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The effect of substrate structure on the chemoselectivity of *Candida antarctica* lipase B-catalyzed acylation of amino-alcohols

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ABSTRACT

The selective acylation of multifunctional compounds exhibiting both alcohol and amino groups gives interesting products with many applications in food, cosmetic and pharmaceutical industries, but it is real challenge. The current work describes the different behavior shown by *Candida antarctica* lipase B (Novozym 435) when catalyzing the O-acylation and N-acylation of bifunctional acyl acceptors. The acylation of three amino-alcohols (alaninol, 4-amino-1-pentanol and 6-amino-1-hexanol) was studied using myristic acid as an acyl donor. To achieve this, a structure-reactivity study was performed in *tert*-amyl alcohol as a solvent, comparing the three amino-alcohols as acyl acceptors and a series of structurally related amines, namely (R)-*sec*-butylamine, 1-methoxy-2-propylamine and 1,2-diaminopropane. These substrates were designed to investigate the effect of the group located in β-position of the amino group on the acyl acceptor: the more nucleophilic the group, the more the apparent maximal velocity ($V_{max,app}$) of N-acylation increases. Moreover, the crucial role of the carbon chain length between the alcohol and amino groups on the chemoselectivity was also demonstrated. The chemoselectivity for the N-acylation was improved when the carbon chain included two carbons (alaninol) whereas the chemoselectivity for the O-acylation was improved when the carbon chain included four carbons or more (4-amino-1-pentanol and 6-amino-1-hexanol).

These results provided new insights for the selective synthesis of amides or esters produced from the acylation of bifunctional substrates.

1. Introduction

The selective acylation of amino-alcohols has applications in many areas, such as pharmaceutics and cosmetics, through the synthesis of ceramides [1,2] as potential anti-viral or anti-tumor drugs [3,4] and anti-oxidant stabilizers [5], or for the environmental, food, and agricultural industries, through the synthesis of glucamide or amino-acid based surfactants [6–8].

Chemical acylation of amino-alcohols is well established but the methods used are faced with several limitations. They need fastidious steps of alcohol group protection and deprotection for the control of chemoselectivity and stereoselectivity. The high temperatures often required for chemical synthesis also preclude the use of fragile molecules and may cause coloration of final products. In addition, the coproduction of salts and the use of toxic solvents (dimethylformamide, methanol, etc.) that must be eliminated at the end of the reaction increase the cost of the processes. The use of biocatalysts can be an interesting alternative, which offers a clean way to perform chemical processes under mild reaction conditions and with a high degree of selectivity. The use of immobilized enzymes in organic media, in particular lipases (*E.C.* 3.1.1.3) provides several advantages such as shifting of the thermodynamic equilibrium in favor of synthesis over hydrolysis reaction, increasing solubility of non-polar substrates, eliminating side reactions, making easier enzyme recovery and increasing enzyme thermostability. Lipases are the most used enzymes for organic synthesis. They have been used to catalyze O-acylation, transesterification and N-acylation reactions to synthesize various multifunctional derivates related to pharmaceuticals, cosmetics and foods. However, despite the large amount of studies on key enzyme properties in biocatalysis, their chemoselectivity is still not completely understood. It is therefore still necessary to optimize the output of lipase-catalyzed reactions and make efforts to understand lipase chemoselectivity.

Despite the attractive properties of lipases in organic solvents, few studies have been devoted to the lipase-catalyzed acylation of bifunctional molecules exhibiting both amino and alcohol groups such as ethanolamine, diethanolamine, 2-amino-1-butanol,

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6-amino-1-hexanol, serine and amino-alcohols with variable carbon chain length [9–14]. In such reactions, the lipase was seen to catalyze O-acylation or N-acylation, with a chemoselectivity which is largely dependent on amino-alcohol structure.

Among lipases used in organic synthesis, *Candida antarctica* lipase B is well known for its ability to convert alcohols and amines into esters and amides in various organic solvents [8,15] and seems to be the ideal enzyme for the acylation of compounds such as amino-alcohols. In the present work, we thus investigated the *C. antarctica* lipase B-catalyzed acylation of various amines and amino-alcohols as acyl acceptors, using myristic acid as an acyl donor. The results obtained under a kinetic approach were analyzed by comparing the apparent kinetic parameters $V_{max,app}$ and $K_{m,app}$ obtained for each acyl acceptor.

2. Materials and methods

2.1. Enzyme and chemicals

Novozym® 435 (immobilized *C. antarctica* lipase B), was kindly provided by Novozymes A/S, Bagsvaerd, Denmark. (±)-Alaninol **1** (98%), (R)-alaninol **(R)-1** (98%), (S)-alaninol **(S)-1** (98%), (R)-*sec*-butylamine **3** (99%), (±)-1-methoxy-2-propylamine **5** (95%), (±)-1,2-diaminopropane **7** (≥98%), (±)-isopropanolamine **9** (93%), and 6-amino-1-hexanol **13** (≥97%), as well as *tert*-amyl alcohol (99%) were purchased from Sigma–Aldrich (St. Louis, USA) while (±)-4-amino-1-pentanol **11** was from Santa Cruz Biotechnology (USA). Myristic acid and acetic acid were from Fluka (St. Quentin-Fallavier, Switzerland). All chemicals were dried over molecular sieves. Pure water was obtained via a Milli-Q system (Millipore, France). Acetonitrile and methanol were purchased from Carlo ERBA (Val-de-Reuil, France).

2.2. Enzymatic reactions

In all cases, reactions were carried out in *tert*-amyl alcohol at 55 °C in screw-capped tubes. 2 ml reaction mixtures containing various amounts of acyl acceptor substrates (25–350 mM) and 175 mM of myristic acid as an acyl donor were incubated for 10 min prior to addition of 50 g l^{-1} of *C. antarctica* lipase B for the acylation of *sec*-butylamine **3** or 5 g l^{-1} of *C. antarctica* lipase B for the acylation of another acyl acceptor. 100 μl samples were taken at intervals and centrifuged at 14,000 rpm. The supernatants were then analyzed by LC–MS, leading to the determination and quantification of remaining substrates and synthesized products. Initial rate measurements were performed according to a previously established procedure [16]. The initial rates were calculated from the linear relationship of the total concentration of products against reaction time (0–2 h).

