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Enzymatic Amidation and Alkoxycarbonylation of Amines using Native and Immobilised Lipases with Different Origins: a Comparative Study

M. Soledad de Castro, Pablo Domínguez and José V. Sinisterra*

Department of Organic and Pharmaceutical Chemistry, Faculty of Pharmacy, Universidad Complutense, Madrid, Spain

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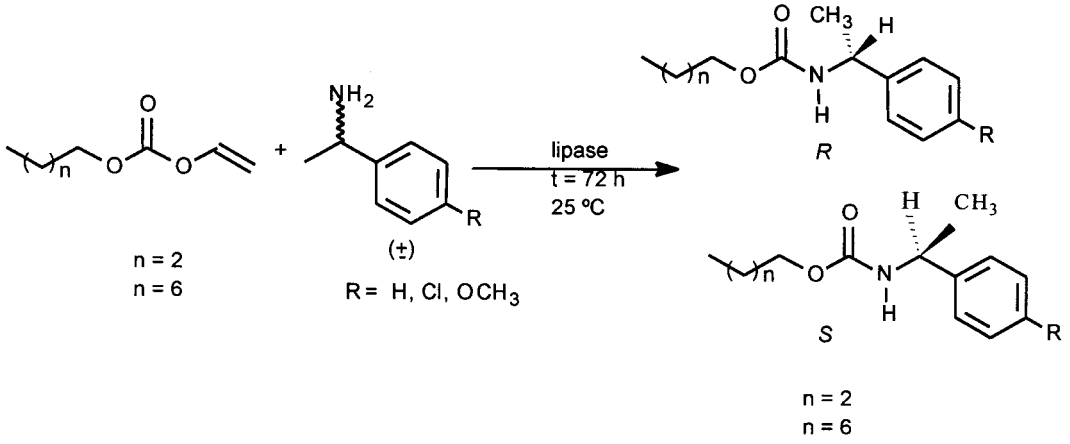
Abstract—Enzymatic alkoxycarbonylation with vinyl carbonates and racemic amines can provide chiral carbamates. In the present paper, we have investigated the catalytic potential of some commercial lipases, with different origins. We have used the alkoxycarbonylation of (*R,S*)-1-phenylethylamine, analysing the influence on the yield and enantioselectivity of some characteristics of the biocatalyts, such as the origin of the lipase and whether the lipase is immobilised or not. We have also investigated the influence on the yield of the chain length of the vinyl carbonate used as the acyl donor. Finally, we have probed this reaction, under the same conditions, with the chiral amines substituted in the aromatic ring, using *p*-chloro and *p*-methoxy-1-phenylethylamine and butyl vinyl carbonate.

Introduction

Lipases have been widely used for the preparation of chiral alcohols, esters and carboxylic acids through the corresponding asymmetric esterification and transesterification reactions.[1,2] Recently, these enzymes have been used in the preparation of some achiral or chiral amides by aminolysis of esters.[3–5] Carbamate derivatives are used in the synthesis of compounds with medical properties and insecticides and pesticides.[6] Due to the interest of the carbamate group, many reagents and synthetic procedures have been developed. In general these processes involve specific reactions, and, unfortunately, in many cases toxic reagents such as phosgene.[7] Therefore, a new enzymatic synthesis of these compounds would be very useful in order to improve the safety of reaction conditions and the enantioselectivity of the process. In this way *Candida antarctica* lipase[8] and esterases[9] have been used to prepare carbamates with high or moderate yields and e.e.'s (Scheme 1).

In the resolution of racemic acids and/or alcohols, many studies have been carried out into the influence on the



Scheme 1.

Keywords: lipase; resolution of amines; enantioselectivity; carbamate; amides.

* Corresponding author. Fax: +9-1-3941822.

