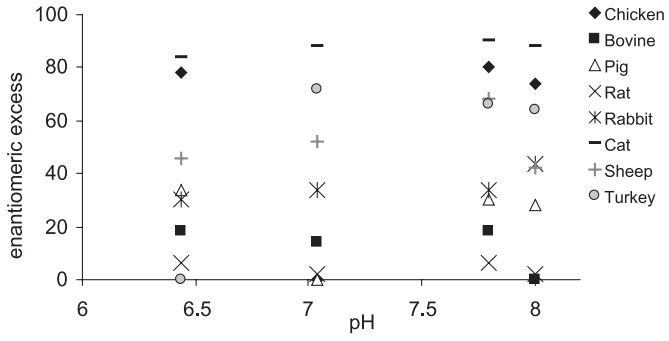
From the above results we decided to use cat LAP to study the effect of the other reaction parameters, because it showed the best results for the hydrolysis of the methyl naproxenate,.

Effect of temperature

We tested three reaction temperatures: room temperature, 45° C and 10° C, the temperatures were selected considering that in aqueous medium the enzymes are more sensitive to temperature. The results showed a very low conversion at 10º C, just 3% after 24 h; at 45° C the conversion was 14%, close to that



Graph 1. Effect of the LAP source and pH in the extent of hydrolysis of the methyl naproxenate.



Graph 2. Effect of LAP source and pH in the enantioselectivity of the hydrolysis of methyl naproxenate.

Table 1. Results of the hydrolysis of methyl and butyl naproxenate using different LAPs.

	Methyl ester				Butyl ester			
LAP	% conv[a]	%ee ester[b]	%ee acid[b]	E[c]	% conv[a]	%ee ester[b]	%ee acid[b]	E[c]
Pig	5.5	6	30	2	47	74	34	4
Sheep*	3	2	68	5	4	2	32	2
Chicken	15	34	80	12	3	6	84	12
Rat	6	4	6	1	5	6	36	2
Bovine	4	6	18	1	4	6.	50	3
Turkey	4	4	66	5	12	34	90	37
Cat	20	32	90	25	17	66	90	37
Rabbit	12	16	34	2	20	16	74	8

Reaction conditions: Phoshpate buffer (pH 7.5), r.t., 10% dioxane, 24 h.
* (S)-enantioselectivity
[a] Determined by GC
[b] Determined by HPLC
[c] E values are calculated using the program "selectivity" by K. Faber and H. Hoening, http://borgc185.kfunigraz.ac.at.

obtained at room temperature. With respect to the enantioselectivity of hydrolysis product, at lower temperature it was a little higher (92% ee), but not much better than the product obtained at room temperature (90% ee). On the other hand at 45° C the ee of the product dropped to 80%, so we decided to continue working at room temperature.

Effect of the co-solvent

In most of the reported LAPs works [6], mainly from Basavaiah [7], the optimum reaction conditions recommended are in a two-phase system, preferentially using ethyl ether as organic solvent mixed with a phosphate buffer solution (at pH 8); although others solvents as DME [6a], CH_2Cl_2 [6d], MTBE [6e], toluene [6b] and cyclohexane [6c] have been also used as the second phase. It is interesting to note that co-solvents, meaning water-miscible solvents, are used in most of the patent applications [8], but not in journal reports.

With this contradictory information about the use of co-solvents or two-phases solvent systems and from our preliminary experiments where we used dioxane as co-solvent [9], we decided to explore the effect of different relative proportions (from 10 to 40%) of this organic, water-miscible solvent, on the reaction and the results are shown in Table 2.

From the results in Table 3 it can be observed that an increase in the amount of co-solvent conduce to a significant drop in the extent of the reaction, specially after 30% (v/v). When the dioxane was present at a concentration of 20% there was a 30% conversion, but there was a significant drop in enantioselectivity, from E=25 to 8.

Since it is necessary the use of a co-solvent because the low solubility of the naproxen esters in water, there was the idea to evaluate other water-miscible solvents, besides the dioxane used initially. The scarce information about the use of water-miscible co-solvent [10], prompted us to use acetonitrile

Table 2. Effect of the co-solvent proportion (dioxane)

	% conv.[a]	%ee ester[b]	% ee acid[b]	E[c]
10%	20	32	90	25
20%	30	20	72	8
30%	14	6.0	60	4
40%	6.0	4.0	30	2

Reaction conditions: Cat LAP, 24 h, rt, phosphates buffer (pH 7.5)
[a]: Determined by GC
[b]:Determined by HPLC
[c]: E values are calculated using the program "selectivity" by K. Faber and H. Hoening, http:// borgc185.kfunigraz.ac.at.