2.3. Evaluation of the chemoselectivity

The chemoselectivity of *C. antarctica* lipase B during the acylation of amino-alcohols was studied by comparing the alcohol group O-acylation and the amino group N-acylation, and then calculated via the apparent catalytic efficiency ratio (Eq. (1)) [17], which was transformed into an apparent maximal velocity ratio (Eq. (2)) owing to the fact that $K_{m,app}$ were identical for a given amino-alcohol.

$$C = \frac{V_{max,app\,O-acylation}/K_{m,app}}{V_{max,app\,N-acylation}/K_{m,app}} \quad (1)$$

$$C = \frac{V_{max,app\,O-acylation}}{V_{max,app\,N-acylation}} \quad (2)$$

Table 1
Elution gradient for HPLC analysis of reaction samples resulting from the acylation of amino-alcohols **1**, **9**, **11** and **13**.

Time (min)	Solvent A: acetonitrile/water/acetic acid (77/23/0.1, v/v/v) (%)	Solvent B: methanol/acetic acid (100/0.1, v/v) (%)
0	100	0
20	100	0
23	0	100
80	0	100
82	100	0
90	100	0

2.4. HPLC and structural analysis

Structural and quantitative analysis of reaction products were conducted using a LC/MS-ES system from Agilent (1100 LC/MSD Trap mass spectrometer VL) with a C18 Prontosil 120-5-C18-AQ reversed-phase column (250 mm × 4 mm, 5 μm; Bischoff chromatography). Products were detected and quantified by differential refractometry and UV detection at 210 nm. An external calibration was performed with pure myristic acid. Then calibrations for individual acylation products were obtained after mass balance in reaction conditions enabling to obtain only these products with myristic acid as an acyl donor. Low-resolution mass spectral analyses were obtained by electrospray in the positive detection mode. Nitrogen was used as the drying gas at 15 l min^{-1} and 350 °C at a nebulizer pressure of 4 bar. The scan range was 50–1000*m*/*z* using five averages and 13,000*m*/*z* per second resolution. The capillary voltage was 4000 V. Processing was done offline using HP Chemstation software.

Various eluent systems were used depending on the acyl acceptor used. Reaction samples resulting from the acylation of (R)-*sec*-butylamine **3** were eluted with acetonitrile/water/acetic acid (90/10/0.1, v/v/v) at room temperature and at a flow rate of 1 ml min^{-1}. Reaction samples resulting from the acylation of 1-methoxy-2-propylamine **5** and 1,2-diaminopropane **7** were eluted with methanol/water/acetic acid (95/5/0.1 and 93/7/0.1, v/v/v, respectively) at room temperature and at a flow rate of 1 ml min^{-1}. The elution of reaction samples resulting from the acylation of amino-alcohols **1**, **9**, **11** and **13**, was carried out at room temperature and at a flow rate of 1 ml min^{-1}, using a gradient that was derived from two eluent mixtures (Table 1).

In order to perform the purification and characterization of acylated products, 20 ml reaction mixtures containing 50 mM of the acyl acceptor and 175 mM of myristic acid in *tert*-amyl alcohol were incubated for 24 h in presence of 15 g l^{-1} of *C. antarctica* lipase B. Purified products were then characterized by ^{1}H NMR and IR after purification via preparative HPLC using a ProntoPrep C18 reversed-phase column (250 mm × 20 mm, 10 μm; Bischoff chromatography) eluted with the gradient given in Table 1, at room temperature and at a flow rate of 5 ml min^{-1}. ^{1}H NMR was recorded on a JEOL-JNM LA400 spectrometer (400 MHz), with tetramethylsilane as an internal reference. Samples were studied as solutions in $CDCl_3$. Infrared (IR) spectra were recorded from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1} using a 100 ATR spectrometer (Perkin-Elmer, United States).

2.4.1. N-myristyl 2-amino-1-propanol 2a

m/*z* (LR-ESI$^+$) $C_{17}H_{35}NO_2$ ($M+H^+$), found: 286.4, calculated for: 286.48. IR ν_{max} (cm^{-1}): 3100–3500 (O—H, alcohol and N—H, amide), 2800–3000 (CH of myristyl chain), 1638 (C=O, amide), 1543 (N—H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, J = 6.06 Hz, —CH_2—$C\underline{H}_3$), 1.17 (d, 3H, J = 6.06 Hz, —CH—$C\underline{H}_3$), 1.25 (m, 20H, —CH_2— of myristyl chain), 1.63 (m, 2H, —$C\underline{H}_2$—CH_2—CO—NH— of myristyl chain), 2.19 (t, 2H, J = 6.06 Hz, —CH_2—$C\underline{H}_2$—CO—NH— of

myristyl chain), 3.04 (s, 1H, —OH), 3.27 (dd, 1H, J = 5 Hz, J = 10 Hz, —CH—C$\underline{H_2}$—OH), 3.46 (dd, 1H, J = 3.7 Hz, J = 11 Hz, —CH—C$\underline{H_2}$—OH), 4.07 (m, 1H, —CH—), 5.7 (s, 1H, —NH—).

2.4.2. N,O-dimyristyl 2-amino-1-propanol 2c

m/z (LR-ESI$^+$) $C_{31}H_{62}NO_3$ (M+Na$^+$), found: 518.6, calculated for: 518.85. IR ν_{max} (cm^{-1}): 3301 (N—H, amide), 2800–3000 (CH of myristyl chain), 1737 (C=O, ester), 1643 (C=O, amide), 1542 (N—H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 6H, J = 7.6 Hz, 2x —CH_2—C$\underline{H_3}$), 1.16 (d, 3H, J = 7.6 Hz, —CH—C$\underline{H_3}$), 1.25 (m, 40H, —CH_2— of myristyl chain), 1.6 (m, 4H, 2x —C$\underline{H_2}$—CH_2—CO— of myristyl chain), 2.14 (t, 2H, J = 7.2 Hz, —CH_2—C$\underline{H_2}$—CO—O— of myristyl chain), 2.32 (t, 2H, J = 7.2 Hz, —CH_2—C$\underline{H_2}$—CO—NH— of myristyl chain), 4 (dd, 1H, J = 4.4 Hz, J = 10.7 Hz, —CH—C$\underline{H_2}$—O—), 4.13 (dd, 1H, J = 4.9 Hz, J = 10 Hz, —CH—C$\underline{H_2}$—O—), 4.29 (m, 1H, —CH—), 5.54 (d, 1H, J = 7.3 Hz, C—N$\underline{H}$—CH_2—).

