The importance of ester and alkoxy type functionalities for the chemo- and enantio-recognition of substrates by hydrolysis with *Candida rugosa* lipase

Francesca Bellezza, Antonio Cipiciani,* Gabriele Cruciani and Francesco Fringuelli

Dipartimento di Chimica, Università di Perugia, Via Elce di Sotto 8, 06100 Perugia, Italy

Received (in Cambridge, UK) 10th July 2000, Accepted 30th October 2000
First published as an Advance Article on the web 30th November 2000

Racemic esters of 1-phenyl- and 1-(pyridyl)ethyl acetates **1a**–**c** (R = Me, Ph) were subjected to hydrolysis in water in the presence of *Candida rugosa* lipase (CRL). 1-Pyridylethyl acetates (**1**, R = Me) are hydrolyzed by crude CRL (C-CRL) and isopropanol (isopropyl alcohol) treated CRL (PT-CRL) at very low rates, and the enantiorecognition was disappointing. By using 1-pyridylethyl benzoates (**1**, R = Ph) the results differed greatly: hydrolysis occurred much faster, and the enantiorecognition was good for 3- and 4-pyridyl derivatives and excellent for 2-pyridyl derivative. Analogous results were obtained by submitting the 1-phenylethanol esters **4** to enzymatic hydrolysis under the same experimental conditions. The hydrolysis of methyl *o*-acetoxybenzoates **7** (R = Me) gave quantitatively the deacetylated methyl *o*-hydroxybenzoates **9** (R = Me) by using either C-CRL or PT-CRL. A complete reversed selectivity is observed in the hydrolysis of phenyl *o*-acetoxybenzoates **7** (R = Ph) catalyzed by PT-CRL. Molecular modeling studies, aimed at probing the substrate specificity and the enantioselectivity of enzyme in terms of its three-dimensional structure is reported.

Introduction

An enzyme is generally useful if it is stable, inexpensive and accepts a broad range of substrates while retaining high selectivity for each one.

Lipase from *Candida rugosa* (CRL) has these qualities and it is extensively used for the hydrolysis and esterification of organic compounds[1] and the knowledge[2] of its crystal structure has greatly contributed to understanding the mechanism of its selective recognition of substrates.[3] In order to improve the chemo-, regio- and diastereoselectivity of the enzyme, commercial CRL (C-CRL), as well as conventional purifications,[4] have been subjected to several treatments that cause molecular (covalent modifications) or conformational (non-covalent modifications) changes. Covalent modifications are caused by immobilizing C-CRL on several supports[5] or by the nitration of tyrosyl residues.[6] Non-covalent modifications are brought about by changing the reaction temperature,[4] pH[7] and solvent,[8,9] by introducing additives such as $CaCl_2$,[10] surfactants,[11] microemulsing agents[12] and carbohydrates[13] and by treating with organic solvents.[14,15]

Surprisingly when the C-CRL does not accept a substrate, no attempt to modify the compound is generally made but the C-CRL is rejected and other lipases are tested.

Following our studies on the use of CRL in organic synthesis,[16] this paper gives three examples that demonstrate that the optical resolution of alcohols *via* hydrolysis of esters and the chemoselective hydrolysis of an alkoxy group in the presence of an ester functionality, depend greatly on the acid and alcoholic residues of the ester function and on whether the CRL is crude or treated.

Results and discussion

Optically active pyridyl-derivatives have received increasing attention as chiral auxiliaries and chiral ligands[17] but the use of systems with a chiral center on the pyridine side is actually limited because the introduction of a chiral center directly attached to the pyridyl ring is difficult to achieve.

Although different strategies have been adopted to obtain chiral pyridyl alcohols with satisfactory enantioselectivities and yields it is still necessary to find new, general and reliable methods. Optically active 1-(pyridyl)ethanols have been prepared by lipase-catalyzed asymmetric acetylation of individual alcohols and currently the reaction has been limited to some 1-(2-pyridyl)ethanols.[18] Among the lipases tested, C-CRL is reported[18b] to be undesirable for the enantioselective transesterification of racemic 1-(pyridyl)ethanols with vinyl acetate.

We have submitted racemic 1-(pyridyl)ethyl acetates (**1**, R = Me) to enzymatic hydrolysis with C-CRL and with isopropanol treated CRL (PT-CRL)[19] at room temperature in aqueous medium at pH 7.2 (Table 1). The hydrolysis was very slow, the 4-pyridyl-derivatives were not accepted and the enantiorecognition was disappointing.[20] By using 1-(pyridyl)ethyl benzoates (**1**, R = Ph), the results were very greatly different; the hydrolysis occurred much faster, the 4-pyridyl-derivatives were accepted and the enantiorecognition was good for 3- and 4-pyridyl-derivatives and excellent for the 2-pyridyl-derivative when hydrolyzed in the presence of C-CRL.

