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The study on efficient hydrolases immobilization for the kinetic resolution of the α-acetoxyamides

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Abstract

Using different immobilization protocols, the lipases from *Pseudomonas cepacia* (PCL) and porcine pancreas (PPL) were immobilised. The catalytic behaviour of the biocatalysts used in the hydrolytic resolution of the target compounds, *viz.*, acetic acid phenyl(3,4,5-trimethoxybenzylcarbamoyl)methyl ester (**3a**) and acetic acid (3,4,5-trimethoxy benzylcarbamoyl)(3,4,5-trimethoxyphenyl)methyl ester (**3b**), in an aqueous environment, was investigated. The native lipases from *P. cepacia* (PCL) and porcine pancreas (PPL) showed low enantioselectivity ($E = 5.1$ and 3.5, respectively). Upon immobilization into a sol–gel matrix, the enantioselectivity of PCL improved significantly (from $E = 5.1$ up to 30.5). The covalent immobilization on Eupergit® substantially increased the enzymatic activity as well as the enantioselectivity of PCL ($E = 34.0$).

Keywords: Enzymatic kinetic resolution; Passerini reaction; Lipases; Enantioselectivity; Immobilization

1. Introduction:

The application of enzymes, especially in organic synthesis, is well documented in the literature [1–3]. In the past decade, a considerable number of processes have been commercialised in industry [4,5]. Hydrolytic enzymes, such as lipases, are frequently used there because of their ability to accept a wide range of substrates and the fact that they often exhibit a high enantioselectivity [6,7]. Most of the documented lipase-catalysed enantioselective hydrolyses have used high concentrations of the soluble enzymes or the enzyme preparations just as received from the supplier.

It has been shown recently that most lipases have a natural tendency to form biomolecular aggregates, by adsorption of the open lipases on the open lipases *via* a large hydrophobic pocket formed around the active centre. These aggregates have completely different catalytic properties from the individual lipases, thus this tendency can be availed to purify the lipases by specific adsorption [8,9] and even to immobilise them [10–13]. In many cases, the over-saturated substrate solutions are utilised and, therefore, the soluble lipases may undergo interfacial activation on the droplets of the substrate [14–18]. The use of the immobilised enzymes instead of the fully or partially soluble preparations brings advantages such as simple process of recovery of the pure products, convenient reuse of biocatalysts, and operational stabilisation of the enzyme [19]. Besides, it is worth noting that the catalytic behaviour of lipases immobilised on the over-saturated substrates in macro-aqueous environments can be quite different from the behaviour of high concentrations of free lipases. The catalytic behaviour of these enzymes might be a direct consequence of the equilibrium between the open-active and the closed-inactive structures of the immobilised lipases. Any change in the equilibrium may strongly influence the activity and the selectivity of the biocatalysts. Thus, the use of the different immobilization strategies may generate biocatalysts of different activity and/or selectivity [20–30].

During our study, we focused on the structural analogues of podophyllotoxin (**I**), which is well-known for its anti-tumour activity [31]. The selection of these analogues (**II**) was based on the fact that they possessed all the structural features essential for the anticancer activity, which were present in podophyllotoxin itself (Fig. 1). For the biological tests, a single enantiomer should be prepared and used separately. An efficient resolution into enantiomers can be achieved using different methods. The data in the literature implies that enantioselective enzymatic hydrolysis can be used for this separation [1,2].

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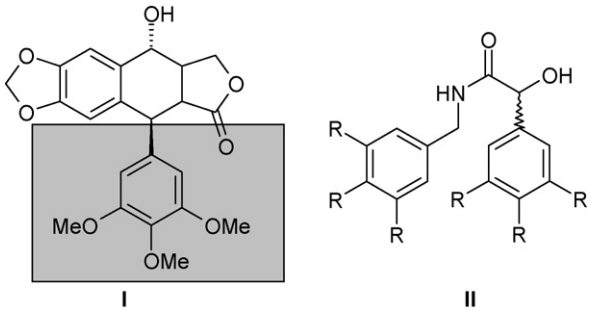