2.4.3. N-myristyl sec-butylamine 4a

m/z (LR-ESI$^+$) $C_{18}H_{37}NO$ (M+H$^+$), found: 284.2, calculated for: 284.51. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.89 (m, 6H, 2x —CH_2—C$\underline{H_3}$), 1.1 (d, 3H, J = 7 Hz, —CH—C$\underline{H_3}$), 1.25 (m, 20H, —CH_2— of myristyl chain), 1.43 (m, 2H, —CH—C$\underline{H_2}$—CH_3), 1.6 (m, 2H, —C$\underline{H_2}$—CH_2—CO—NH— of myristyl chain), 2.12 (st, 2H, J = 7 Hz, —CH_2—C$\underline{H_2}$—CO—NH— of myristyl chain), 3.9 (st, 1H, J = 6.8 Hz, —CH—), 5.17 (s, 1H, —NH—).

2.4.4. N-myristyl 1-methoxy-2-propylamine 6a

m/z (LR-ESI$^+$) $C_{18}H_{37}NO_2$ (M+H$^+$), found: 300.3, calculated for: 300.51. IR ν_{max} (cm^{-1}): 3304 (N—H, amide), 2800–3000 (CH of myristyl chain), 1634 (C=O, amide), 1544 (N—H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, J = 6.79 Hz, —CH_2—C$\underline{H_3}$), 1.17 (d, 3H, J = 6.17 Hz, —CH—C$\underline{H_3}$), 1.26 (m, 20H, —CH_2— of myristyl chain), 1.62 (m, 2H, —C$\underline{H_2}$—CH_2—CO—NH— of myristyl chain), 2.15 (t, 2H, J = 6.8 Hz, —CH_2—C$\underline{H_2}$—CO—NH— of myristyl chain), 2.19 (t, 1H, J = 6.8 Hz, —CH—C$\underline{H_2}$—OCH_3), 3.36 (m, 3H, —OC$\underline{H_3}$), 4.06 (t, 1H, J = 6.45 Hz, —CH—C$\underline{H_2}$—OCH_3), 4.16 (m, 1H, —CH—), 5.62 (d, 1H, J = 5.84 Hz, —NH—).

2.4.5. 1-N-myristyl 1,2-diaminopropane 8a

m/z (LR-ESI$^+$) $C_{17}H_{36}N_2O$ (M+H$^+$), found: 285.4, calculated for: 285.49. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, J = 6.67 Hz, —CH_2—C$\underline{H_3}$), 1.22 (d, 3H, J = 6.67 Hz, —CH—C$\underline{H_3}$), 1.25 (m, 20H, —CH_2— of myristyl chain), 1.61 (q, 2H, J = 6.67 Hz, —C$\underline{H_2}$—CH_2—CO—NH— of myristyl chain), 1.96 (s, 2H, —NH_2), 2.19 (t, 2H, J = 7.3 Hz, —CH_2—C$\underline{H_2}$—CO—NH— of myristyl chain), 3.21 (st, 1H, J = 5.9 Hz, —CH—), 3.27 (qd, 1H, J = 2.6 Hz, J = 11.8 Hz, —CH—C$\underline{H_2}$—NH—), 3.46 (qd, 1H, J = 3.24 Hz, J = 13.6 Hz, —CH—C$\underline{H_2}$—NH—), 7.22 (t, 1H, J = 4.42 Hz, —NH—).

2.4.6. N-myristyl 1-amino-2-propanol 10a

m/z (LR-ESI$^+$) $C_{17}H_{35}NO_2$ (M+H$^+$), found: 286.4, calculated for: 286.48. IR ν_{max} (cm^{-1}): 3100–3500 (O—H, alcohol and N—H, amide), 2800–3000 (CH of myristyl chain), 1619 (C=O, amide), 1571 (N—H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, J = 6.55 Hz, —CH_2—C$\underline{H_3}$), 1.19 (d, 3H, J = 5.61 Hz, —CH—C$\underline{H_3}$), 1.25 (m, 20H, —CH_2— of myristyl chain), 1.64 (m, 2H, —C$\underline{H_2}$—CH_2—CO—NH— of myristyl chain), 2.21 (t, 2H, J = 7.49 Hz, —CH_2—C$\underline{H_2}$—CO—NH— of myristyl chain), 2.72 (d, 1H, J = 3.74 Hz, —OH), 3.13 (m, 1H, —CH—C$\underline{H_2}$—NH—), 3.44 (m, 1H, —CH—C$\underline{H_2}$—NH—), 3.92 (m, 1H, —CH—), 5.95 (s, 1H, —NH—).

2.4.7. N,O-dimyristyl 1-amino-2-propanol 10c

m/z (LR-ESI$^+$) $C_{31}H_{62}NO_3$ (M+Na$^+$), found: 518.6, calculated for: 518.85. IR ν_{max} (cm^{-1}): 3291 (N—H, amide), 2800–3000 (CH of myristyl chain), 1725 (C=O, ester), 1641 (C=O, amide), 1557 (N—H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 6H, J = 6.55 Hz, 2x —CH_2—C$\underline{H_3}$), 1.22 (d, 3H, J = 6.39 Hz, —CH—C$\underline{H_3}$), 1.25 (m, 40H, —CH_2— of myristyl chain), 1.61 (m, 4H, 2x —C$\underline{H_2}$—CH_2—CO— of myristyl chain), 2.16 (t, 2H, J = 6.62 Hz, —CH_2—C$\underline{H_2}$—CO—O— of myristyl chain), 2.3 (t, 2H, J = 6.62 Hz, —CH_2—C$\underline{H_2}$—CO—NH— of myristyl chain), 3.37 (m, 1H, —CH—C$\underline{H_2}$—NH—), 3.45 (m, 1H, —CH—C$\underline{H_2}$—NH—), 4.99 (m, 1H, —CH—), 5.69 (s, 1H, C—N$\underline{H}$—CH_2—).