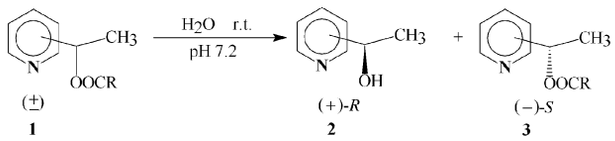
Analogous results were obtained by submitting the 1-phenylethanol esters **4** to enzymatic hydrolysis under the same experimental conditions (Table 2). The C-CRL and PT-CRL do not show enantiopreference for the acetate **4** (R = Me) while they preferentially hydrolize the (+)-*R* benzoate **4** (R = Ph).

o-Acetoxybenzoates **7** are potential precursor of aspirin. To reduce or block the side effects of aspirin (gastric irritation and bleeding) numerous chemical derivatives of acetylsalicylic acid (precursor of aspirin) have been tested[22] but it is still an open problem.

Moreover hydrolases play an important role in the metabolism of many xenobiotics, and humans have been shown to express hydrolases in the liver, plasma, intestine, brain, stomach colon, macrophage and monocytes. Although hydrolases activities vary quite strongly between species, the active site region of the enzymes is sometimes conserved and the mechanism of hydrolysis shows a similar pattern. Thus the results of this study can be easily extended to check the role in the

DOI: 10.1039/b005512n

Table 1 C-CRL and PT-CRL catalyzed hydrolysis of 1-(pyridyl)ethanol esters



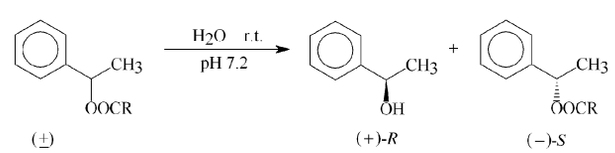
1 **2** **3**

2-Py = **a** 3-Py = **b** 4-Py = **c** R = Me, Ph

Entry	Enzyme	Substrate	*t*/h	Conv. (%)[a]	**2** (ee)[b]	**3** (ee)[b]	**E**[c]
1	C-CRL	**1a** R = Me	120	70	27	64	3
2	PT-CRL	**1a** R = Me	76	33	24	29	2
3	C-CRL	**1a** R = Ph	7	48	91	97	100
4	PT-CRL	**1a** R = Ph	2	39	75	68	15
5	C-CRL	**1b** R = Me	408	35	49	36	9
6	PT-CRL	**1b** R = Me	309	41	46	61	5
7	C-CRL	**1b** R = Ph	22	49	83	80	27
8	PT-CRL	**1b** R = Ph	10	44	83	65	21
9	C-CRL	**1c** R = Ph	54	43	84	65	26
10	PT-CRL	**1c** R = Ph	29	45	81	75	22

[a] Conversion of reaction after consumption of the required amount of NaOH 0.2 M. [b] Enantiomeric excess (%) determined by measuring the specific optical rotation. [c] Enantioselectivity factor.

Table 2 C-CRL and PT-CRL catalyzed hydrolysis of 1-phenylethanol esters



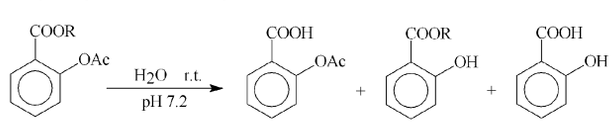
4 **5** **6**

R = Me, Ph

Entry	Enzyme	R	*t*/h	Conv. (%)[a]	**5** (ee)[b]	**6** (ee)[b]	**E**[c]
1	C-CRL	Me	31	46	44	39	4
2	PT-CRL	Me	22	51	29	43	3
3	C-CRL	Ph	36	45	81	77	20
4	PT-CRL	Ph	21	47	70	58	10

[a] For footnotes *a*,*b*,*c* see Table 1.

Table 3 C-CRL and PT-CRL catalyzed hydrolysis of *o*-acetoxybenzoates



7 **8** **9** **10**

Entry	Enzyme	R	*t*/h	Conv. (%)[a]	**8**[b]	**9**[b]	**10**[b]
1	C-CRL	Me	3	100	—	100	—
2	PT-CRL	Me	3	100	—	100	—
3	C-CRL	Ph	60	85	50	—	50
4	PT-CRL	Ph	11	100	100	—	—

[a] See Table 1. [b] Determined by GC.

pharmacokinetic behaviour of most therapeutic agents containing acetoxybenzoate-like compounds.

Using C-CRL or PT-CRL, the hydrolysis of methyl *o*-acetoxybenzoates **7** (R = Me) at room temperature in aqueous medium at pH 7.2 gave quantitatively the deacetylated methyl *o*-hydroxybenzoates **9** (R = Me) (Table 3). A completely reversed selectivity is observed in the hydrolysis of phenyl *o*-acetoxybenzoates **7** (R = Ph) catalyzed by PT-CRL. Only the hydrolysis of carboxylate function was observed and the *o*-acetoxybenzoic acid **8** was obtained with high yield and high chemoselectivity. Phenyl *o*-acetoxybenzoate **7** (R = Ph) is therefore an excellent precursor of aspirin in the presence of PT-CRL, while less than 50% of the compound acts as precursor of aspirin in the presence of C-CRL.