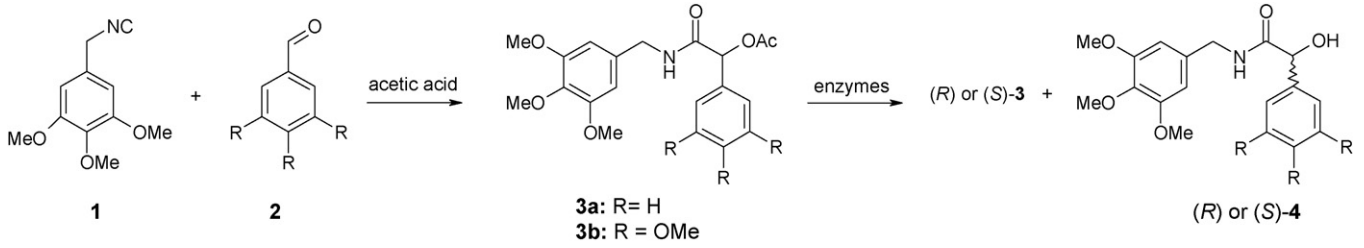
Fig. 1. Biologically active podophyllotoxin (**I**) containing the biaryl system with methoxy groups and its structural analogue **II**.

2. Results and discussion

The structural analogues of podophyllotoxin, *viz.*, acetic acid aryl(3,4,5-trimethoxy benzylcarbamoyl)methyl esters 3, were readily obtained *via* Passerini reaction from the 3,4,5-trimethoxybenzyl isocyanide (**1**) and the corresponding aryl aldehydes **2** (Scheme 1) in the yield of 73% and 63%, respectively. Both these products were obtained as racemates.

In the preliminary study, nine commercially available enzymes were screened as biocatalysts for the kinetic resolution of acetic acid phenyl(3,4,5-trimethoxy benylcarbamoyl)methyl ester (**3a**) and acetic acid (3,4,5-trimethoxybenzylcarbamoyl)(3,4,5-trimethoxy phenyl)methyl ester (**3b**). These reactions were conducted at pH 7.4 in phosphate buffer at 36 °C. The first substrate had a very low solubility; therefore, it was essential to use a great excess of an organic co-solvent, which undoubtedly influenced the enzyme activity. According to literature, the organic solvent can affect not only the enzyme activity, but also the stability and, in many cases, the enantioselectivity [32,33].

Three different esterases [rabbit liver esterase (RLE), porcine liver esterase (PLE)] and pig liver acetone powder (PLAP), five native lipases [from: *Rhizopus arhizeus* (RAL), *Pseudomonas cepacia* (PCL), porcine pancreas (PPL), wheat germ (WGL), hog pancreas (HPL)] and one protease [Amano AK protease] were tested as biocatalysts. It has been already known that the porcine pancreas lipase may exhibit the esterase activity [34–37]. The commercially available enzymes were used in the condition as received from the suppliers, without being purified before the immobilization.



Scheme 1. Enzymatic kinetic resolution of aryl-(3,4,5-trimethoxybenzylcarbamoyl)methyl ester **3a** and **3b**.

Table 1
Results of the enzymatic hydrolysis of **3a** and **3b**[a]

Entry	Enzyme	Product	*c* (%)[b]	Time (h/day)	% ee[c]	*E*[d]
1	RLE	(*S*)-**4a**	0	–/1	nd	nd
2	PLE	(*S*)-**4a**	70	–/1	34	4.5
3	PLAP	(*S*)-**4a**	36	–/1	26	1.9
4	*Rhizopus arhizeus*	(*S*)-**4a**	72	–/1	41	10
5	*P. cepacia*	(*S*)-**4a**	15	–/8	64	5.1
6	Porcine pancreas	(*S*)-**4a**	0	–/1	nd	nd
7	Wheat germ	(*S*)-**4a**	0	–/1	nd	nd
8	Hog pancreas	(*S*)-**4a**	0	–/1	nd	nd
9	Amano protease AK	(*S*)-**4a**	27	–/1	76	9.7
10	RLE	**4b**	0	–/1	nd	nd
11	PLE	**4b**	0	–/1	nd	nd
12	PLAP	**4b**	0	–/1	nd	nd
13	*R. arhizeus*	**4b**	0	–/1	nd	nd
14	*P. cepacia*	**4b**	5	–/5	89	17
15	Porcine pancreas	**4b**	48	7/–	42	3.5
16	Wheat germ	**4b**	25	9/–	70	7.1
17	Hog pancreas	**4b**	40	9/–	37	2.7
18	Amano protease AK	**4b**	41	–/2	15	1.5