2.4.8. N-myristyl 4-amino-1-pentanol 12a

m/z (LR-ESI$^+$) $C_{19}H_{39}NO_2$ (M+H$^+$), found: 314.2 calculated for: 314.53. IR ν_{max} (cm^{-1}): 3200–3500 (O—H, alcohol and N—H, amide), 2800–3000 (CH of myristyl chain), 1639 (C=O, amide), 1545 (N—H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, J = 6.58 Hz, —CH_2—C$\underline{H_3}$), 1.14 (d, 3H, J = 6.23 Hz, —CH—C$\underline{H_3}$), 1.25 (m, 20H, —CH_2— of myristyl chain), 1.53 (m, 4H, —CH—C$\underline{H_2}$—C$\underline{H_2}$—CH_2—OH), 1.63 (m, 2H, —C$\underline{H_2}$—CH_2—CO—NH— of myristyl chain), 2.14 (t, 2H, J = 7.27 Hz, —CH_2—C$\underline{H_2}$—CO—NH— of myristyl chain), 2.94 (s, 1H, —OH), 3.67 (m, 2H, —CH_2—C$\underline{H_2}$—OH), 4.06 (m, 1H, —CH—), 5.28 (s, 1H, —NH—).

2.4.9. O-myristyl 4-amino-1-pentanol 12b

m/z (LR-ESI$^+$) $C_{19}H_{39}NO_2$ (M+H$^+$), found: 314.2 calculated for: 314.53. IR ν_{max} (cm^{-1}): 3291 (N—H, amine), 2800–3000 (CH of myristyl chain), 1736 (C=O, ester), 1557 (N—H, amine). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, J = 6.99 Hz, —CH_2—C$\underline{H_3}$), 1.14 (d, 3H, J = 8 Hz, —CH—C$\underline{H_3}$), 1.25 (m, 20H, —CH_2— of myristyl chain), 1.52 (m, 4H, —CH—C$\underline{H_2}$—C$\underline{H_2}$—CH_2—O—), 1.62 (m, 2H, —C$\underline{H_2}$—CH_2—CO—O— of myristyl chain), 2.22 (t, 1H, J = 7.16 Hz, —C$\underline{H_2}$—C$\underline{H_2}$—CO—O— of myristyl chain), 2.29 (t, 1H, J = 7.5 Hz, —CH_2—C$\underline{H_2}$—CO—O— of myristyl chain), 3.43 (m, 1H,–CH_2—C$\underline{H_2}$—O—), 3.69 (m, 1H, —CH_2—C$\underline{H_2}$—O—), 4.1 (m, 1H, —CH—).

2.4.10. N,O-dimyristyl 4-amino-1-pentanol 12c

m/z (LR-ESI$^+$) $C_{33}H_{66}NO_3$ (M+Na$^+$), found: 546.2 calculated for: 546.9. IR ν_{max} (cm^{-1}): 3304 (N—H, amide), 2800–3000 (CH of myristyl chain), 1732 (C=O, ester), 1640 (C=O, amide), 1546 (N—H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 6H, J = 7.43 Hz, 2x —CH_2—C$\underline{H_3}$), 1.14 (d, 3H, J = 6.83 Hz, —CH—C$\underline{H_3}$), 1.25 (m, 40H, —CH_2— of myristyl chain), 1.53 (m, 4H, —CH—C$\underline{H_2}$—C$\underline{H_2}$—CH_2—O—C), 1.6 (m, 4H, 2x —C$\underline{H_2}$—CH_2—CO— of myristyl chain), 2.14 (t, 4H, J = 7.08 Hz, —CH_2—C$\underline{H_2}$—CO— of myristyl chain), 3.68 (m, 2H, —CH_2—C$\underline{H_2}$—O—C), 4.07 (m, 1H, —CH—), 5.27 (d, 1H, J = 6.86 Hz, —NH—).

2.4.11. N-myristyl aminohexanol 14a

m/z (LR-ESI$^+$) $C_{20}H_{41}NO_2$ (M+H$^+$), found: 329.5 calculated for: 328.56. IR ν_{max} (cm^{-1}): 3385 (O—H, alcohol), 3314 (N—H, amide), 2800–3000 (CH of myristyl chain), 1634 (C=O, amide), 1534 (N—H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, J = 7.5 Hz, —CH_2—C$\underline{H_3}$), 1.25 (m, 20H, —CH_2— of myristyl chain), 1.51 (m, 2H, —C$\underline{H_2}$—CH_2—CO—O— of myristyl chain), 1.59 (m, 4H, —CH_2—C$\underline{H_2}$—C$\underline{H_2}$—CH_2—OH), 2.26 (t, 2H, J = 7.65 Hz, —CH_2—C$\underline{H_2}$—CO—OH of myristyl chain), 2.72 (s, 1H, —OH), 3.25 (t, 2H, J = 7.07 Hz, —CH_2—C$\underline{H_2}$—OH), 3.63 (t, 2H, J = 7.29 Hz, —CH_2—C$\underline{H_2}$—NH—CO—CH_2), 5.41 (s, 1H, —NH—).

2.4.12. O-myristyl aminohexanol 14b

m/z (LR-ESI$^+$) $C_{20}H_{41}NO_2$ (M+H$^+$), found: 329.5 calculated for: 328.56. IR ν_{max} (cm^{-1}): 3400 (N—H, amine), 2800–3000 (CH of myristyl chain), 1736 (C=O, ester), 1544 (N—H, amine). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 3H, J = 7.28 Hz, —CH_2—C$\underline{H_3}$), 1.25 (m, 20H, —CH_2— of myristyl chain), 1.55 (m, 2H, —C$\underline{H_2}$—CH_2—CO—O— of myristyl chain), 1.62 (m, 4H, —CH_2—C$\underline{H_2}$—C$\underline{H_2}$—CH_2—NH_2), 2.28 (t, 2H, J = 7.65 Hz, —CH_2—C$\underline{H_2}$—CO—O— of myristyl chain), 2.81 (s, 2H, —NH_2),

3.64 (t, 2H, J = 6.47 Hz, $-CH_2-\underline{CH_2}-NH_2$), 4.04 (t, 2H, J = 6.47 Hz, $-CH_2-\underline{CH_2}-O-CO-CH_2$).