To probe the substrate specificity and the enantioselectivity of C-CRL we carried out molecular modeling studies related to the hydrolysis of 1-(pyridyl)ethyl acetates and benzoates **1a**–**c** in terms of their three-dimensional structure.

Fig. 1 reports an example of the structure of the complex between *R*- and *S*-enantiomers of compound **1a** (R = Ph) and the CRL structure obtained by docking experiments.

Amino acids forming the catalytic triad of the enzyme, as well as those amino acids in contact with **1a** are highlighted. The hydrophobic regions calculated by GRID are shown in grey. Three hydrophobic regions are present in the active site of the enzyme. Region 1 generated by Phe-296 and Phe-345 (not shown) is used by both enantiomers to interact with the enzyme. This interaction is particularly important for both enantiomers of all the compounds **1a**–**c** (R = Ph) because the phenyl ligand of the benzoyl group is positioned exactly in the above mentioned region. Conversely, enantiomers of compounds **1a**–**c** (R = Me) only partially interact with the methyl moiety of acetyl groups in this region, thereby recovering a little stabilization energy. The hydrophobic region 2 is generated by Val-127 and Phe-296 and is important for discriminating between the selective interaction with the *R*- and *S*-enantiomers. In fact, compound *R*-**1a** (R = Ph) can favourably position the methyl moiety of the chiral center, in this region (see Fig. 1-A), while the *S*-**1a** enantiomer tends to orient the methyl group out of this hydrophobic region and toward the catalytic histidine side-chain His-449 (see Fig. 1-B). The latter orientation tends to disfavour the binding energy as well as the catalytic activity thereby disrupting the exact orientation of the catalytic triad. Hydrophobic region 3, close to Leu-302 (not shown), is not used by either of the two enantiomers of **1a** and therefore cannot be responsible for the observed enantioselectivity.

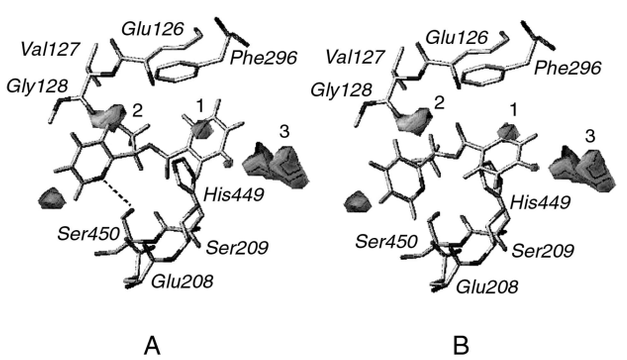


Fig. 1

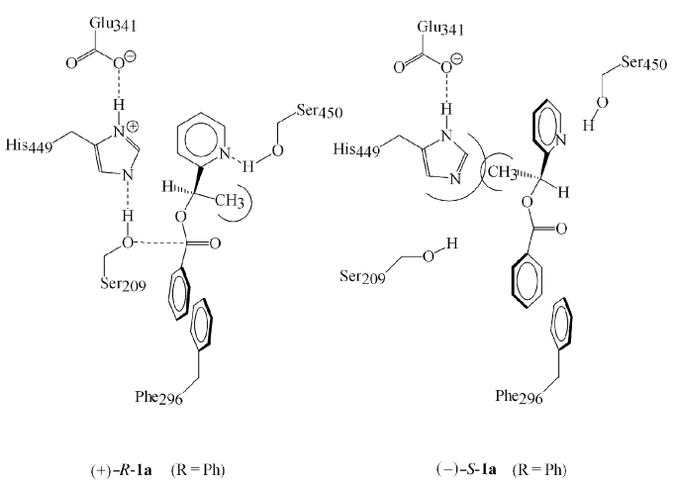
Ser-450 plays a fundamental role in the enantioselection of the enzyme. Scheme 1 illustrates the complexes (A and B) of C-CRL with the two enantiomers (+)-*R*-**1a** and (−)-*S*-**1a**. Serine 450 is able to form a strong hydrogen bond (about −4 kcal mol^{-1}) with the pyridine nitrogen of the *R*-enantiomer (complex A). The distance between the nitrogen and serine hydroxy is 3.2 Å and the orientation of the serine hydrogen and pyridine nitrogen lone pair are optimal for interaction.

Conversely, the distance between the nitrogen and hydroxy group of serine for the *S*-enantiomer is about 4.0 Å (complex B) and the pyridyl ring is not optimally oriented for hydrogen bonding, thus leading to a very weak bond (about 0.3 Kcal mol^{-1}). The hydroxy group of Ser-209 therefore, preferentially attacks the carbonyl of (+)-*R* **1a** (R = Ph) in agreement with experimental findings. The pyridyl rings of both enantiomers of **1b** (R = Ph) and **1c** (R = Ph) are not oriented so as to form strong hydrogen bonding with Ser-450 and, consequently, a minor enantioselection is observed. This also accounts for the low enantioselection observed in the hydrolysis of 1-phenylethyl benzoate **4**, (R = Ph) which cannot form a hydrogen bond with Ser-450.