[a] Conditions: **3a** (0.02 mmol, 8.0 mg), phosphate buffer pH 7.4 (0.7 mL), acetonitrile (0.6 mL), the enzyme (2.0 mg), 36 °C; **3b** (0.02 mmol, 8.0 mg), phosphate buffer pH 7.4 (0.8 mL), acetone (0.2 mL), the enzyme (2.0 mg), 36 °C.

[b] Determined by HPLC on the reverse phase column.

[c] Determined by HPLC on the Daicel Chiracel OD-H column.

[d] Calculated according to Chen et al. [38], using the equation: $E = (\ln[1 - c(1 + ee_p)])/(\ln[1 - c(1 - ee_p)])$.

Table 2
Results of the enzymatic hydrolysis of **3a** and **3b** by the immobilised enzymes

Entry	Immobilised enzyme	*c* (%)	Time (h/day)	(*R*)-**3a** % ee[a]	(*S*)-**4a** % ee[a]	*E*[c]	*c* (%)	Time (h/day)	**3b** % ee[a]	**4b** % ee[a]	*E*[c]
1	Native PCL	23	–/10	78 (68)	62 (19)	5.1	15	–/10	15 (80)	89 (11)	17
2	PCL-Eupergit®	58	–/8	99 (40)	72 (56)	34	0	–/1	nd	nd	nd
3	PCL-Ca alginate	39	–/8	30	54	4.6	0	–/1	nd	nd	nd
4	PCL-Celite® 545	70	–/8	28	23	2.5	0	–/1	nd	nd	nd
5	PCL-sol–gel, *i*-BTMS	41	–/8	48	70	9.1	0	–/1	nd	nd	nd
6	PCL-sol–gel, PTMS, Celite® 545	40	–/8	87	75	11.4	0	–/1	nd	nd	nd
7	PCL-sol–gel, *i*-BTMS, Celite® 545	42	–/8	89 (56)	86 (41)	25.1	0	–/1	nd	nd	nd
8	PCL-sol–gel, PTMS, inprint.	20	–/8	78 (76)	80 (18)	10.9	10	–/5	10 (88)	91 (8)	23.4
9	PCL-sol–gel, PTMS, Tween 80®	39	–/7	87 (59)	89 (36)	30.5	0	–/1	nd	nd	nd
10	native PPL	0	–/1	nd	nd	nd	48	–/7	43	42	3.5
11	PPL-Eupergit®	0	–/1	nd	nd	nd	25	–/6	12	27	1.9
12	PPL-Ca alginate	0	–/1	nd	nd	nd	41	20/–	40	56	5.1
13	PPL-Celite® 545	0	–/1	nd	nd	nd	53	5/–	55	46	4.4
14	Native WGL	0	–/1	nd	nd	nd	25	9/–	26[b]	70[b]	7.1
15	WGL-sol–gel, PTMS, Celite® 545	0	–/1	nd	nd	nd	23	–/6	11	40	2.6
16	WGL-Ca alginate	0	–/1	nd	nd	nd	37	20/–	37[b]	65[b]	6.8
17	Native HPL	0	–/1	nd	nd	nd	40	9/–	29	37	2.7
18	HPL-Eupergit®	0	–/1	nd	nd	nd	32	–/6	17	36	2.5
19	HPL-sol–gel, PTMS, Celite® 545	0	–/1	nd	nd	nd	42	–/6	3	13	1.4

[a] ee determined by HPLC on the Chiracel OD-H column. The isolated yields are given in brackets.
[b] Opposite absolute configuration.
[c] Calculated according to Chen et al. [38], using the equation: $E = (\ln[1 - c(1 + ee_p)])/(\ln[1 - c(1 - ee_p)])$.