2.4.13. N,O-dimyristyl aminohexanol 14c

m/z (LR-ESI$^+$) $C_{34}H_{67}NO_3$ (M+Na$^+$), found: 560.7, calculated for: 560.93. IR ν_{max} (cm^{-1}): 3298 (N—H, amide), 2800–3000 (CH of myristyl chain), 1726 (C=O, ester), 1635 (C=O, amide), 1547 (N—H, amide). ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm): δ 0.88 (t, 6H, J = 6.48 Hz, 2x $-CH_2-\underline{CH_3}$), 1.25 (m, 40H, $-CH_2-$ of myristyl chain), 1.5 (m, 4H, $-\underline{CH_2}-CH_2-CO-$ of myristyl chain), 1.6 (m, 4H, $-CH_2-\underline{CH_2}-\underline{CH_2}-CH_2-O-C$), 2.15 (t, 2H, J = 7.8 Hz, $-CH_2-\underline{CH_2}-CO-NH-$), 2.29 (t, 2H, J = 7.8 Hz, $-CH_2-\underline{CH_2}-CO-NH-$), 3.24 (q, 2H, J = 6.5 Hz, $-CH_2-\underline{CH_2}-NH-$), 4.06 (t, 2H, J = 5.9 Hz, $-CH_2-\underline{CH_2}-O-CO-CH_2$), 5.4 (s, 1H, $-NH-$).

2.5. Analysis of the ionization state of substrates

The ionization state of myristic acid in *tert*-amyl alcohol was investigated using infrared spectroscopy analysis. Infrared (IR) spectra of samples containing 175 mM myristic acid and from 0 to 250 mM alaninol were recorded from 1500 to 1800 cm^{-1} with a resolution of 4 cm^{-1} using a 100 ATR spectrometer (Perkin-Elmer, United States). Before the interpretation of data, a treatment (base line correction, smoothing and normalization min–max) was applied to spectra.

3. Results and discussion

In order to explore the chemoselectivity of the enzyme, kinetic studies were investigated for the acylation of several acyl acceptors including various amines, methoxyamine and amino-alcohols (Table 2), catalyzed by *C. antarctica* lipase B using myristic acid (175 mM) as an acyl donor and *tert*-amyl alcohol as a solvent. From the LC–MS analysis, the decrease in myristic acid concentration was always seen to be concomitant with the synthesis of acylated products. The purification and the structural elucidation by mass spectroscopy, IR and NMR analyses led to identify the structure of the acylated products described in Table 2. In absence of enzyme, no product was detected within 2 days.

The acylation of (±)-alaninol (amino-alcohol **1**, Table 2, entry 1) was chosen as the model reaction to study the kinetic behavior of *C. antarctica* lipase B when catalyzing the acylation of amino-alcohols. To achieve this, we determined the apparent kinetic parameters of the amide and ester synthesis from myristic acid and (±)-alaninol. As no mono-O-acylation product was detected under our experimental conditions, systematic analysis of the rates of myristic acid conversion and (±)-alaninol N-acylation were conducted, by varying (±)-alaninol concentration. This analysis revealed Lineweaver–Burk reciprocal plots presented in Fig. 1. The intercepts of the y-axis and the x-axis gave the values of $V_{max,app}$ and $K_{m,app}$. The $V_{max,app}$ were found to be 4.9 mmol h^{-1} g^{-1} for myristic acid conversion and 4.3 mmol h^{-1} g^{-1} for (±)-alaninol N-acylation. The $V_{max,app}$ of O-acylation of the N-acylated product **2a** was extremely low (0.3 mmol h^{-1} g^{-1}), which explained the similarities observed between the reciprocal rate values of myristic acid conversion and (±)-alaninol N-acylation. The $K_{m,app}$ value of (±)-alaninol was found to be 182 mM.

For high concentrations of amino-alcohol (superior to 100 mM), a decrease in initial rates was observed. This phenomenon was most likely due to an inhibitor effect similar to an excess substrate inhibition probably due to an interaction between myristic acid and the amino group of alaninol. Indeed, the presence of an amino substrate and a fatty acid in an organic solvent generally leads to the formation of an ion-pair complex between both substrates, depending on the acido-basic conditions of the medium [16,18,19].

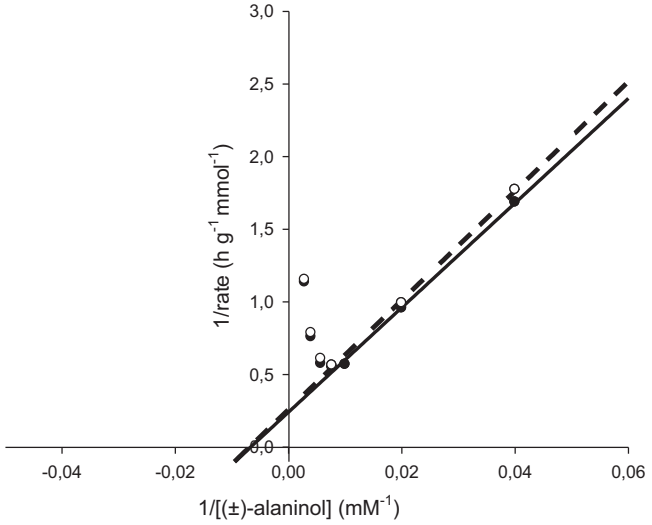


Fig. 1. Reciprocal initial rates of myristic acid conversion (●) and (±)-alaninol N-acylation (○) versus reciprocal (±)-alaninol concentrations. Reactions were carried out at 55 °C using a fixed concentration of myristic acid (175 mM) and 5 g l^{-1} of *C. antarctica* lipase B in 2 ml of *tert*-amyl alcohol. The data represent the averages of triplicate runs whose standard deviations were always lower than 15%.

