Stereoselectivity of Chemically Modified α-Chymotrypsin and Immobilized Lipases

José Vicente Sinisterra,* Emilio Fidel Llama, Carmen del Campo, María José Cabezas, José María Moreno and Miguel Arroyo
Organic & Pharmaceutical Chemistry Department, Faculty of Pharmacy, Universidad Complutense, Madrid 28040, Spain

The chemical modification of α-chymotrypsin by monomethoxypolyethylene glycol and the immobilization of lipases from *Candida rugosa* and *C. antarctica* on several supports changes the enantioselectivity of the enzymatic derivatives compared with the behaviour of the native enzymes. Hydrolysis and synthesis of esters have been used as reaction tests. This alteration could be related to the rigidification of the 'h' subsite of the enzyme by the effect of those processes. Technical variables such as time, temperature, stirring speed *etc.* do not alter the observed enantioselectivity. The presence or absence of water changes slightly the enantioselectivity in the synthesis of esters catalysed by immobilized lipases in dry isooctane.

The immobilization or the chemical modification of enzymes changes the microenvironment and the tertiary structure of the protein compared with its native state. This fact can change the conformer or the configuration of the substrate that is recognized by the protein, changing the enzymatic activity and the enantioselectivity of some enzymes such as proteases[1] or lipases.[2] This fact could be related to the slight alterations of structural parameters—which control the binding of substrates to an enzyme and its catalytic activity—caused by the effect of those methodologies.

In this way, we have described the alteration of the chemoselectivity of chemically modified α-chymotrypsin (α-CT) in the synthesis of peptides or in the hydrolysis of esters of amino acids[3,4] showing that the native and the modified enzyme do not use the same conformer of the ester of the amino acid to carry out the kinetically controlled peptide synthesis or the hydrolysis of these esters. Sih *et al.*[5] have reported similar effects in the case of lipase of *Candida rugosa* (LCR) by the effect of selective modifications of internal residues using several modifying agents. Similar observations have been described for the immobilized enzymes.[6]

In order to analyse if the chemical modification or the immobilization methodologies described previously by us[7,8] could change the stereoselectivity of the enzymes, two different enzymatic derivatives were analysed: (*i*) chemically modified α-chymotrypsin with monomethoxypolyethylene glycol with respect to the native enzyme (PEG-CT). (*ii*) Lipases from *C. rugosa* (LCR) and *C. antarctica* (LCA) immobilized on several supports with respect to the native enzyme.

Three reactions have been chosen as reaction test: (*i*) enantioselective hydrolysis of (*R,S*)-*N*-benzoylphenylalanine methyl ester in the case of PEG-CT. (*ii*) Enantioselective hydrolysis of (*R,S*)-2-phenylpropionic acid ethyl ester [lipase *C. rugosa* (LCR)]. (*iii*) Enantioselective esterification of (*R,S*)-ibuprofen [(*R,S*)-2-(4-isobutylphenylpropionic acid)] with propanol [lipase *C. antarctica* (LCA)].

The first reaction test allows us to explore the ar, am and h subsites both in the native and chemically modified enzyme.[9,10] The hydrolysis of the esters of (*R,S*)-2-phenylpropionic acid and the enantioselective synthesis of the ester of ibuprofen let us analyse the steric parameters of the active site in the immobilized lipases—both in hydrolysis and in synthesis—and, furthermore, compare these parameters with those from native lipases whose active site has been described such as lipase of *Pseudomonas cepacia*[11,12] and other lipases.[13]

Results and Discussion

Hydrolysis of Esters Catalysed by Proteases.—In order to analyse if the chemical modification changes the stereoselectivity of the α-CT, the hydrolysis of (*R,S*)-*N*-benzoylphenylalanine methyl ester was carried out.