We found that all the tested enzymes exhibited an activity towards hydrolysis of the target compounds. However, only the lipases from *R. arhizeus*, *P. cepacia*, porcine pancreas, wheat germ, and the Amano AK protease exhibited good enantioselectivity in the kinetic resolution of the compounds **3a** and **3b** (Table 1).

As a general rule, enantioselectivities below 15 are unacceptable for practical purposes. Enantioselectivities in the range of 15–30 may be regarded as good, while enantioselectivities over 30 are excellent [2]. Unfortunately, the enzymes selected by the preliminary screening exhibited low levels of enantioselectivity. In order to increase the stereoselectivity of these biocatalysts, four different immobilization techniques were used: covalent immobilization on Eupergit® [39], adsorption on **Celite**® 545 [39], encapsulation in Ca-alginate beads [40] and in a sol–gel matrix [41,42]. Table 2 shows the results obtained for the immobilization of the selected enzymes. The immobilization yields were above 43%.

The immobilization of the *P. cepacia* lipase on Eupergit® C enhanced the enantioselectivity by a factor of 7 ($E = 34$, Entry 2, Table 2). Application of this biocatalyst allowed us to prepare the ester **3a** in a good chemical yield and with a very good enantiomeric excess of 99%. The immobilization on the other supports was less efficient, while the same procedure using Celite® 545 was a complete failure, since the enantioselectivity was very low ($E = 2.5$, Entry 4, Table 2). When the substrate was replaced with the compound **3b**, the porcine pancreas lipase turned out to be a good biocatalyst of this reaction. The reaction lasted 7 h but the enantioselectivity was also low ($E = 3.5$; Entry 10, Table 2). During the immobilization of this enzyme on Eupergit® C towards **3b**, its stereoselectivity was almost completely lost, when compared to **3a**. Although the time of the reaction increased to 6 days, the enantioselectivity was very low ($E = 1.9$, Entry 11, Table 2). The immobilization of the porcine pancreas lipase on Ca-alginate gel beads was more promising. The reaction time decreased to 20 h and the selectivity was slightly better ($E = 5.1$, Entry 12, Table 2). The best result, although still not satisfactory, was obtained upon the immobilization of the enzyme on Celite® 545. The reaction time decreased to 5 h and the selectivity increased to 4.4 (Entry 13, Table 2). The best enantioselectivity enhancement was obtained for the *P. cepacia* lipase immobilised in the sol–gel matrix (Entry 8, Table 2). Native wheat germ and hog pancreas lipases exhibited low enantioselectivity (Entries 14 and 17, Table 2)—the immobilization of these enzymes did not enhance their enantioselectivities (Entries 15–16 and 18–19, Table 2). Unfortunately, the immobilization of the Amano AK protease (Entry 18, Table 1) resulted in a complete loss of activity (data not shown).

3. Conclusions

The lipase catalysed kinetic resolution of aryl-(3,4,5-trimethoxybenzylcarbamoyl)methyl esters afforded low enantioselectivities with the native enzymes. The enantioselectivity was significantly increased and the reaction time decreased upon proper immobilization of the enzymes. The best results were obtained for the immobilization of the *P. cepacia* lipase both on Eupergit® C and in a sol–gel matrix. Our research demonstrated the significance of a proper enzyme immobilization procedure for the synthesis of new bioactive compounds.

4. Experimental

4.1. General

The NMR spectra were recorded in $CDCl_3$ with TMS as an internal standard using a 200 MHz Varian Gemini 200 spectrometer. The chemical shifts are reported in ppm and the coupling constants (J) are given in hertz (Hz). The MS spectra were recorded on an API-365 (SCIEX) apparatus. The IR spectra were recorded in $CHCl_3$ on a Perkin-Elmer FT-IR Spectrum 2000 apparatus. The HPLC analyses were performed on a Chiracel OD-H column (4.6 mm × 250 mm, from Diacel Chemical Ind., Ltd.) equipped with a pre-column (4 mm × 10 mm, 5 μm) using an LC-6A Shimadzu apparatus with UV SPD-6A detector and Chromatopac C-R6A analyser. The elemental analyses were performed on CHN Perkin-Elmer 240 apparatus. The melting points were uncorrected. All the reactions were monitored by TLC on Merck silica gel Plates 60 F_{254}.