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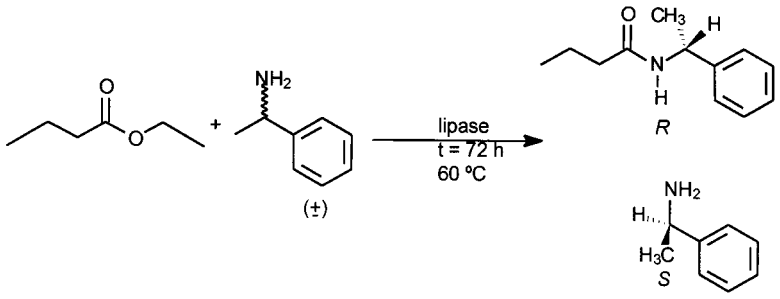

Scheme 2.

Table 1. Synthesis of *N*-1-phenylethyl butyl carbamate. Vinyl carbonate=1.2 mmol, (*R*,*S*)-1-phenylethylamine=2 mmol, 200 mg of solid biocatalyst, *V*=15 ml of hexane, *T*=25°C, *t*=72 h

Lipase	Yield (%)[a]	e.e. (%)[b]	Configuration
PS	25	40	*S*>*R*
AK	47	99	*R*⩾*S*
Newlase F	50	99	*S*⩾*R*
SP524	80	12	*R*>*S*
IM20	39	94	*R*>*S*
SP523	64	90	*R*>*S*
CRL	77	30	*R*>*S*
SP525	68	54	*R*>*S*
SP526	13	99	*R*⩾*S*
NOV-435	79	75	*R*>*S*
PPL	71	33	*R*>*S*

[a] Calculated by HPLC.
[b] Determined by ^{1}H NMR.

yield and/or the e.e. of: (i) the technical variables;[10] (ii) the purity degree of the enzymes;[11] (iii) the origin of the lipase[12] and (iv) the structure of the substrates.[10,13] Nevertheless, there are very few articles concerning these topics as applied to the resolution of racemic amines by the enzymatic alkoxycarbonylation or amidation.[14] In this paper we investigate the catalytic potential of some commercial lipases, with different origins, in the alkoxycarbonylation of racemic amines. We have analysed the influence on the yield and enantioselectivity of the carbamate synthesis, of some biotechnological properties of the biocatalyts such as: (i) isoenzymes, (ii) origin of the lipase and (iii) nature of the support. The results are compared to the classic aminolysis of esters (Scheme 2).[14]

Table 2. Synthesis of *N*-1-phenylethylamine octyl carbamate. Vinyl carbonate=1.2 mmol, (*R*,*S*)-1-phenylethylamine=2 mmol, 200 mg of solid biocatalyst, *V*=15 ml hexane

Lipase	Yield (%)[a]	e.e. (%)[b]	Configuration
PS	39	90	*S*>*R*
AK	37	95	*R*>*S*
Newlase F	38	95	*S*>*R*
SP524	86	14	*R*>*S*
IM20	64	80	*R*>*S*
SP523	74	44	*R*>*S*
CRL	41	<10	*R*>*S*
SP525	45	20	*R*>*S*
SP526	52	>95	*R*>*S*
NOV-435	69	<10	*R*>*S*
PPL	60	<10	*R*>*S*

[a] Calculated by HPLC.
[b] Determined by ^{1}H NMR.

The lipases selected were:

1. **Fungal lipases:** *Rhizomucor miehei* lipase, native lyophilised (**SP524**) and immobilised by adsorption on anionic resin (Duolite A568) (**IM20**). Native lyophilised lipase from *Rhizopus niveus* (Newlase F). Native lyophilised *Humicola lanuginosa* lipase (**SP523**).
2. **Yeast lipases:** *Candida antarctica* lipase B, native lyophilised (**SP525**) or adsorbed on Lewatit E (**Novozym 435**). Native lyophilised lipase A from *C. antarctica* (**SP526**), to explore the different activity of both isoenzymes of the *C. antarctica.* Native lyophilised *Candida rugosa* lipase (**CRL**).
3. **Bacterial lipases:** native lyophilised *Pseudomonas cepacia* lipase (**PS**) and native lyophilised *Pseudomonas fluorescens* lipase (**AK**).
4. **Mammalian lipase:** native lyophilised porcine pancreatic lipase (**PPL**).