The acid, obtained in the reaction, was extracted as described above and analysed by two methods:

(*a*) Polarimeter (Perkin-Elmer 8012) in MeOH as solvent. The obtained values for α were: $[\alpha]^{25} = -0.106$, (c = 10.3 mg cm^{-3} MeOH) in the hydrolysis catalysed by PEG-CT. $[\alpha]^{25} = -0.02$, (c = 4.3 mg cm^{-3} MeOH) in the hydrolysis of the (*R,S*)-*N*-benzoylphenylalanine methyl ester catalysed by the native α-CT.

From these results we can conclude that the acids obtained in the hydrolysis of the ester of *N*-benzoyl-L-phenylalanine using modified and native enzyme is levogyrous. Nevertheless, a greater enantiomeric excess (e.e.) seems to be present in the case of the acid obtained with PEG-CT than in the acid obtained with native enzyme (α-CT). This result agrees with the results obtained by Zerner *et al.*,[14] which report a moderate stereoselectivity *vs.* the (L) enantiomer in the case of the hydrolysis of esters of amino acids catalysed by native α-CT.

(*b*) ^{1}H NMR spectra. The values of the enantiomeric excess of the acids produced in the hydrolysis or remaining after the esterification were analysed by ^{1}H NMR spectroscopy using (*R,R*)-1,2-diphenylethane-1,2-diamine (0.08 mol dm^{-3}) as chiral agent. This compound complexes the acid in a molar ratio amine–acid = 1:2, shifting the *S*-enantiomer signals of CH_2 to higher δ values than the ones of the *R*-enantiomer.[15] The ^{1}H NMR spectra of the complexes are shown in Fig. 1.

We can observe that the hydrolysis catalysed by PEG-CT (Scheme 1) practically produces only one isomer (*S*) [because two clear quartets are observed, Fig. 1(*b*)]. The enantiomeric ratio was (*S*:*R*) = 98:2 as was calculated from the integral curve using the calculus program of the apparatus. The native α-CT gives both enantiomers [Fig. 1(*c*)], and we obtained a ratio (*R*:*S*) = 50:50 from the integral curve. These results agree with those obtained in the polarimeter. Therefore we can conclude that the chemical modification increases the enantioselectivity of the enzyme.

One explanation for this observation could be deduced that PEG-CT has a more rigid active site than the native α-CT, because only the interaction described by the conformer **I**—reported by Hansch *et al.*[16] for the (*S*)-isomer—is reasonable, the only conformation used in the interaction of the ester with

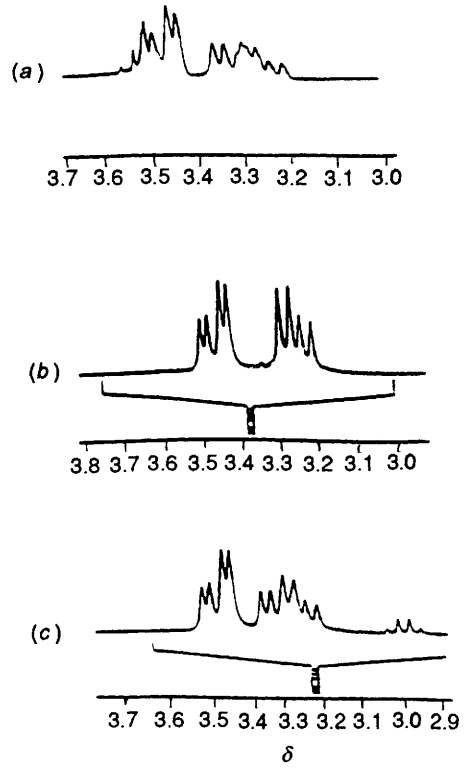
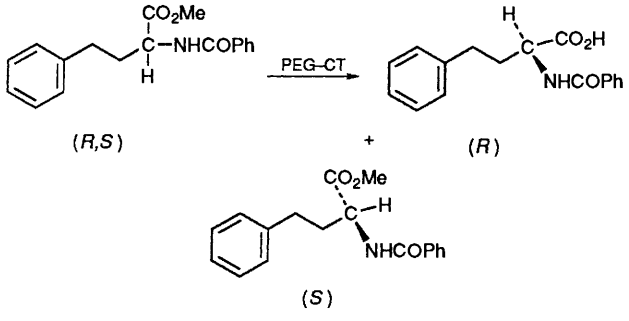