The lipases from *R. arhizeus*, porcine pancreas, hog pancreas, and the esterases from rabbit liver and porcine liver were purchased from Fluka. The lipases from wheat germ and *P. cepacia* were purchased from Sigma. The Amano AK protease was purchased from Aldrich. The pig liver esterase was prepared in our laboratory.

All the chemicals were obtained from commercial chemical sources. The solvents were of analytical grade.

4.2. Protein determination

In order to determine the loading efficiency (the amount of the immobilised enzyme), the Lowry Protein Assay Kit of Sigma was used. Accordingly, the solutions of each respective enzyme were incubated for 30 min and then the absorbance was measured at room temperature using a UV–vis spectrophotometer at 595 nm according to the Technical Bulletin of Sigma. Distilled water was used as a reference solution, and BSA was used as a standard.

The loading efficiency was calculated from the difference between the initial and final concentrations of the enzyme, i.e., before and after the immobilization.

Before the immobilization, the powdered commercial enzymes (50–150 mg) were suspended in 50 mM phosphate buffer (1 mL, pH 7.4) for 20 min. The supernatant obtained after 15 min of centrifugation at 4000 rpm at 4 °C served as the source of the enzyme in question.

4.3. The immobilization of enzymes on Eupergit® C:

The enzyme supernatant (0.8 mL) was diluted with 50 mM phosphate buffer (1 mL, pH 7.4). Beads of the epoxy-activated Eupergit®C resin (150 mg) were added to the enzyme solu-

tion at room temperature and left for 1 day. The immobilised enzyme was separated by vacuum filtration and rinsed on the filter with water (2× 10 mL). The filtered solution and washings were collected for determination of the loading efficiency.

The loading efficiency of the *P. cepacia* lipase was found to be 82%. The loading efficiency of the porcine pancreas lipase was estimated to be 80%.

4.4. The immobilization of the lipases within calcium alginate beads

The enzyme solution (0.8 mL, 0.9–4 mg protein/mL) was mixed with aqueous sodium alginate (8 mL, 2%, v/v), then stirred thoroughly to ensure thorough mixing. As the mixed solution was added dropwise through a syringe into 10 mL of $CaCl_2$ solution (300 mM), the calcium alginate beads formed. The bead size was altered by using different needle diameters. After 30 min of curing, the beads were separated from the calcium chloride solution by vacuum filtration. They were rinsed on the filter with water (2 ×10 mL). The filtered solution and washings were collected for determination of the loading efficiency.

The loading efficiencies of the lipases from *P. cepacia*, porcine pancreas, and wheat germ were determined as 94%, 74%, and 82%, respectively.

4.5. The immobilization on Celite® 545

At room temperature, an aqueous enzyme solution (0.2 mL, 0.225 mg/mL) in carbonate buffer (0.8 mL, 50 mM, pH 9.4) was mixed with Celite® 545 (100 mg). The mixture was shaken for 30 min and dried under vacuum at room temperature. The catalyst was stored as a dry material at 4 °C until activity was determined. The loading efficiency of porcine pancreas lipase was 43%.

4.6. Entrapment of the lipase in a sol–gel matrix with Celite® 545

At room temperature, 0.7 mmol of the mixture of PTMS or iBTMS and TMOS at a molar ratio of the organic silane to TMOS of 4, and 2 μL of 40 mM HCl were mixed in a glass vial in order to obtain a homogeneous solution. Then 1 mL of 50 mM phosphate buffer (pH 7.4) was added to the mixture at the temperature of 36 °C, and then 0.7 mL of enzyme solution was mixed with the buffered solution. The resultant mixture was blended with 250 mg of the Celite® 545 powder in a Petri dish, and left for 1 day at room temperature. The obtained solid mass was rinsed with water (2× 10 mL) on a filter twice, and then dried under vacuum. The filtered solution and washings were collected for determination of the loading efficiency. The loading efficiency of the *P. cepacia* lipase on PTMS and iBTMS turned out to be 88 and 90%, respectively. The loading efficiency of wheat germ lipase on PTMS was 65%; the loading efficiency of hog pancreas lipase was 75%.