Results and Discussion

In order to directly compare all the commercial biocatalysts, the same amount of solid was used and all the reactions were stopped at 72 h (Tables 1 and 2). The direct aminolysis of esters (Table 3) was performed under more severe conditions than the alkoxycarbonylation but the aminolyses were also stopped at 72 h. We have selected this philosophy of using the same amount of lyophilised powder, although we know that the specific activities of each biocatalyst is different.[15] We have proved that the number of units of each catalyst varies depending on the nature of the considered

Table 3. Aminolysis of ethyl butyrate by (*R*,*S*)-1-phenylethyl amine, 5 mmol of ester and 3.5 mmol of amine. *V*=30 ml hexane, 100 mg of solid biocatalyst, *T*=60°C, *t*=72 h

Lipase	Yield (%)[a]	e.e. (%)[b]	Configuration
PS	22	80	*R*>*S*
Newlase F	23	99	*R*⩾*S*
SP524	41	62	*R*>*S*
IM20	30	99	*R*⩾*S*
SP523	10	99	*R*⩾*S*
CRL	20	99	*R*⩾*S*
SP525	23	99	*R*⩾*S*
SP526	59	95	*R*>*S*
NOV-435	66	82	*R*>*S*
PPL	9	92	*R*>*S*

[a] Calculated by HPLC.
[b] Determined by ^{1}H NMR.

reaction—hydrolysis or synthesis in organic media—and the structure of the substrates and the fermenter conditions.[16,17]

In Tables 1–3 we can observe that very different yields and e.e.'s are obtained depending on the origin of the lipase. Generally the carbamate with the *R*-configuration is obtained as the main product, as observed for the aminolysis of ethyl butyrate (Table 3). Only the lipases of *P. cepacia* (PS) and of *R. niveus* (Newlase) showed the opposite enantiopreference to that described for the aminolysis of ethyl butyrate (Table 3). If we compare the results with those obtained for the preparation of octyl carbamate (Table 2) we can observe that the enantiopreference is the same in both cases. Therefore, we can conclude that the enantiopreference observed for one lipase in a concrete aminolysis reaction cannot be extrapolated to all the aminolysis reactions catalysed by this lipase because the structure of the acyl donor plays an important role. This situation is different to that reported in the resolution of racemic secondary alcohols by alcoholysis of acids or esters where the (*R*) ester is produced.[13] We observed the same (*R*)-enantiopreference in the aminolysis of esters (Table 3) as described in literature.[18] Pozo and Gotor[8] have also described, *using only immobilised C. antarctica lipase*, SP435A the formation of octyl or butyl carbamates with *R*-configuration.[19] These results were assumed as absolute and *R*-stereopreference is indicated in the biotransformation literature for the aminolysis reactions. Nevertheless, the stereochemistry of the process is controlled by the origin of the enzyme as shown in Tables 1 and 2, and it was described by Klibanov's group[20] in the acylation of (*R*,*S*)-α-methylbenzylamine with trifluoroethyl butyrate in octane. These authors described different ratios V_S/V_R, for several enzymes (subtilisine Carlsberg=1.4, Porcine Pancratic=1.5, *C. cylindracea*=0.88, *Chromobacterium viscosum*=0.66). On the other hand, Maestro et al.[21] described a change in the enantioselectivity observed in the aminolysis process, depending on the process and the substrates, using *C. antarctica* lipase. The alkoxycarbonylation of (*R*,*S*)-2-aminocyclohexanol gave the carbamate (1*S*,2*S*) but the acylation of the same amine with dimethyl malonate or dimethyl glutarate gave (1*R*,2*R*) amide. Therefore, the *R*-enantiopreference of the aminolysis reaction is not absolute.