This salt complex makes the ion forms of both substrates non reactive (NH_3^+ amine form and COO^- fatty acid form) and therefore leads to the overestimation of the substrate concentrations that are really available for the enzyme in the reaction medium. This ion-pair complex was already described by Maugard et al. [16] as a limiting factor of the lipase-catalyzed acylation under conditions where it was less soluble than free substrates. To ascertain this hypothesis, the composition of the medium, especially the carbonyl species, was analyzed by infrared spectroscopy at the start of the reaction, for four concentrations of (±)-alaninol within the range 50–250 mM (Fig. 2). When only myristic acid was solubilized in *tert*-amyl alcohol, only one carbonyl band was observed at 1710 cm^{-1}, corresponding to the acid form. When the concentration of (±)-alaninol was increased, the carbonyl acid band disappeared in favor of a band at 1562 cm^{-1} corresponding to a carboxylate ion. This additional band demonstrated the formation of an ion-pair complex between myristic acid and alaninol when using an (±)-alaninol concentration superior or equal to 100 mM.

The kinetic studies resulting from the acylation of other acyl acceptors (Table 2) were determined using the same method as the one used above for the acylation of (±)-alaninol. In all kinetic profiles, a decrease in initial rates was observed for high concentrations of amino substrates, most probably due to the formation of an ion-pair complex between substrates similar to the complex described above in case of (±)-alaninol acylation. The apparent kinetic parameters $K_{m,app}$ and $V_{max,app}$ resulting from the acylation of all acyl acceptors were determined and are given in Table 3.

Firstly, we compared the acylation resulting from a series of amines (**3**, **5** and **7**) structurally related to amino-alcohol **1** (alaninol) with different groups in β position of the amino group (Fig. 3 and Table 2, entries 1–4).

The $K_{m,app}$ of amine **3** ((R)-*sec*-butylamine) (619 mM; Table 3, entry 4) was 3-fold and 5-fold higher than the $K_{m,app}$ of amino-alcohols **1** and **(R)-1** ((±)-alaninol and (R)-alaninol) (182 mM and 118 mM; Table 3, entries 1 and 2), respectively. This showed a better affinity of *C. antarctica* lipase B toward a bifunctional amino-alcohol rather than a monofunctional amine. On the other hand, the $K_{m,app}$ values of bifunctional compounds **1** ((±)-alaninol), **5** ((±)-1-methoxy-2-propylamine) and **7** ((±)-1,2-diaminopropane), structurally related substrates with two carbons between the two functional groups, were of the same order ($K_{m,app}$ equal

Table 2
Structure of substrates and products resulting from the acylation of various acyl acceptors catalyzed by *C. antarctica* lipase B using myristic acid as an acyl donor in *tert*-amyl alcohol.

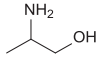
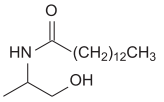
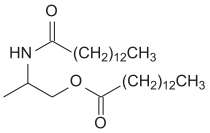
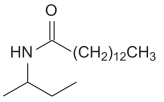
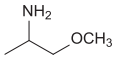
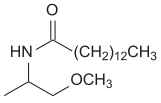
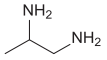
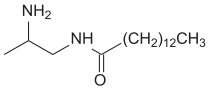
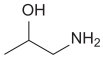
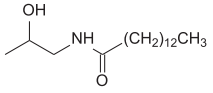
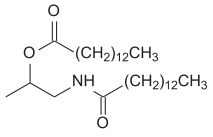
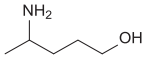
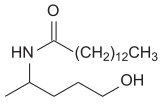
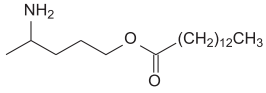
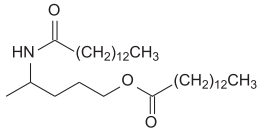
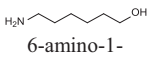
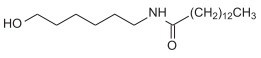
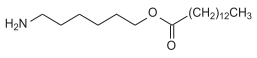
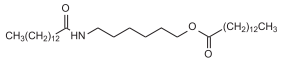
Entry	Acyl acceptor	Products		
		Amide	Ester	Amido-ester
1	 alaninol (**1**)	 N-myristyl 2-amino-1-propanol (**2a**)	–	 N,O-dimyristyl 2-amino-1-propanol (**2c**)
2	 (R)-*sec*-butylamine (**3**)	 N-myristyl *sec*-butylamine (**4a**)	–	–
3	 **(±)**-1-methoxy-2-propylamine (**5**)	 N-myristyl 1-méthoxy-2-propylamine (**6a**)	–	–
4	 **(±)**-1,2-diamino propane (**7**)	 1-N-myristyl 1,2-diaminopropane (**8a**)	–	–
5	 isopropanolamine (**9**)	 N-myristyl 1-amino-2-propanol (**10a**)	–	 N,O-dimyristyl 1-amino-2-propanol (**10c**)
6	 **(±)**-4-amino-1-pentanol (**11**)	 N-myristyl 4-amino-1-pentanol (**12a**)	 O-myristyl 4-amino-1-pentanol (**12b**)	 N,O-dimyristyl 4-amino-1-pentanol (**12c**)
7	 6-amino-1-hexanol (**13**)	 N-myristyl aminohexanol (**14a**)	 O-myristyl aminohexanol (**14b**)	 N,O-dimyristyl aminohexanol (**14c**)

Table 3
Apparent kinetics parameters for the N-acylation and O-acylation of various acyl acceptors using *C. antarctica* lipase B in *tert*-amyl alcohol at 55 °C. Myristic acid (175 mM) was used as an acyl donor.