4.7. Entrapment of the lipase in a sol–gel matrix

At room temperature, 0.37 mL of the enzyme solution was mixed with an additive of Tween 80®, 0.18 mL and D,L-phenylalanine methyl ester hydrochloride, 20 mg. Next, 0.1 mL of aqueous PVA (4%, w/v), aqueous sodium fluoride (50 μL of a 1 M solution) and isopropyl alcohol (0.1 mL) were added. The mixture was blended thoroughly. Then the alkylsilane (2.5 mmol) and TMOS (0.5 mmol) were added, and the mixture was agitated for 15 min, and let dry overnight in an open flask. Isopropyl alcohol (10 mL) was added and the gel was washed with distilled water (10 mL) and hexane (10 mL). The lipase immobilizate was dried in the air at room temperature. The filtered solution and washings were collected for determination of the loading efficiency. The loading efficiency of the *P. cepacia* lipase was found to be 71% for PTMS with D,L-phenylalanine methyl ester hydrochloride, 65% for iBTMS, and 60% for iBTMS with Tween 80®.

*4.8. Synthesis of 3,4,5-trimethoxybenzyl isocyanide (**1**)*

A solution of 3,4,5-trimethoxybenzylamine (2 mL, 12 mmol) in ethyl formate was heated under reflux for 30 h. The mixture was concentrated under vacuum, dissolved in dichloromethane (10 mL), washed three times with 5% aq. Na_2CO_3 (10 mL), 5% HCl (20 mL) and distilled water. The organic layer was dried over anhydrous $MgSO_4$ and the solvent was evaporated under vacuum. The product was purified by crystallisation from hexane/ethyl acetate, and was obtained in 96% yield as yellow crystals: *mp* 71–73 °C; ^{1}H NMR δ 3.77 (s, 3H), 3.79 (s, 6H), 4.35 (d, J = 5.86 Hz, 2H), 6.45 (s, 2H), 8.22 (s, 1H); ^{13}C NMR δ 42.7, 56.4, 61.1, 104.1, 105.0, 133.7, 137.4, 153.5, 161.3. A solution of the obtained amide (1.28 g, 5.7 mmol) in dry dichloromethane (3 mL) with dry triethylamine (3 mL, 22 mmol) was cooled to −20 °C. Then the solution of phosphorus oxychloride (0.58 mL, 6.3 mmol) in dry dichloromethane (1 mL) was added carefully. The mixture was blended for 2 h at 5 °C. Distilled water (2 mL) was added at the same temperature and the mixture was extracted three times with dichloromethane (20 mL). The combined organic layers were washed with 10% sodium hydrogen carbonate (10 mL) and distilled water (20 mL), dried over anhydrous $MgSO_4$ and passed through silica gel. The solvent evaporated under vacuum and the product was obtained in 90% yield in form of white crystals: *mp* 47–50 °C; ^{1}H NMR δ 3.84 (s, 3H), 3.87 (s, 6H), 4.57 (s, 2H), 5.13 (s, 1H), 6.54 (s, 2H); ^{13}C NMR δ 45.8, 46.1, 56.5, 61.2, 104.0, 128.1, 138.2, 153.9; Anal. Calcd. for $C_{11}H_{13}NO_3$: C, 63.76; H, 6.32; N, 6.76; Found: C, 63.79; H, 6.30; N, 6.82.

*4.9. The general procedure 1 for the synthesis of the α-acetoxyamides **3**:*

To the solution of acetic acid (3.3 mmol) in dichloromethane (3 mL), the corresponding aldehyde **2** (3.0 mmol), and 3,4,5-trimethoxybenzyl isocyanide (**1**) (3.3 mmol) were added at room temperature. The reaction mixture was stirred for 24 h at room temperature and then concentrated *in vacuo*. The crude

product was purified by silica gel flash chromatography using hexane/ethyl octane (6:4, v/v) as an eluent to afford the corresponding product **3**.