Fig. 1 ^{1}H NMR spectra of *N*-benzoylphenylalanine with (*R*,*R*)-1,2-diphenylethane-1,2-diamine. (*a*) Complex of racemic *N*-benzoyl-(±)-phenylalanine, (*b*) *N*-benzoylphenylalanine produced in the hydrolysis of the ester by PEG-CT, (*c*) *N*-benzoylphenylalanine produced in the hydrolysis of the ester by native α-CT.

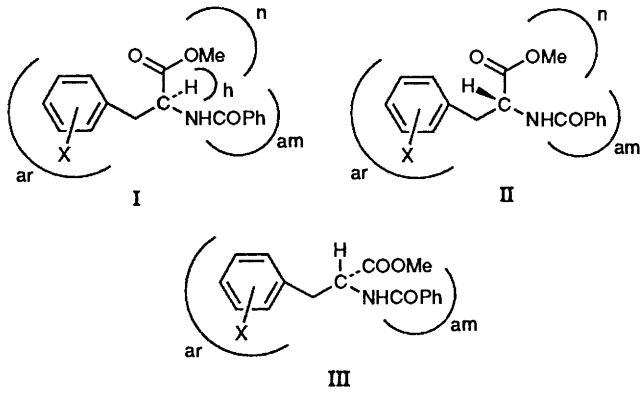


Scheme 1

the enzyme. The interactions described by the conformers **II** and **III**[16] leading to the hydrolysis of the (*R*)-enantiomers would not be useful in the case of PEG-CT.

These results can be explained by assuming a strong rigidification of the h subsite (by the effect of this chemical modification) that cannot accept another group different from hydrogen, and so, only (*S*)-ester is hydrolysed by PEG-CT.

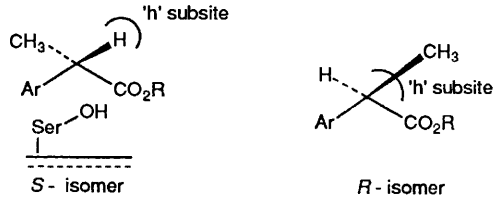
Hydrolysis of Esters Catalysed by Lipases.—A similar qualitative behaviour has been observed in the hydrolysis of (±)-2-phenylpropionic ethyl ester using LCR immobilized on agarose or alumina. In Fig. 2 we show the ^{1}H NMR spectrum of the complex formed between the hydrolysis products in each hydrolysis and (*R*,*R*)-1,2-diphenylethane-1,2-diamine. The samples were obtained in the hydrolysis of the (±)-ethyl 2-phenylpropionate using native LCR (47%); immobilized LCR on agarose (48%) and on alumina (48%). The methyl groups of both stereoisomers are not equivalent in the presence of the amine, and so, two doublets are observed in the presence of the (*R*,*R*)-1,2-diphenylethane-1,2-diamine. We can see in the case of native LCR two broad bands that can be resolved using a



deconvolution program in two doublets. In this case 70% *S*-(+) and 30% *R*-(−) were obtained (e.e. 40%). This value is very similar to that obtained with the same enzyme and (±)-methyl 2-chloropropionate (e.e. 31%) and the one described for this compound by Sih *et al.*[5] (e.e. 30–40%).

Nevertheless both immobilized enzymes are more stereoselective because only one clear doublet is obtained in these cases. This doublet resonates at lower frequency than in the absence of chiral amine. Therefore we could conclude that only the *S*-(+) isomer is produced with the immobilized enzymes.