The results can be explained considering that: (i) the phenyl of the benzoyl group [compounds **1a**–**c** (R = Ph); **4** (R = Ph)] interacts with hydrophobic region 1 more favourably than the methyl group of the acetatye [compounds **1a**–**c** (R = Me); **4** (R = Me)]; (ii) the pyridyl group can form a hydrogen bond with Ser-450 and this is particularly advantageous when the heterocyclic ring is α-substituted; and (iii) the enantioselection depends on the orientation of the methyl of the chiral center towards a close hydrophobic region 2 of the active site of the enzyme.

It has been reported[14,15] that the purification of crude CRL (C-CRL) by treatment with simple aliphatic alcohols play an important role in the increase of enantioselection of esters containing stereocenters in the acid portion of the ester. In the present paper we put in evidence that propan-2-ol-treated CRL (PT-CRL) is less enantioselective than C-CRL toward esters containing stereocenters in the alcohol portion of the ester. This result is in agreement with the finding of Kazlauskas and co-workers[14b] in the hydrolysis of (±)-menthyl acetate and (±)-(2-acetoxy-1-naphthyl)methylphenylphosphine oxide. The results from a broader spectrum of compounds are necessary to determine the general applicability of this rule.

In conclusion, CRL is not always able to accept and selectively recognize the methyl ester function derived from a carboxylic group or the acetoxy function derived from a hydroxy group of alcohols and phenols (this is the common



Scheme 1

way to functionalize the substrate submitted to enzymatic hydrolysis); no improvement is observed by using non-covalent modification of the enzyme (PT-CRL). The problem can be overcome by derivatising the carboxylic acid by phenylation instead of methylation and by using the benzyloxy derivative of an alcohol instead of its acetate.

In accord with the molecular modeling related to the hydrolysis of 1-(pyridyl)ethyl acetates and benzoates a qualitative suggestion can be advanced to explain the complete chemoselectivity of PT-CRL observed in the hydrolysis of phenyl *o*-acetoxybenzoate **7** (R = Ph) with respect to methyl *o*-acetoxybenzoate **7** (R = Me). The phenyl (ligand) of the benzoyl group of **7** (R = Ph), and **1a**–**c** (R = Ph) compounds, likes to be positioned in the region 1 and the phenyl of phenoxy group prefers to be positioned in the hydrophobic region 2 so that only the benzoate function is favourably oriented for hydrolysis. Conversely, methyl *o*-acetoxybenzoate **7** (R = Me) only partially interacts with the methyl moiety of the acetyl group in the region 1, thereby recovering a little stabilization energy. Moreover the substrate may change the orientation and the position in the active site thus favoring the hydrolysis of the acetate function.

The higher activity and chemoselectivity of PT-CRL, compared to C-CRL, in the hydrolysis of phenyl *o*-acetoxybenzoate **7** (R = Ph) probably occurs because the propan-2-ol treatment causes a conformational change[14,23] of the lipase which influences the structure of the active site and the flexibility of the enzyme. It is therefore possible that the enzyme becomes more flexible[15] and the substrate **7** (R = Ph) can be positioned favourably in the active site (induced fit enzyme[24]) for the hydrolysis.

The use of CRL has been extended which is of practical interest because it is commercially available, stable and inexpensive. Moreover the proposed model should also be useful in estimating the mechanism of hydrolysis in the presence of CRL for pro-drug or soft-drug candidates ahead of their synthesis.

Experimental section

General

Candida rugosa lipase (crude CRL E.C.3.1.1.13 type VII) was purchased from Sigma. PT-CRL was purified as recently described.[15a] 2-, 3-, 4-Acetylpyridine and 1-phenylethanol were obtained from Aldrich Chemical Co. Compound **7** was prepared according to the literature.[16a] Compounds **2a**–**c** are not well characterized in the literature; compounds **3a**–**c** (R = Ph) and **4** (R = Ph) are new compounds. The optical rotations of **2a**–**c**, **3a**–**c** **5** and **6** are known.[18a,24] All the organic solvents were of reagent grade and used without further purification (except for pyridine which was distilled over KOH). ^{1}H-NMR spectra were recorded in $CDCl_3$ solution on a Bruker AC 200 MHz spectrometer. GC analyses were performed on a Hewlett-Packard 5890 chromatograph with HP-5-fused silica capillary column (30 m, 0.25 internal diameter, 0.25 μm film thickness), an "on-column" injector system, a FID detector and H_2 as gas carrier. Mass spectra were recorded at 70 eV on a GC-MS apparatus. The specific optical rotations were measured on a JASCO-DIP 360 polarimeter in $CHCl_3$ or MeOH solution.