4.10. Acetic acid phenyl-(3,4,5-trimethoxybenzylcarbamoyl)methyl ester (*3a*)

The ester **3a** was obtained according to the general procedure 1 in 73% yield as white crystals (EtOAc/hexane): *mp* 179–180 °C; $R_f = 0.30$ (EtOAc/hexane, 1:1, v:v); ^{1}H NMR δ 2.18 (s, 3H), 3.76 (s, 6H), 3.81 (s, 3H), 4.25–4.56 (m, 2H), 6.08 (s, 1H), 6.38 (s, 2H), 6.48 (s, 1H), 7.30–7.54 (m, 5H); ^{13}C NMR δ 21.4, 43.7, 56.4, 61.2, 76.0, 104.6, 127.4, 127.5, 129.1, 129.3, 153.6, 168.6, 169.5; IR (film) ν_{max} cm^{-1}: 1661 (CONH), 1729 (CO); HPLC analysis [hexane/*i*-PrOH 6:4; $\lambda = 223$ nm; 1.0 mL/min; t_R (*R*) = 9.35 min, t_R (*S*) = 12.05 min]; HPLC analysis [acetonitrile/H_2O; 6:4; v:v; $\lambda = 223$ nm; 1.0 mL/min] $t_R = 9.50$ min; Anal. Calcd. for $C_{20}H_{23}NO_6$: C, 64.33; H, 6.21; N, 3.75; Found: C, 64.20; H, 6.51; N, 3.83. (*R*)-**3a** was obtained from (*R*)-O-acetylmandelic chloride and benzylamine; $[\alpha]_D^{25} = -90.2$ (c 1.0, $CHCl_3$); HPLC analysis [hexane/*i*-PrOH 6:4; $\lambda = 223$ nm; 1.0 mL/min; t_R (*R*) = 9.35 min] 100% ee;

4.11. Acetic acid (3,4,5-trimethoxybenzylcarbamoyl)(3,4,5-trimethoxyphenyl)methyl ester (*3b*)

The ester **3b** was obtained according to the general procedure 1 in 63% yield as white crystals (EtOAc/hexane): *mp* 130–132 °C; ^{1}H NMR δ 2.19 (s, 3H), 3.75-3.87 (m, 18H), 4.28-4.58 (m, 2H), 5.99 (s, 1H), 6.34–6.48 (m, 3H), 6.66 (s, 2H); ^{13}C NMR δ 21.4, 43.8, 56.6, 61.2, 76.0, 104.8, 105.0, 114.9, 131.0, 153.7, 168.4, 169.6; HPLC analysis [hexane/*i*-PrOH 6:4; v:v; $\lambda = 223$ nm; 0.8 mL/min; $t_{R1} = 15.80$ min, $t_{R2} = 20.70$ min]; HPLC analysis [acetonitrile/H_2O 6:4; $\lambda = 223$ nm; 1.0 mL/min] $t_R = 6.88$ min; Anal. Calcd. for $C_{23}H_{29}NO_9$: C, 59.60; H, 6.31; N, 3.02; Found: C, 59.31; H, 6.29; N, 3.12.

4.12. The general procedure 2 for the synthesis of the α-hydroxyamides *4*:

One molar of NaOH solution (10 mL) was added to the solution of the α-acetoxyamide **3** (3.3 mmol) in methyl alcohol (3 mL). The reaction mixture was sonicated at room temperature for 30 min in an ultrasound washer, then methyl alcohol was evaporated under reduced pressure. The mixture was extracted three times with chloroform and the solvent was evaporated under reduced pressure. The crude product was purified by silica gel flash chromatography using hexane/ethyl octane (6:4, v/v) as an eluent to afford the corresponding product **4**.

4.13. 2-Hydroxy-2-phenyl-N-(3,4,5-trimethoxybenzyl)acetamide (*4a*)