(AcCN), dimethylsufoxide (DMSO), *N,N*-dimethylformamide (DMF), tetrahydrofuran (THF), methanol (MeOH), and isopropyl ether (*i*PrE), although the last one is water-immiscible, is soluble enough to not form a second phase at this concentration, all of them were used at 10% (v/v). The results are shown in Table 3.

From Table 3 the best result is given by DMSO as co-solvent, in both conversion (43%) and enantioselectivity (E=84), followed by AcCN, DMF and dioxane, the worst being isopropyl ether. As stated before the literature reports [6, 7] indicate that the best conditions for this kind of hydrolytic reactions are in two-phase systems, but in our hands, using a 1:1 ratio of buffer solution (pH 7.5)/isopropyl ether, there was practically no conversion, at the reaction time set in our study (24 h).

Effect of pig LAP/substrate ratio

In Table 4 are displayed the results of the effect of different ratios pig LAP/substrate (w/w) on the reactivity and enantiose-

Tabla 3. Effect of the different co-solvent in the reaction.

	Cat LAP				
	Co-solvent (10%)	conv. (%)[a]	%ee ester[b]	% ee acid[b]	E[c]
	Dioxane	20	32	90	25
	AcCN	30	32	94	44
	DMSO	43	54	96	84
	DMF	18	10	90	20
	THF	4	4	74	7
i	PrE	10	4	6	1
	MeOH	24	24	72	8

Reaction conditions: phosphate buffer (pH=7.5), r.t., 24 h.
[a]: Determined by GC
[b]: Determined by HPLC
[c]: E values are calculated using the program "selectivity" by K. Faber and H. Hoening, http:// borgc185.kfunigraz.ac.at

Table 4 Effect of different ratios pig LAP/Substrate on the hydrolysis of butyl naproxenate.

LAP (w/w)	conv. (%)[a]	%ee ester[b]	% ee acid[b]	E[c]
0.25:1	8.0	24	99	251
0.5:1	24	76	92	55
1.5:1	51	74	80	19
2.5:1	65	74	64	10
5:1	49	72	70	12

Reaction conditions: buffer pH 7.5, r.t.,10% DMSO, pig LAP, 5 h
[a]: Determined by GC
[b]: Determined by HPLC
[c]: E values are calculated using the program "selectivity" by K. Faber and H. Hoening, http:// borgc185.kfunigraz.ac.at

lectivity of the butyl ester hydrolysis. From Table 4 is evident that increasing the amount of biocatalysts the rate of reaction is increased, but this brings a dramatic drop of enantioselectivity, being the best results between 0.25-0.5:1 LAP/substrate ratio (w/w).

In conclusion, although the vast information about the use of LAPs there is not possible to make a generalization of the reaction conditions or parameters that affect their use, it seems that the optimization of the reaction conditions has to be done for each particular case. We found that the reaction in two-phases did not work, as mentioned by Basavaiah. The recommendation seems to us awkward, because it has been proposed that the hydrolytic activity of LAPs is due to an esterase [11], and this enzyme should work without the so called "interfacial activation" [12], which it is favoured under two-phase reaction conditions. Besides, DMSO probed to be the solvent that gave the best enantioselectivities, with regard to the length of the alkyl chain, a longer one let to have better enantioselectivities, meaning that with the butyl ester the hydrolysis, in general, was better than with the methyl ester.

Experimental

The different livers were purchased from local market or donated from University animal facilities. *n*-Butanol was analytical grade, methanol and hexanes were HPLC grade (Tecsiquim, México). ^{1}H-NMR spectra were determined in $CDCl_3$ (Aldrich) using a Varian Mercury VX 400 MHz spectrometer, with TMS as internal standard. Infrared spectroscopy was determined in a Paragon 1000 spectrometer (Perkin-Elmer) as a film or KBr disk. The reactions were monitored by GC using an Agilent Chromatograph Mod. 6890 with a HP-5 column (Agilent Technologies) and a FID detector, injector temperature at 250°, nitrogen was the carrier gas, at 1 mL/min. The reactions were monitored by HPLC using an Agilent 1100 Chromatograph, with a Chiracel OD column (Chiral Technologies), with detection at 260 nm, with hexanes (0.25% trifluoroacétic acid)/isopropyl alcohol, 95:5 as eluent, at 25° C and at 0.8 mL/min.