We can observe that isoenzyme A from *C. antarctica* is a good biocatalyst in both reactions, which is according to literature,[14] where this isoenzyme presents better enzymatic activity than isoenzyme B in non-conventional reactions. The difference between both isoenzymes is larger in the synthesis of carbamates (Tables 1 and 2)—especially with the largest acylating agent—than in the synthesis of amides (Table 3). *C. antarctica* lipase B (SP525) shows lower stereoselectivity than isoenzyme A (SP526). These differences must be related to the acylating agent recognition subsite of both isoenzymes. This result agrees with the different specificity for triglycerides described for both isoenzymes.[22,23] The isoenzyme A (SP526) accepts larger triglycerides than isoenzyme B (SP525). Besides SP526 is very sensitive to the hydrophobicity of the acyl donor because ethyl butyrate (hydrophobic) is better recognised (59% yield Table 3) than butyl vinyl carbamate (13% yield Table 1), which has a similar molecular size but a different polarity. Finally, SP526 is strongly stereoselective in the amine recognition sub-site because excellent e.e.'s are obtained in all cases (Tables 1–3). Therefore, we observed that SP525 is more stereoselective (small pocket) than SP526 (large pocket)[22,23] in the resolution of small racemic acids such as ethyl (*R*,*S*)-2-methyl-butyrate via aminolysis reaction (SP525 75% yield 86% e.e. and SP526 80% yield 46% e.e.).[14] On the other hand both isoenzymes show the same *R*-enantiopreference.

The immobilisation of SP525 by adsorption—Novozym 435—slightly increases the yield and e.e., but the same *R*-enantiopreference remains because the active site of the enzyme is not affected by the adsorption immobilisation methodology. The increase in the yield (Novozym 435 versus SP525) is dramatic in the aminolysis of ester (Table 3) that was performed at 60°C. This is explained because SP525 is not very thermostable[19] and it is stabilised after the immobilisation process.

In general, the immobilised lipase from *C. antarctica* B (Novozym 435) and *R. miehei* lipase (IM20) are more active as biocatalysts than the same crude enzymes SP525 (*C. antarctica* B) or SP524 (*R. miehei* lipase).[24] We also observe that longer the size of the acyl donor, the more active the *R. miehei* lipase free (SP524) or immobilised (IM20) is versus the *C. antarctica* lipase B free (SP525) and immobilised (Novozym 435). This result is according to the relative size reported in the literature[25] of the recognition site for *R. miehei* lipase, larger than that of *C. antarctica* lipase B.

Other interesting lipases are SP523 (*H. lanuginosa*) for the synthesis of butyl carbamate (Table 2) but not for the larger

Table 4. Synthesis of *N*-1-*p*-chloro-phenylethyl butyl carbamate. Butyl carbamate, vinyl carbonate=1.2 mmol, (*R*,*S*)-4-X-1-phenylethylamine=2 mmol, 200 mg of solid biocatalyst, *V*=15 ml hexane

	X=4-Cl		X=4-MeO		X=H	
Lipase	Yield (%)[a]	e.e. (%)[b]	Yield (%)[a]	e.e. (%)[b]	Yield (%)[a]	e. e. (%)[b]
CRL	28	95	–	–	77	30
NOV-435	34	99	34	99	79	75
SP523	0	n. d.	–	–	64	90
SP525	17	99	<5	–	68	54
PS	0	n. d.			23	40

[a] Calculated by HPLC.
[b] Determined by ^{1}H NMR.

octyl carbamate (Table 3). PS (*P. cepacia*) shows the opposite specificity (Tables 1 and 2).

Finally, we can say that a quantitative study of the enzymatic load–activity is not possible in these commercial lipases owing to the fact that different lots have not been tested. We have reported[16] that it is necessary to test different lots of the same commercial enzyme to get quantitative relationships due to the heterogeneity of many commercial samples.

To explore the influence of the substituent in the *para* position of the aromatic ring of the amine, the synthesis of butylcarbamates using *p*-chloro, *p*-methoxy and *p*-nitro (*R,S*)-1-phenylethylamine was performed using several lipases with different origins that showed sensitivity to the steric hindrance (Table 4).