This improvement in the stereoselectivity of the LCR by the effect of the immobilization must be related to an alteration of either the conformation that interacts with the immobilized enzyme or the microenvironment of the immobilized enzyme produced by the immobilization process. This effect is being studied in our group. A similar situation has been reported by Sih *et al.*[5] in the selective modification of LCR using several selective modifiers. Therefore we can assume—as in the case of the chemically modified α-CT—that the immobilization process produces a conformational change in the active site that makes the h subsite more rigid. Thus, it only accepts a hydrogen atom and not the methyl group, and the enantioselectivity increases significantly.



Finally we have analysed if the variation in the experimental conditions could affect the stereoselectivity of lipases in the hydrolysis and in the synthesis of esters. In this way we have used LCR immobilized on alumina and LCA adsorbed on anionic resin. The first enzymatic derivative was used in the stereoselective hydrolysis of (*R*,*S*)-ethyl 2-phenylpropionate and the second one in the stereoselective synthesis of (*R*)-ibuprofen propyl ester (Scheme 2).

The obtained results in the first case are shown in Table 1. The e.e. values were obtained by ^{1}H NMR spectroscopy as described above. We can observed a high enantioselectivity when the conversion is near 50% (entries 1–4). This value diminishes when the conversion increases. We can also observe that the experimental variables do not seem to influence the enantiomeric excess.

In the second case (Table 2), we observe that only the addition of water to the organic medium seems to increase the enantiomeric excess of the acid that remains unreacted, but only to a very small extent. This result does not agree with the data reported by Carrea *et al.*[17] who did not observe a large

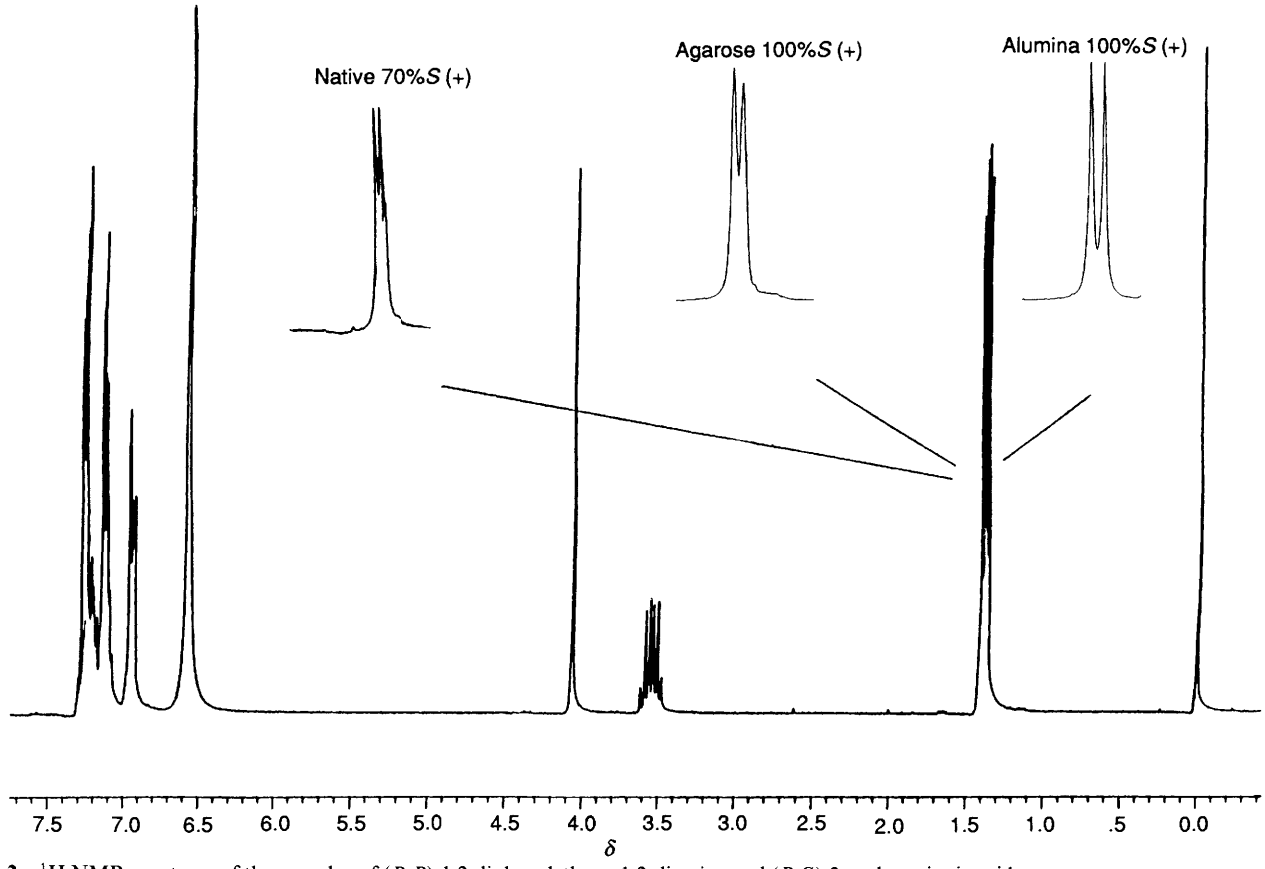
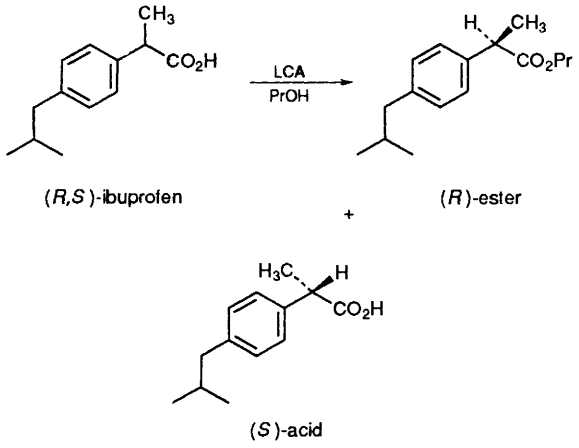