Liver acetone powder (LAP) preparation. In a blendor vessel were added approximately 250 g of the liver and covered completely with acetone, the mixture was grounded at high speed. The brown mash was filtered and the residue subjected to the same process twice; the filtrate was discharged. The solid residue was left in the fume hood for complete evaporation of the residual acetone, yielding a light brown fine powder. The crude material was kept in tightly close jars in the freezer (4° C).

General procedure for ester preparation: Racemic naproxen (1 g) was dissolved in the corresponding alcohol (methanol or butanol, 5 mL), stirred and then thionyl chloride (1 mL) was slowly added. The reaction mixture was stirred at room temperature for 24h. After that time the mixture was diluted with 20 mL of hexane and cooled for complete crystallization.

Methyl ester: white powder; yield: 87%; mp: 64-65° C; IR n 2976, 1738, 1605, 1201 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 7.70 (d, *J* = 8.6 Hz, 2H), 7.65 (d, *J* = 2.0 Hz, 1H), 7.39 (dd, *J* = 2.0, 8.6 Hz, 1H), 7.14 (d, *J* = 2.8 Hz, 1H), 7.11 (dd, *J* = 2.8, 6.8 Hz, 1H), 3.90 (s, 3H), 3.87 (q, *J* = 7.6 Hz, 1H), 3.66 (s, 3H), 1.57 (d, *J* = 6.8 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 174.7, 157.2, 135.3, 133.3, 128.9, 128.6, 126.8, 125.9, 125.6, 118.7, 105.3, 55.1, 51.9, 45.2, 18.5; HPLC: R_t: 7.0 (*R*-) and 7.7 min (*S*-); GC: R_t 4.3 min.

Butyl ester: white powder; yield: 57%; mp: 50-52° C; IR ν 2954, 1726, 1606, 1193 cm^{-1}; ^{1}H-NMR ($CDCl_3$) : δ 7.69 (d, *J* = 8.4, 2H), 7.66 (d, *J* = 1.6 Hz, 1H), 7.40 (dd, *J* = 1.6, 8.4 Hz, 1H), 7.14 (d, *J* = 2.4 Hz, 1H), 7.11 (dd, *J* = 1.6, 5.6 Hz, 1H), 4.08 (m, 2H), 3.91 (s, 3H), 3.84 (q, *J* = 7.6 Hz, 1H), 1.57 (d, *J* =

7.6 Hz, 3H), 1.55 (m, 2H), 1.28 (m, 2H), 0.85 (t, J = 7.2 Hz, 3H); ^{13}C-NMR ($CDCl_3$): δ 174.3, 157.2, 135.5, 133.3, 128.9, 128.6, 126.7, 125.9, 125.6, 118.6, 105.2, 64.5, 55.1, 45.5, 30.5, 19.0, 18.5, 13.6; HPLC: R_t 6.0 (*R*-) and 6.5 min (*S*-); GC: R_t 8.3 min.

General procedure for enzymatic hydrolysis: In a 30 mL vial, the racemic ester (100 mg) was dissolved in dioxane (1.5 mL), then phosphate buffer (13.5 mL) was added under stirring, followed by the corresponding LAP (100 mg). The reaction mixture was magnetically stirred at room temperature for 24h. The reaction mixture was filtered over celite, and washed three times with dichloromethane (5 mL). The phases were separated and the aqueous layer was extracted three times with dichloromethane (5 mL); the combined organic extracts were dried over sodium sulphate, filtered and evaporated. The crude product was analyzed by GC and HPLC.