We can see that the process is very sensitive to the steric hindrance in the *para* position of the aromatic ring of the amine. The 4-NO_2 amine was not recognised by the enzymes and in the case of 4-MeO- and 4-Cl amine, only *C. antarctica* lipase, Novozym 435 and SP525 yielded product. CRL only was active with the H- and 4-Cl amine but not in the other cases.

Finally, and according to previous literature,[25] different studies with (*R,S*)-1-phenylethylamine with different substituents in the aromatic ring showed that the presence of this substituent has no influence on the enantioselectivity of the process. All the reactions were *R*-stereoselective.

Experimental

Enzymes

C. rugosa lipase Type VII crude (CRL) and *Porcine pancreatic* lipase Type II crude (PPL) were from Sigma Chemical Co. *C. antarctica* lipases (SP525, SP526 and Novozym-435), *R. miehei* lipases (SP524 and IM20) were a gift of Novo Nordisk Bioindustrial (Spain). *R. niveus* lipase (Newlase F), *P. cepacia* lipase (PS) and *P. fluorescens* lipase (AK) were from Amano Pharmaceutical Co.

Materials

All the reagents were of commercial quality and were purchased from Aldrich. For column chromatography, Merck silica gel 7-230 mesh was used. Optical rotations measured using a Perkin–Elmer 241 polarimeter. IR spectra were recorded on a Perkin–Elmer Paragon 1000 spectrophotometer. ^{1}H and ^{13}C NMR were obtained with TMS as internal standard, using a Bruker AC-250 (^{1}H, 250 MHz and ^{13}C, 62.89 MHz) spectrometer. Analytical HPLC was performed on an LDC chromatograph using a Nucleosil C_8 120 (20×0.4 cm, 10 μm) column.

Synthesis of vinyl carbonates

Vinyl chloroformate (50 mmol) was slowly added to a solution of the appropriate alcohol (35 mmol) in dry pyridine (4 ml). The solution was stirred for 2 h and then was acidified with HCl (3N) and extracted with dichloromethane; the organic layer was dried over sodium sulphate and submitted to chromatography on neutral silica using hexane–ethyl acetate as eluent (9:1 (v/v)).

***n*-Butyl vinyl carbonate.** IR (KBr) $\nu_{C=O}$: 1763 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ (ppm): 7.10 (dd, 1H, J=13.8 and 6.1 Hz, =CH–), 4.90 (dd, 1H, J=13.8 Hz, =CH_2), 4.60 (dd, 1H, J=6.1 Hz, =CH_2), 4.20 (t, 2H, J=6.6 Hz, CH_2–O–), 1.70 (m, 2H, CH_2), 1.45 (m, 2H, CH_2), 1.00 (t, 3H, J=7.3 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 152.68 (C=O), 142.52 (CH), 97.39 (CH_2), 68.63 (CH_2), 36.40 (CH_2), 18.73 (CH_2), 13.47 (CH_3).Anal. Calcd for $C_7H_{12}O_3$: 58.30% C 8.39% H. Found: 58.17% C 8.41% H.

***n*-Octyl vinyl carbonate.** IR (KBr) $\nu_{C=O}$: 1765 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ (ppm): 7.10 (dd, 1H, J=13.9 and 6.2 Hz, –CH=), 4.90 (dd, 1H, J=13.8 Hz, =CH–), 4.50 (dd, 1H, J=6.2 Hz, –CH=), 4.20 (t, 2H, J=6.6 Hz, CH_2–O–), 1.70 (m, 2H, CH_2), 1.40–1.10 (m, 10H), 0.90 (t, 3H, J=7.1 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 152.68 (C=O), 142.52 (CH), 97.23 (CH_2), 68.61 (CH_2), 31.65 (CH_2), 31.49 (CH_2), 29.04 (CH_2), 28.44 (CH_2), 25.53 (CH_2), 22.51 (CH_2), 13.89 (CH_3). Anal. Calcd for $C_{11}H_{20}O_3$: 65.77% C 9.98% H. Found: 65.39% C 10.11% H.