The hydroxyamide **4a** was obtained according to the general procedure 2 in 95% yield as a colourless oil; ^{1}H NMR δ 3.84 (s, 6H), 3.89 (s, 3H), 4.49 (d, $J = 6$ Hz, 2H), 5.20 (s, 1H), 6.43 (s, 2H), 6.51 (s, 1H), 7.40-7.58 (m, 5H); ^{13}C NMR δ 43.8, 56.3, 61.1, 74.5, 104.5, 126.7, 128.9, 129.1, 133.8, 139.8, 153.6, 172.5; HPLC analysis [hexane/*i*-PrOH 6:4; $\lambda = 223$ nm; 1.0 mL/min] t_R (*R*) = 7.95 min, t_R (*S*) = 10.18 min; HPLC analysis [acetonitrile/H_2O; 6:4; v:v; $\lambda = 223$ nm; 1.0 mL/min] $t_R = 4.50$ min; ESI-MS (*m*/*z*): Calcd. for $C_{18}H_{21}NO_5$ ($[M+Na]^+$): 354.1317; Found 354.1312; (*R*) **4a** was obtained by hydrolysis of (*R*)-**3a** with 1M NaOH; $[\alpha]_D^{25} = -76.9$ (c 1.0, $CHCl_3$); HPLC analysis [hexane/*i*-PrOH; 6:4; v:v; $\lambda = 223$ nm; 1.0 mL/min] t_R (*R*) = 7.95 min, 100% ee.

4.14. 2-Hydroxy-2-N-(3,4,5-trimethoxybenzyl)-2-(3,4,5-trimethoxyphenyl)acetamide (*4b*)

The hydroxyamide **4b** was obtained according to the general procedure **2** in 90% yield as a colourless oil; ^{1}H NMR δ 3.80–4.00 (m, 18H), 4.38–4.60 (m, 2H), 5.11 (s, 1H), 6.48 (s, 2H), 6.56 (s, 1H), 6.71 (s, 2H), 7.34 (s, 1H); ^{13}C NMR δ 43.9, 56.4, 61.1, 74.5, 103.9, 104.8, 108.5, 133.8, 135.4, 153.7; HPLC analysis [hexane/*i*-PrOH 6:4; $\lambda = 223$ nm; 0.8 mL/min] $t_{R1} = 14.80$ min, $t_{R2} = 21.33$ min; HPLC analysis [acetonitrile/H_2O; 6:4; v:v; $\lambda = 223$ nm; 1.0 mL/min] $t_R = 3.93$ min; Anal. Calcd. for $C_{21}H_{27}NO_8$: C, 59.85; H, 6.46; N, 3.32; Found: C, 59.87; H, 6.61; N, 3.27.

4.15. Enzymatic kinetic resolution of the α-acetoxyamides *3a*

The activity of the immobilised enzymes was assayed by adding 20–30 mg of a biocatalyst to 13 mL of the substrate **3a** (100 mg, 0.27 mmol) in 50 mM sodium phosphate with 50% (v/v) acetonitrile and shaking at 36 °C. Blank experiments were performed by using suspensions of the support material. The reactions were terminated by filtering off the enzyme. The aqueous solution was extracted with chloroform (3× 5 mL) and the combined organic layers were evaporated under reduced pressure. The mixture was separated on silica gel flash chromatography using hexane/ethyl octane (6:4, v/v) as an eluent.

The degree of hydrolysis was followed by a reverse-phase HPLC (LC-6A Shimadzu apparatus with UV SPD-6A detector and Chromatopac C-R6A analyser) on a Kromasil C18 (250 mm × 4 mm) column supplied by Amersham. Each assay was performed at least three times. The experimental error was approx. 5%. The mobile phase consisted of acetonitrile (60%) and distilled water, at a flow rate of 1.0 mL/min. The elution was monitored by recording the absorbance at 223 nm. The retention time of the ester **3a** was 9.50 min and retention time of the alcohol **4a** was 4.50 min.

Under the researched conditions, both the spontaneous hydrolysis and the adsorption of the substrate/product on the immobilization matrices were found to be lower than the experimental error.

4.16. The enzymatic kinetic resolution of the α-acetoxyamides *3b*

The immobilised enzymes (20–30 mg) were added to 13 mL of the compound **3b** (100 mg, 0.21 mmol) in 50 mM sodium

phosphate with 20% acetone (v/v) under shaking at 36 °C. The reactions were terminated by filtering off the enzyme. The aqueous solution was extracted with chloroform (3× 5 mL), and the combined organic layers were evaporated under reduced pressure. The components of the mixture were separated by a silica gel flash chromatography using hexane/ethyl octane 6:4 (v:v) as an eluent.

The retention time of the ester **3b** was 6.88 min and retention time of the alcohol **4b** was 3.93 min.

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