Entry	Acyl acceptor	$K_{m,app}$ (mM)	$V_{max,app}$ of N-acylation (mmol h^{-1} g^{-1})	$V_{max,app}$ of O-acylation (mmol h^{-1} g^{-1})
1	(±)-Alaninol **1**	182	4.3	–
2	(R)-alaninol **(R)-1**	118	5.5	–
3	(S)-alaninol **(S)-1**	122	5.4	–
4	(R)-*sec*-butylamine **3**	619	0.2	–
5	(±)-1-Methoxy-2-propylamine **5**	185	1.6	–
6	**(**±)-1,2-Diaminopropane **7**	252	7.6	–
7	(±)-Isopropanolamine **9**	103	2.5	–
8	(±)-4-Amino-1-pentanol **11**	75	1.1	7.3
9	6-Amino-1-hexanol **13**	63	1	10.1

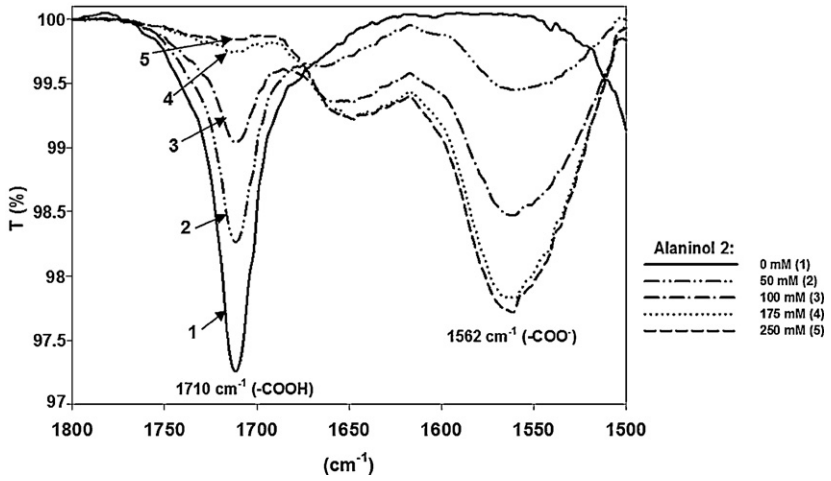
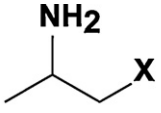


Fig. 2. IR analysis of mixtures containing 175 mM of myristic acid and various concentrations of (±)-alaninol in *tert*-amyl alcohol.

to 182 mM, 185 mM and 252 mM; Table 3, entries 1, 5 and 6). On the contrary, $V_{max,app}$ values of N-acylation for compounds **1**, **5** and **7** were variable (from 1.6 to 7.6 mmol h^{-1} g^{-1}), which showed that *C. antarctica* lipase B catalytic efficiency was mainly depending on its differential catalytic activity for these substrates. Furthermore, in a previous work we have compared the acylation of mono-amine **3** ((R)-*sec*-butylamine) and a structurally similar secondary alcohol: (R)-2-butanol. We noticed that the $V_{max,app}$ of O-acylation of (R)-2-butanol was 23-fold higher than the $V_{max,app}$ of N-acylation of amine **3** [20]. These results were in contrast with those obtained for the acylation of (R)-alaninol, for which no mono-O-acylation product was detected and a value of $V_{max,app}$ equal to 5.5 mmol h^{-1} g^{-1} was obtained for N-acylation. Moreover, it was shown that the stereochemistry of compounds did not influence the chemoselectivity during the acylation of alaninol because similar results were observed for the racemic (±)-alaninol and the R and S pure enantiomers of alaninol: no mono-O-acylation product was detected and values of $V_{max,app}$ of N-acylation close to 5 mmol h^{-1} g^{-1} were obtained in all cases. Furthermore, by comparing the acylation of (±)-alaninol **1** (2-amino-1-propanol) with the acylation of (±)-isopropanolamine **9** (1-amino-2-propanol), for which the positions of the alcohol and amine functions are interchanged (Table 2, entries 1 and 5), similar results were obtained: $V_{max,app}$ of N-acylation of the same order were observed, equal to 4.3 mmol h^{-1} g^{-1} and 2.5 mmol h^{-1} g^{-1}, respectively (Table 3, entries 1 and 7) and no mono-O-acylation product was detected. These results seem to indicate that the position (1 or 2) of the alcohol and amine functions has no impact on the chemoselectivity of the lipase. Finally, to better understand the influence of substrate structure on the chemoselectivity of *C. antarctica* lipase B, we compared the $V_{max,app}$ of mono-N-acylation of structurally related amines (Fig. 3, compounds **1**, **3**, **5**, **7** and **9**, Table 3, entries 1 and 4–7). The order of $V_{max,app}$ values was found to be: diamine **7** (7.6 mmol h^{-1} g^{-1}) > amino-alcohol **1** and **9** ((±)-alaninol and (±)-isopropanolamine) (4.3 mmol h^{-1} g^{-1} and 2.5 mmol h^{-1} g^{-1}) > methoxyamine **5** (1.6 mmol h^{-1} g^{-1}) ≫ amine **3** (0.2 mmol h^{-1} g^{-1}). The first substrate diamine **7**, which exhibits the highest $V_{max,app}$ values was mono-N-acylated only in position 1. Indeed, only mono-amide **8a** was detected (Table 2, entry 4). The last substrate amine **3** is a monofunctional amine with no nucleophilic group in β-position of an amino group. From these results, we could thus conclude that the presence of a nucleophilic group (alcohol group, methoxy group or a second amino group) in β-position of the acyl-acceptor amino group was responsible for the enhancement of $V_{max,app}$ of N-acylation of this amino group and that the more nucleophilic group (—NH_2 > —OH > —OCH_3) in β-position, the higher the reactivity for the amino group.



X = OH (1), CH_3 (3), OCH_3 (5) or NH_2 (7)

Fig. 3. Alaninol (**1**) and structurally related amines (**3**, **5**, **7**).