Fig. 2 ^{1}H NMR spectrum of the complex of (*R*,*R*)-1,2-diphenylethane-1,2-diamine and (*R*,*S*)-2-arylpropionic acid



Scheme 2

modification of the enantioselectivity of the native lipase of *P. cepacia* with the variation of the water activity. The small positive effect observed in our case can be explained by the drastic variation of the amount of water used by us: 0.0 or 300 mm^3. This variation is different to that used by Carrea *et al.*[17] ($0.1 < a_w < 0.53$), where the water is present in all cases.

In the absence of water, only the water molecules of the enzymatic derivative can be used to generate the oil/water interphase—leading to strong dehydration of the enzyme. This fact produces a strong deactivation of the immobilized enzyme as observed by us in the case of the α-CT immobilized on agarose.[18] In the second case, the oil/water interphase is produced from the water molecules added to the reaction mixture without alteration of the enzymatic derivative. It is clear, then, that the conformation of the enzyme would be different in both cases and thus, the stereoselectivity would be slightly different.

Therefore we can conclude that the immobilization or the chemical modification of the enzymes changes the stereoselectivity of the organic reaction catalysed by the derivatives.

Experimental

Materials.—α-Chymotrypsin type VII (α-CT) and lipase from *C. rugosa* type VII (LCR) (1010 units mg^{-1} solid using olive oil) were from Sigma Chemicals (St. Louis, MO). Immobilized lipase from *C. antarctica* (SP 435A) (LCA) was kindly supplied by Novo Nordisk (Denmark). Monomethoxypolyethylene glycol (PEG, *P*m = 5000) was from Sigma Chemicals and trichlorotriazine was from Merck. Alumina-60 (70 Å pore size) was from Merck (Germany).

The synthesis of (*R*,*S*)-*N*-benzoylphenylalanine methyl ester was described before.[19]

Enzymatic Derivatives.—The chemical modification of α-CT was carried out according to the methodology previously described in the literature[1] using the trichlorotriazine–monomethoxypolyethylene glycol procedure. The modified enzyme (PEG-CT) was lyophilized during 48 h and stored at 4 °C. This derivative retains 45% activity compared to the native α-CT using the hydrolysis of the *N*-benzoyltyrosine ethyl ester as standard reaction.