Preparation of racemic pyridylethanols 2a–c

$NaBH_4$ (52 mmol) was added to a stirred solution of acetylpyridine (41 mmol) in MeOH (20 ml) at room temperature. After being stirred for 5 h, the mixture was poured into water and extracted with EtOAc. The organic layer was dried with Na_2SO_4 and then evaporated at reduced pressure. The alcohol was obtained with GC purity 95% and then used without further purification. Chemical yields and spectroscopic data are described as follows:

1-(2-Pyridyl)ethanol 2a. Yield 70%, colourless crystals, mp 27–28 °C (from *n*-hexane), MS (EI) *m*/*z* (rel. intensity) 52 (33), 80 (50), 108 (100), 121 (70); ^{1}H-NMR δ 1.15 (d, 3H, CH_3, J = 6.3 Hz), 4.32 (br s, 1H, OH), 4.90 (q, 1H, CH, J = 6.3 Hz), 7.19–7.23 (m, 1H, H_{arom}), 7.29 (d, 1H, H_{arom}, J = 7.9 Hz), 7.70 (ddd, 1H, H_{arom}, J = 7.7, 7.7, 1.7 Hz), 8.55 (d, 1H, H_{arom}, J = 4.9 Hz).

1-(3-Pyridyl)ethanol 2b. Yield 97%, oil, MS (EI) *m*/*z* (rel. intensity) 53 (20), 80 (80), 108 (100), 123 (M^+, 35); ^{1}H-NMR δ 1.52 (d, 3H, CH_3, J = 6.4 Hz), 2.17 (br s, 1H, OH), 4.97 (q, 1H, CH, J = 6.4 Hz), 7.29 (dd, 1H, H_{arom}, J = 8.0, 5.0 Hz), 7.74 (ddd, 1H, H_{arom}, J = 7.9, 1.7, 1.7 Hz), 8.52 (dd, 1H, H_{arom}, J = 4.9, 1.3 Hz), 8.60 (s, 1H, H_{arom}).

1-(4-Pyridyl)ethanol 2c. Yield 83%, colourless crystals, mp 55–58 °C (from *n*-hexane), MS (EI) *m*/*z* (rel. intensity) 43 (60), 51 (70), 78 (100), 106 (90), 121 (75); ^{1}H-NMR δ 1.48 (d, 3H, CH_3, J = 6.5 Hz), 3.5 (br s, 1H, OH), 4.88 (q, 1H, CH, J = 6.5 Hz), 7.29 (dd, 2H, H_{arom}, J = 4.5, 1.6 Hz), 8.45 (dd, 2H, H_{arom}, J = 4.5, 1.6 Hz).

Preparation of racemic esters 1a–c (R = Ph, Me) and 4 (R = Ph, Me)

The alcohol (40 mmol) and dry pyridine (8 ml) were mixed at 0 °C under N_2 atmosphere. The acetyl or benzoyl chloride (60 mmol) was then added dropwise during 5–10 min. The mixture was left at room temperature for 1 h and then quenched with water and extracted with EtOAc. The organic layer was washed with water (3 × 20 ml), dried over Na_2SO_4 and evaporated at reduced pressure. The residue was purified by column chromatography on silica gel eluted with ethyl ether to give acetates and with ethyl ether–petroleum ether 1 : 1 to give benzoates. Chemical yields and spectroscopic data are described as follows:

1-(2-Pyridyl)ethyl acetate 1a, (R = Me). Yield 95%, oil, MS (EI) *m*/*z* (rel. intensity) 43 (50), 78 (33), 105 (80), 123 (100), 165 (M^+, 30); ^{1}H-NMR δ 1.60 (d, 3H, CH_3, J = 6.7 Hz), 2.12 (s, 3H, $COCH_3$), 5.92 (q, 1H, CH, J = 6.7 Hz), 7.23–7.27 (m, 1H, H_{arom}), 7.35 (dm, 1H, H_{arom}, J = 7.8 Hz), 7.68 (ddd, 1H, H_{arom}, J = 7.8, 7.8, 1.8 Hz), 8.60 (dm, 1H, H_{arom}, J = 4.5 Hz).

1-(3-Pyridyl)ethyl acetate 1b, (R = Me). Yield 85%, oil, MS (EI) *m*/*z* (rel. intensity) 43 (50), 78 (45), 105 (85), 123 (100), 165 (M^+, 20); ^{1}H-NMR δ 1.57 (d, 3H, CH_3, J = 6.6 Hz), 2.08 (s, 3H, $COCH_3$), 5.90 (q, 1H, CH, J = 6.6 Hz), 7.25–7.32 (m, 1H, H_{arom}), 7.66 (dm, 1H, H_{arom}, J = 7.9 Hz), 8.55 (dd, 1H, H_{arom}, J = 4.8, 1.7 Hz), 8.61–8.62 (m, 1H, H_{arom}).