Secondly, we compared the acylation of three amino-alcohols that exhibited a variable carbon chain length between the amino and alcohol groups: amino-alcohols **1** (two carbons), **11** (four carbons) and **13** (six carbons) (Table 2, entries 1, 6 and 7). In terms of $K_{m,app}$ values, the $K_{m,app}$ of amino-alcohol **11** (75 mM; Table 3, entry 8) was in the same order than the $K_{m,app}$ of amino-alcohol **13** (63 mM; Table 3, entry 9), whereas the $K_{m,app}$ of amino-alcohol **1** (182 mM; Table 3, entry 1) was higher. This pointed out a better affinity of *C. antarctica* lipase B toward long chain amino-alcohols **11** and **13** than for short amino-alcohols **1**. On the other hand, the acylation of long chain amino-alcohols **11** and **13** by *C. antarctica* lipase B gave $V_{max,app}$ of N-acylation of 1.1 and 1 mmol h^{-1} g^{-1} (Table 3, entries 8 and 9), respectively, as short chain amino-alcohol **1** was N-acylated 4-fold faster ($V_{max,app}$ of N-acylation: 4.3 mmol h^{-1} g^{-1}; Table 3, entry 1) than long chain amino-alcohols. In contrast, $V_{max,app}$ of O-acylation of 7.3 mmol h^{-1} g^{-1} for amino-alcohol **11** and 10.1 mmol h^{-1} g^{-1} for amino-alcohol **13** (Table 3, entries 8 and 9) were obtained, whereas no mono-O-acylated product and only trace amounts of amido-ester **2c** were detected during the acylation of short chain amino-alcohol **1**. This was attributed to the fact that the reaction could take place at the amino group (N-acylation) and/or alcohol group (O-acylation) of long chain amino-alcohols **11** and **13**, giving either N- or O-acylated products **12a**, **12b**, **14a** and **14b** (Table 2, entries 6 and 7), while the mono-O-acylation of short chain amino-alcohol **1** did not occur. Starting from these results, we could calculate the chemoselectivity ratio (Eq. (2)) of the *C. antarctica* lipase B-catalyzed acylation of long chain amino-alcohols **11** and **13**, which was close to 6.6 and 10.1, respectively. From these results, we could conclude that the increase in the carbon chain length between the alcohol and amino groups of long chain amino-alcohols was concomitant with the increase in the chemoselectivity of *C. antarctica* lipase B for the O-acylation of these substrates.

Up to now in the literature, it has been suggested that the lipase-catalyzed chemoselective N-acylation of short chain amino-alcohols proceeds through an initial O-acylation followed by spontaneous O- to N-acyl migration to give the N-acylated product [12,13]. According to this hypothesis, the acyl migration would be slower when the carbon chain length of the amino alcohol increases, which could be in accordance with the experimental results obtained for the acylation of amino-alcohols **1**, **9**, **11** and **13**. However, the increase in the N-acylation rate observed for the acylation of bifunctional compounds, which are structurally related to alaninol **1** but exhibit no alcohol group in their structure (1-methoxy-2-propylamine **5** and (±)-1,2-diaminopropane **7**) and thus cannot possibly be O-acetylated, seems to rule out this hypothesis. On the other hand, several works have demonstrated that spontaneous acyl migration in 1,2-amino-alcohol substrates proceeds with a rate much lower than $1\,min^{-1}$ [21,22]. Thus, according to the experimental data of alaninol **1**, reaction rates for spontaneous O- to N-acyl migrations are at least one to two orders of magnitude slower than reaction rates for enzyme-catalyzed acylation of this amino-alcohol (N-acylation of alaninol: $V_{max,app} = 4.3\,mmol\,h^{-1}\,g^{-1}$ which corresponds to $k_{cat} = 50\,min^{-1}$). Under our experimental conditions, if lipase-catalyzed N-acylation of alaninol only occurred through an initial O-acylation followed by spontaneous O- to N-acyl migration, the O-acylated intermediate would accumulate and reach a steady-state concentration up to values within the range 1–100 mM. But, no O-acylated product was found despite the detection limit of the LC/MS-ES system being 2 μM. These data show that the hypothesis of acyl migration alone does not enable to explain experimental results resulting from the acylation of structurally related amines (**1**, **3**, **5** and **7**) and amino-alcohols with various carbon chain length (**1**, **11** and **13**). For this reason, we formulated the following postulate: the presence of a nucleophilic group in β-position of the amino group of the acyl acceptor amine would result in the enhancement of the $V_{max,app}$ of N-acylation of this amino group. This might be due to the formation of an intramolecular interaction between the amino group and the nucleophilic group located in β-position, which is strengthened by the fact that this interaction could not occur for long chain amino-alcohols **11** and **13**, considering the longer distance between both functional groups, giving as a result a decrease in the $V_{max,app}$ of N-acylation. In order to confirm and develop this new postulate, molecular modeling studies are currently in progress to precisely understand the *C. antarctica* lipase B-catalyzed N-acylation mechanism using a methoxy-amine, a di-amine, a short chain amino-alcohol or a long chain amino-alcohol as an acyl acceptor.

4. Conclusion

In this work, we investigated the *C. antarctica* lipase B-catalyzed acylation of various amines and amino-alcohols as acyl acceptors, using myristic acid as an acyl donor, and showed that the presence of a nucleophilic group (—NH_2 or —OH or —OCH_3) in β-position of the amino group of the acyl acceptor enhances the $V_{max,app}$ of N-acylation and thus the enzyme activity. Moreover, the crucial role of the carbon chain length between the alcohol and amino groups was highlighted in the *C. antarctica* lipase B-catalyzed acylation of amino-alcohols. The $V_{max,app}$ of N-acylation was indeed improved when the carbon chain included two carbons (alaninol **1** and isopropanolamine **9**) whereas the $V_{max,app}$ of O-acylation was improved when the carbon chain included four carbons or more (4-amino-1-pentanol **11** and 6-amino-1-hexanol **13**). The present investigation demonstrated the great influence of substrate structure on the chemoselectivity of *C. antarctica* lipase B, providing new insights for the selective synthesis of amides or esters produced from the acylation of bifunctional substrates.

Finally, the ability to understand and control the chemoselectivity of *C. antarctica* lipase B, apart from its interest in the specific acylation of bifunctional substrates, constitutes a promising enzymatic way to acylate other heterofunctional compounds such as precursors of ceramide synthesis (e.g. sphingoid bases or other amino-polyols) or precursors of amino-acid based surfactant synthesis (e.g. amino-acids or peptides).

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