Immobilization of LCR. The activation of agarose was carried out according to a modified tosylation methodology[20] and the immobilization methodology has been reported previously.[8] The activation methodology of inorganic supports, using trichlorotriazine methodology has been described by the

Table 1 Hydrolysis of (±)-ethyl 2-phenylpropionate using LCR immobilized on alumina, T = 35 °C; V = 15 cm^3

Entry	pH	I^a/mol dm^{-3}	[Ester]/mol dm^{-3}	Speed[b]/rpm	Yield (%)[c]	E.e.(S) (%)[d]
1	7.0	0.0	0.05	300	50	95
2	8.5	0.7	0.05	300	43	96
3	8.5	0.0	0.05	700	48	96
4	8.5	0.7	0.1	700	50	95
5	7.0	0.7	0.1	700	54	60
6	7.0	0.0	0.05	700	69	44

[a] Ionic strength. [b] Stirring speed. [c] Yield in acid at 168 h; determined by HPLC (4% error). [d] Enantiomeric excess of (S)-acid (5% error) S(%) − R(%).

Table 2 Stereoselective esterification of (±)-ibuprofen with propanol, catalysed by LCA, 66 mmol dm^{-3} ibuprofen, V = 10 cm^3 of isooctane

Entry	H_2O/μl	T/°C	Speed[a]/rpm	Enz./mg	Ibu:PrOH[b]	t/h	Yield (%)	E.e. (%)[c]
1	0.0	50	500	100	1:1	3	44	6
2	0.0	50	100	500	1:4	3	49	9
3	300	24	500	500	1:1	3	34	29

[a] Stirring speed. [b] (±)-Ibuprofen:propanol molar ratio. [c] Enantiomeric excess of substrate after the reaction S(%) − R(%).

authors.[8] The immobilization of LCR was carried out at 4 °C and has been described in a previous paper.

Reactions.—Hydrolysis of (R,S)-N-*benzoylphenylalanine methyl ester.* The hydrolysis of (*R,S*)-*N*-benzoylphenylalanine methyl ester (Phe-OMe) ($2 \times 10^{-5} - 3.5 \times 10^{-3}$ mol dm^{-3}) was carried out using 125 μg cm^{-3} of native α-CT or 1.25 mg of PEG-CT (equiv. to 125 μg cm^{-3} of native enzyme). T = 25 °C, t = 24 h, V = 10 cm^3 of phosphate buffer with 10% MeOH to solubilize the ester.

Hydrolysis of (R,S)-*ethyl 2-phenylpropionate.* The hydrolysis of (*R,S*)-ethyl 2-phenylpropionate was carried out in a shaken batch reactor at 35 °C with 2 g of immobilized LCR on alumina or agarose, in 15 cm^3 of buffer Tris–HCl 0.1 mol dm^{-3} with different amounts of ester. The mixture was emulsified in an ultrasonic bath (2 min at 20 W). The reactions were stopped at 168 h with the addition of 1 cm^3 of 0.1 mol dm^{-3} HCl. The yield (%) was determined by HPLC with a C_8 column (5 μ Nucleosil 120).

Esterification of (R,S)-*ibuprofen.* Ester synthesis was carried out in a shaken flask. 10 cm^3 of dry isooctane containing 0.066 mol dm^{-3} ibuprofen acid were mixed with dry propanol, the immobilized derivative (LCA) and water in different amounts. The reaction mixture was incubated for 3 h. The reaction yield was determined by GC using a SPB-1 sulfur 15 m × 0.32 mm capilar column under isothermal conditions (T = 180 °C) using nitrogen as carrier gas. The reaction was stopped as above.