Synthesis of racemic carbamates

To a solution of (*R,S*)-1-phenylethylamine (5.2 mmol) and triethylamine (5.2 mmol) in 20 ml of dichloromethane was slowly added the corresponding vinyl chloroformate (5.2 mmol) at 0°C. The solution was stirred for 12 h and then was acidified with HCl (3N). The organic layer was dried over $MgSO_4$ and the solvent was evaporated in vacuum.

***N*-(*R,S*)-1-phenylethyl butyl carbamate.** IR (KBr) $\nu_{C=O}$: 1692 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ (ppm): 7.40 (m, 5H, arom.), 5.20 (bs, 1H, NH), 480, (bs, 1H, CH), 4.00 (m, 2H, CH_2), 1.70–1.20 (m, 7H) 0.90 (t, 3H, J=7.5 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 154.84 (C=O), 143.62 (C), 128.36 (2×CH), 126.98 (2×CH), 125.73 (CH), 64.51 (CH_2), 50.34 (CH), 30.86 (CH_2), 22.27 (CH_3), 18.85 (CH_2), 13.53 (CH_3). Anal. Calcd for $C_{13}H_{19}NO_2$: 70.54% C 8.66% H 6.33% N. Found: 70.45% C 8.67% H 6.34% N.

***N*-(*R,S*)-1-Phenylethyl octyl carbamate.** IR (KBr) $\nu_{C=O}$: 1694 cm^{-1}, ^{1}H NMR ($CDCl_3$) δ(ppm): 7.30 (m, 5H, arom.), 5.00 (bs, 1H, NH), 4.85 (bs, 1H, CH), 4.00 (m, 2H, CH_2), 1.60 (m, 2H, CH_2), 1.50 (d, 3H, J=6.9 Hz, CH_3), 1.40–1.20 (m, 10H),0.90 (t, 3H, J=6.9 Hz, CH_3); ^{13}C NMR ($CDCl_3$) δ (ppm): 155.85 (C=O), 143.61 (C), 128.47 (2×CH), 127.12 (2×CH), 125.80 (CH), 64.95 (CH_2), 50.40 (CH), 30.86 (CH_2), 31.66 (CH_2), 29.12 (CH_2), 29.09 (CH_2), 28.89(CH_2), 25.73 (CH_2), 22.53 (CH_2), 22.34 (CH_3), 13.99 (CH_3). Anal. Calcd for $C_{17}H_{27}NO_2$: 73.59% C 9.82% H 5.05% N. Found: 73.69% C 9.81% H 5.06% N.

Lipase reaction conditions

To a solution of 1.2 mmol of vinyl carbonate and 2 mmol of amine in 15 ml of hexane was added 200 mg of catalyst. The

temperature was 25°C. After 72 h the enzyme was removed by filtration and the solvent was evaporated. The conversion was determined by HPLC. The chromatographic separation on neutral silica of the resulting residue yielded the carbamate (eluent hexane–ethyl acetate (7:3)) and the e.e. was determined by ^{1}H NMR.

Analytical HPLC

HPLC was performed on an LDC chromatograph using a Nucleosil C_{18} 120 (25×0.46 cm, 5 μm) column with MeOH/H_2O as eluent; flow rate 0.3 ml min^{-1} with UV detector λ=254 nm.

Determination of enantiomeric excess

Determination of the enantiomeric excess was achieved by ^{1}H NMR spectroscopy using the chiral shift reagent tris-[3-(heptafluoropropylhydroxy-methylene)-(+)-camphorate] europium(III) derivative. The molar ratios carbamate/Eu-derivatives were 1:0.4 (butyl carbamate) and 1:0.3 (octyl carbamate). The absolute configuration of the carbamate was assigned by comparing their optical rotations with authentically chiral carbamates.[8]

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