1-(4-Pyridyl)ethyl acetate 1c, (R = Me). Yield 80%, oil, MS (EI) *m*/*z* (rel. intensity) 43 (50), 78 (35), 106 (50), 123 (100), 165 (M^+, 20); ^{1}H-NMR δ 1.50 (d, 3H, CH_3, J = 6.7 Hz), 2.11 (s, 3H, $COCH_3$), 5.83 (q, 1H, CH, J = 6.7 Hz), 7.24 (d, 2H, H_{arom}, J = 5.7 Hz), 8.58 (d, 2H, H_{arom}, J = 5.7 Hz).

1-(2-Pyridyl)ethyl benzoate 1a, (R = Ph). Yield 82%, oil, MS (EI) *m*/*z* (rel. intensity) 51 (20), 77 (45), 106 (65), 122 (100), 182 (10); ^{1}H-NMR δ 1.75 (d, 3H, CH_3, J = 6.65 Hz), 6.19 (q, 1H, CH, J = 6.65 Hz), 7.15–7.25 (m, 1H, H_{arom}), 7.42–7.49 (m, 3H, H_{arom}), 7.54–7.58 (m, 1H, H_{arom}), 7.68 (ddd, 1H, H_{arom}, J = 7.8, 7.8, 1.8 Hz), 8.12 (dm, 2H, H_{arom}, J = 6.9 Hz), 8.66 (dm, 1H, H_{arom}, J = 5.0 Hz).

1-(3-Pyridyl)ethyl benzoate 1b, (R = Ph). Yield 72%, oil, MS (EI) *m*/*z* (rel. intensity) = 51 (35), 77 (65), 105 (100), 122 (40), 227 (M^+, 65); ^{1}H-NMR δ 1.7 (d, 3H, CH_3, J = 6.64 Hz), 6.15 (q, 1H, CH, J = 6.64 Hz), 7.25–7.57 (m, 4H, H_{arom}), 7.76

(dm, 1H, H_{arom}, J = 7.9 Hz), 8.06 (dm, 2H, H_{arom}, J = 6.9 Hz), 8.62–8.63 (m, 1H, H_{arom}), 8.72 (s, 1H, H_{arom}).

1-(4-Pyridyl)ethyl benzoate 1c, (R = Ph). Yield 77%, oil, MS (EI) *m*/*z* (rel. intensity) 51 (35), 77 (65), 105 (100), 122 (40), 227 (M^+, 65); ^{1}H-NMR δ 1.65 (d, 3H, CH_3, J = 6.65 Hz), 6.45 (q, 1H, CH, J = 6.65 Hz), 7.30 (dd, 2H, H_{arom}, J = 4.5, 1.6 Hz), 7.40–7.54 (m, 3H, H_{arom}), 8.08 (dm, 2H, H_{arom}, J = 6.9 Hz), 8.59 (dd, 2H, H_{arom}, J = 4.5, 1.6 Hz).

1-Phenylethyl acetate 4, (R = Me). Yield 88%, oil, MS (EI) *m*/*z* (rel. intensity) 43 (82), 51 (31), 77 (54), 104 (88), 122 (100), 165 (M^+, 0.5); ^{1}H-NMR δ 1.56 (d, 3H, CH_3, J = 6.6 Hz), 2.09 (s, 3H, OCH_3), 5.91 (q, 1H, CH, J = 6.6 Hz), 7.30–7.39 (m, 5H, H_{arom}).

1-Phenylethyl benzoate 4, (R = Ph). Yield 86%, oil, MS (EI) *m*/*z* (rel. intensity) = 51 (25), 77 (55), 105 (100), 226 (M^+, 35); ^{1}H-NMR δ 1.67 (d, 3H, CH_3, J = 6.6 Hz), 6.14 (q, 1H, CH, J = 6.6 Hz), 7.32–7.56 (m, 8H, H_{arom}), 8.08 (dm, 2H, H_{arom}, J = 6.9 Hz).

Enzymatic hydrolysis in presence of crude CRL

In a standard experiment, crude CRL (120 mg) was suspended in water (12 ml) and phosphate buffer (NaH_2PO_4–Na_2PO_4, 0.1 M, pH 7.2, 2 ml), stirred at room temperature and the pH adjusted to 7.2. Racemic ester (1 mmol) was added and the mixture was maintained at pH 7.2 by automatic titration with NaOH 0.2 M using a Mettler DK pH-stat under vigorous stirring. When the hydrolysis reached the conversion indicated in the tables, the reaction was stopped adding a saturated solution of NaCl (10 ml), the solution was filtered through Celite and the mixture was extracted with EtOAc (3 × 20 ml). The organic layer was dried with Na_2SO_4 and concentrated *in vacuo*. Alcohol and ester were separated by column chromatography on silica gel using eluents described above.