Enantiomeric Excess Determination.—The reaction was stopped by acidification (pH = 1–2) with 0.1 mol dm^{-3} H_2SO_4. The free acid was extracted by diethyl ether (2×100 cm^3), using conventional methodology. The organic layer was dried at vacuum and the acids were analysed using a polarimeter Perkin-Elmer 8012 (MeOH) or by ^1H NMR spectroscopy using a Bruker spectrometer 250 MHz in [$^2H_5$]pyridine and (*R,R*)-1,2-diphenylethane-1,2-diamine as chiral agent.[15]

Acknowledgements

This work has been supported by the grant PB090-010 from *Ministerio de Educación y Ciencia* of Spain.

References

1 H. Gaertner, T. Watanabe, J. V. Sinisterra and A. Puigserver, *J. Org. Chem.*, 1991, **56**, 3149.
2 Q.-M. Gu and C. J. Sih, *Biocatalysis*, 1992, **6**, 115.
3 M. J. Cabezas, E. F. Llama, C. Campo and J. V. Sinisterra, *Biocatalysis in Non-conventional Media*, ed. J. Tramper and M. H. Vermue, Elsevier Science, Amsterdam, 1992, pp. 67–74.
4 J. V. Sinisterra, in *Biocatalysis in Non-conventional Media*, ed. J. Tramper and M. H. Vermue, Elsevier Science, Amsterdam, 1992, pp. 75–82.
5 S. H. Wu, Z. W. Guo and C. J. Sih, *J. Am. Chem. Soc.*, 1990, **112**, 1990.
6 D. Salvador, J. V. Sinisterra and J. M. Guisan, *J. Mol. Catal.*, 1990, **62**, 93.
7 M. J. Cabezas, C. del Campo, E. F. Llama, J. V. Sinisterra and J. M. Guisan, *J. Mol. Catal.*, 1991, **62**, 353.
8 J. M. Moreno, M. Arroyo and J. V. Sinisterra, in *Stability and stabilization of enzymes*, ed. W. J. J. van den Tweel, A. Harder and R. M. Buitelaar, Elsevier, Netherlands, 1993, pp. 22–25.
9 D. M. Blow, *Enzymes*, ed. P. D. Boyer, Academic Press, New York, 1971, vol. III, pp. 185–246.
10 J. B. West and C. H. Wong, *J. Org. Chem.*, 1986, **51**, 2728.
11 Ch. Exl, H. Hönig, G. Renner, R. Rogi-Kohlenprath, V. Seebauer and P. Seufer-Wasserthal, *Tetrahedron: Asymmetry*, 1992, **3**, 1391.
12 K. K. Kim, K. Y. Hwang, H. S. Jeon, S. Kim, R. M. Sweet, Ch. H. Yang and S. W. Suh, *J. Mol. Biol.*, 1992, **227**, 1258.
13 M. Ahmar, C. Girard and R. Bloch, *Tetrahedron Lett.*, 1989, **30**, 7053.
14 B. Zerner, R. P. M. Bond and M. L. Bender, *J. Am. Chem. Soc.*, 1964, **86**, pp. 3674–3679.
15 R. Fulwood and D. Parker, *Tetrahedron: Asymmetry*, 1992, **3**, 25.
16 C. Hansch, C. Grieco, C. Sillipo and A. Vittoria, *J. Med. Chem.*, 1977, **20**, 1420.
17 G. Carrea, R. Bovara, G. Ottolina and S. Riva, *Biotechnol. Lett.*, 1993, **15**, 169.
18 M. T. Martín, J. V. Sinisterra and A. Heras, *J. Mol. Catal.*, 1993, **80**, 127.
19 J. F. Bello, E. F. Llama, C. del Campo, M. J. Cabezas and J. V. Sinisterra, *J. Mol. Catal.*, 1993, **78**, 91.
20 A. Alcántara, A. Ballesteros and J. V. Sinisterra, *Applied Biochem. Biotechnol.*, ed. H. Weetall, The Humana Press Inc., 1990, pp. 297–310.

Paper 3/07193F
Received 6th *December* 1993
Accepted 17th *February* 1994