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References

1. Patel, R. N. *Stereoselective biocatalysis* Marcel Dekker Inc., Ed. New York: 2000.
2. a) Patel, R. N. *Adv. Synth. Catal.* **2004**, *343*, 527-546; b) Ikunaka, M. *Catalysis Today* **2004**, *96*, 93-102. c) Leuenberger, H.G.W. in: *Microbial Reagents in Organic Synthesis*, Servi, S., Ed., Kluwer Academic Publishers, Netherland, **1992**, p.149-158.
3. Some references on the applications of LAPs. a) Basavaiah, D.; Rama Krishna P.; Bharathi, T.K. *Tetrahedron Lett.* **1990**, *31*, 4347-4348; b) Basavaiah, D.; Bhaskar Raju, S. *Tetrahedron.* **1994**, *50*, 4137-4148; c) Basavaiah, D.; Dharma Rao, P. *Synth. Comm.* **1994**, *24*, 925-929; d) Comini, A.; Forzato, C.; Nitti, P.; Pitacco, G.; Valentin, E. *Tetrahedron: Asymmetry* **2004**, *15*, 617-625; Basavaiah, D.; Rama Krishna, P. *Pure & Appl. Chem.* **1992**, *64*, 1067-1072.
4. Some patent applications of LAPs. a) Holton, R.A.; Vu, Ph. *US patent 6,548,293* **2003**; b) Goswami, A. *US patent 5,281,534* **1994**; c) Liu, K.K. *US patent 6,828,134* **2004**; d). Flavin, M.T; Xu, Z-Q; Khilevich, A.; Rizzo, J.D; Chen, W.; Lin, L.; Kucherenko, A.; Sheinkman, A.K;. Boulander, W.A *US patent 6,043,271* **2000**; e) Raju, M.S.; Huh, N. *US patent 5,348,973* **1994**.
5. a). Liu, K.K *US Patent* 6,828,134 **2001**; b) Raju, M., Huh, N. *US Patent* 5,348,973 **1974**; c) Goswani, A. *US Patent* 5,281,534 **1994**.
6. a) Holla, W. *US Patent* 6,406,912 **2000**; b) Holton, R.A. *US Patent* 6,548,293 **2000**; c) Iding, H., Wuirz, B., Zutter, V. *US Patent* 6,518,048 **2003**; d) Liu, K.K. *US Patent* 6,828,134 **2004**; e) Zard, L., Tixidre, A. *US Patent* 5,677,168 **1997**.
7. a) Basavaiah, D.; Rama Krisna, P.; Bharathi, T.K. *Tetrahedron Lett*. **1990**, *31*, 4347-4348; b) Basavaiah, D., Rama Krisna, P. *Indian J. Chem.* **1973**, *32*B, 131-134; c) Basavaiah, D., Bhaskar Raju, S. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 955-958.
8. a) Jogham, S.; Koening, J.J.; Puech, F.; Burnier, P.; Zard, L. *US Patent* 5,641,785 **1995**; b) Crosby, J.; Pittam, J.D.; Halt, R. *US Patent* 6,261,830 **2000**; c) Chiarello, J.F., Buckwalter, B.L.; Barden, T.C. *US Patent* 6,770,463 **2004**; d) Ghorpade, S.R.; Kalkote, U.R.; Chavan, S.P.; Bhide, S.R.; Ravinidranathan, R. *US Patent* 6,417,374 **2001**; e) George, P.; Froissant, J.; Tixidre, A. *US Patent* 5,244,901 **1993**; f) Comini A.; Forzato, C.; Nitti, P.; Pitacco, G.; Valentin, E. *Tetrahedron: Asymmetry* **2004**, *15*, 617-625; g) Felluga, F.; Fermeglia, M.; Ferrone, M.; Pitacco, G.; Priel, S.; Valentin, E. *Tetrahedron: Asymmetry* **2002**, *13*, 475-486.
9. Sánchez, R.; Luna, H.; Pérez, H. I.; Manjarrez, N.; Solís, A. *Tetrahedron: Asymmetry*. **2001**, *12*, 1399-1401.
10. a) Goswami, A. *US Patent* 5,281,534 **1994**; b) Holla, W. *US Patent* 6,406,912 **2000**; c) Yoshida, N.; Sugiura, T.; Koizumi, Y. *US Patent* 5,518,903 **1996**.
11. a) Seebach, D.; Eberle, M. *Chimia* **1986**, *40*, 315-318; b) Basavaiah, D.; Dharma Rao, P. *Synth. Commun*. **1994**, *24*, 925-929.
12. Faber, K. *Biotransformations in Organic Chemistry* Springer-Verlag, 4Th Ed., Berlin, 2000.