Enzymatic hydrolysis in presence of PT-CRL

12 ml of solution of PT-CRL (225 units with p-NPA assay) and phosphate buffer (NaH_2PO_4–Na_2PO_4, 0.1 M, pH 7.2, 2 ml) were stirred at room temperature and the pH adjusted to 7.2. Racemic ester (1 mmol) was added and the mixture was maintained at pH 7.2 by automatic titration with NaOH 0.2 M under vigorous stirring. When the hydrolysis reached the conversion indicated in the tables, the reaction mixture was worked up as described above.

Determination of the enantiomeric excess

The ee of all alcohols (+)-**2a**–**c** and acetates (−)-**3a**–**c** (R = Me) obtained in the enzymatic hydrolysis was determined by known specific optical rotation.[18a,25] The acetate (−)-**6** (R = Me) and the benzoates (−)-**3a**–**c** (R = Ph) and **6** (R = Ph) were first hydrolyzed to the corresponding alcohols (+)-**5** and (+)-**2** respectively by treatment with NaOH 0.5 M for 3 h at reflux and the ee were determined by comparing them with known specific optical rotation.[18a,25]

Molecular modeling

Atomic coordinates of CRL protein were obtained from Brookhaven National Laboratories Protein Databank PDB (structure code 1CRL at 2.0 Å resolution). Water molecules were deleted from the model. The global charge of the enzyme target was −4. Since uncharged systems are preferable for the analysis, potassium counterions were added to the CRL protein. To perform this task a GRID[26] map with potassium cations was prepared, and the utility programs Minim and Filmap[27] were then used in tandem. Minim finds and lists all the energy minima in the grid map for the potassium counterions. Filmap then used a simulated annealing procedure to postprocess the list of the minima in order to select the minima locations subset that gives the most favourable overall interaction energy with the counterions layer. Hydrophobic interaction in the active site of the protein was computed by the GRID program. GRID basically is a computational procedure for detecting energetically favourable binding sites on macromolecules. The program works by defining a 3D grid of points that contains the chosen substrate-binding site. At each node of the grid, the energy between the probe and the target is calculated as the electrostatic, hydrogen bond and Lennard–Jones interactions of chemically selective probes with the chosen target structure. For hydrophobic interactions a methyl probe group interacting with the enzyme was used. Ligand structures (compound **1a** (R = Ph, *R*- and *S*-enantiomers)) were generated with the SYBYL[28] program and energy-minimised using the Amsol[29] force field to which solvation energy was added. Conformational analysis was not required. After conversion to the appropriate format, the output conformers of compounds **1a** and **2a** and the enzyme structure were used by the program Autodock to find the docked conformations of compounds **1a** and **2a** in the active site of CRL enzyme. Autodock is a state of the art tool for identifying possible binding interactions between macromolecules one of which is considered rigid (the protein) and the other (the ligand) is considered to have a flexible structure. Autodock automatically performs conformational analysis of the ligand when docking procedure is applied. During the docking operations, Autodock[30] moves the ligand into the site, modifies the conformation of the ligand molecule and evaluates the energy of the various complexes as generated. In our work, only the minimum energy complexes were selected and reported for interpretation purposes. It is interesting to note that this procedure is relatively independent of the initial orientation and conformation of the modeled docked ligands.

Acknowledgements

The Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) Consiglio Nazionale delle ricerche (CNR, Rome) and Università degli Studi di Perugia are thanked for financial support.

References

1 C. H. Wong and G.-M. Whitesides, *Enzymes in Organic Chemistry*, Pergamon, New York, 1994.
2 P. Grochulski, Y. Li, J. D. Schrag, F. Bouthillier, P. Smith, D. Harrison, B. Rubin and M. Cygler, *J. Biol. Chem.*, 1993, **268**, 12843; P. Grochulski, Y. Li, J. D. Schrag and M. Cygler, *Protein Sci.*, 1994, **3**, 82.
3 M. Cygler, P. Grochulski, R. J. Kazlauskas, J. D. Schrag, F. Bouthillier, B. Rubin, A. N. Serreqi and A. K. Gupta, *J. Am. Chem. Soc.*, 1994, **116**, 3180.
4 M. L. Rua, T. Diaz-Maurino V. M. Fernandez, C. Otero and A. Ballesteros, *Biochim. Biophys. Acta*, 1993, **1156**, 181.
5 J. V. Sinisterra, E. F. Llama, C. Del Campo, M. J. Cabezas, J. M. Moreno and M. J. Arroyo, *J. Chem. Soc.*, *Perkin Trans. 2*, 1994, 1330; B. Berger and K. Faber, *J. Chem. Soc.*, *Chem. Commun.*, 1991, 1198.
6 Q. M. Gu and C. J. Sih, *Biocatalysis*, 1992, **6**, 115.
7 L. Lam, R. Hui and J. B. Jones, *Eur. J. Org. Chem.*, 1998, 1441.
8 T. Miyazawa, S. Kurita, S. Ueji, T. Yamada and S. Kuwata, *Biotechnol. Lett.*, 1992, **14**, 941.
9 S. Parida and J. S. Dordick, *J. Am. Chem. Soc.*, 1991, **113**, 2253.
10 E. Holmberg, M. Holmquist, E. Hedenstrom, P. Berglund, T. Norin, H. E. Hogberg and K. Hult, *Appl. Microbiol. Biotechnol.*, 1992, **35**, 572.
11 A. Bhaskar Rao, H. Rehaman, B. Krishnakumari and J. S. Yadav, *Tetrahedron Lett.*, 1994, **35**, 2611.
12 G. Hedstrom, M. Backlund and J. P. Slotte, *Biotechnol. Bioengineer.*, 1993, **42**, 618.

13 J. M. Sànchez-Montero, V. Hamon, D. Thomas and M. D. Legoy, *Biochim. Biophys. Acta*, 1991, **1078**, 345.
14 (*a*) S. H. Wu, Z. H. Guo and C. J. Sih, *J. Am. Chem. Soc.*, 1990, **112**, 1990; (*b*) I. J. Colton, N. A. Sharmin and R. J. Kazlauskas, *J. Org. Chem.*, 1995, **60**, 212.
15 (*a*) A. Cipiciani, M. Cittadini and F. Fringuelli, *Tetrahedron*, 1998, **54**, 7890; (*b*) A. Cipiciani, F. Bellezza, F. Fringuelli and M. Stillitano, *Tetrahedron: Asymmetry*, 1999, **10**, 4599.
16 (*a*) A. Cipiciani, F. Fringuelli and A. M. Scappini, *Tetrahedron*, 1996, **52**, 9869; (*b*) A. Cipiciani, F. Fringuelli, V. Mancini, O. Piermatti, A. M. Scappini and R. Ruzziconi, *Tetrahedron*, 1997, **53**, 11853.
17 M. R. Uskokovic, R. L. Lewis, J. J. Partridge, C. W. Despreaux and D. L. Pruess, *J. Am. Chem. Soc.*, 1979, **101**, 6742; A. Franck and C. Ruchardt, *Chem. Lett.*, 1984, 1431; M. Amat, M. D. Coll, D. Passarella and J. Bosh, *Tetrahedron: Asymmetry*, 1996, **7**, 2775.
18 (*a*) M. Seemayer and M. P. Schneider, *Tetrahedron: Asymmetry*, 1992, **3**, 827; (*b*) K. Kano, S. Negi, A. Kawashima and K. Nakamura, *Enantiomer*, 1997, **2**, 261; (*c*) J. Uenishi, T. Hiraoka, S. Hata, K. Nishiwaki, O. Yonemitsu, K. Nakamura and H. Tsukube, *J. Org. Chem.*, 1998, **63**, 2481.
19 PT-CRL was selected for this study because the isopropanol treatment is easy, gives synthetically useful amounts of enzyme and increases the enantiopreference of enzyme towards esters. (I. J. Colton, N. A. Sharmin and R. J. Kazlauskas, *J. Org. Chem.*, 1995, **60**, 212).
20 Experiments at 40 °C in aqueous medium at pH 7.2 and, for substrate **1a** (R = Me), also in water–organic solvent 10 : 1 v/v (organic solvent: acetone, acetonitrile, benzene, cyclohexane, *n*-hexane), give very low E values.
21 C. S. Chen, Y. Fujimoto, G. Girdauskas and C. J. Sih, *J. Am. Chem. Soc.*, 1982, **104**, 7294.
22 N. M. Nielsen and H. Bunbgaard, *J. Pharm. Sci.*, 1988, **77**, 285; M. Ankersen, K. K. Nielsen and A. Senning, *Acta Chem. Scand.*, 1989, **43**, 213; M. Hundewadt and A. Senning, *J. Pharm. Sci.*, 1991, **80**, 545.
23 M. Botta, E. Cernia, F. Corelli, F. Manetti and S. Soro, *Biochim. Biophys. Acta*, 1997, **1337**, 302.
24 F. Theil, *Chem. Rev.*, 1995, **95**, 2203.
25 D. Battail-Robert and D. Ganaire, *Bull. Soc. Chim. France*, 1965, 208.
26 P. J. Goodford, *J. Med. Chem.*, 1985, **28**, 849; D. N. A. Bobbyer, P. J. Goodford and P. M. McWhinnie, *J. Med. Chem.*, 1989, **32**, 1083.
27 GRID v. 17, Molecular Discovery Ltd., Oxford, G.B., 1999.
28 SYBYL, Tripos Associates, St. Louis, Missouri, USA.
29 C. J. Cramer, G. D. Hawikins, G. C. Lynch, D. J. Giesen, I. Rossi, W. J. Storer, D. G. Thrular and D. A. Liotard, *QCPE Bull.*, 1995, **15**, 41.
30 G. M. Morris, D. S. Goodsell, R. S. Halliday, R. Hvey, W. E. Hart, R. K. Betew and A. J. Olson, *J. Comput. Chem.*, 1998, **19**, 1639.