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The effect of substrate structure on the chemoselectivity of \textit{Candida antarctica} lipase B-catalyzed acylation of amino-alcohols

Florian Le Joubioux, Yesmine Ben Henda, Nicolas Bridiau, Oussama Achour, Marianne Graber, Thierry Maugard*

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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 a real challenge. The current work describes the different behavior shown by \textit{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 \textit{tert}-amyl alcohol as a solvent, comparing the three amino-alcohols as acyl acceptors and a series of structurally related amines, namely (R)-\textit{sec}-butylamine, 1-methoxy-2-propylamine and 1,2-diaminopropane. These substrates were designed to investigate the effect of the group located in $\beta$-position of the amino group on the acyl acceptor: the more nucleophilic the group, the more the apparent maximal velocity ($V_{\text{max,app}}$) 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.

**Keywords:** N-acylation; O-acylation; \textit{Candida antarctica} lipase B; Organic solvent; Chemoselectivity
1. Introduction

The selective acylation of amino-alcohols have 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, …) 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, 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 *Candida 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 $V_{\text{max},\text{app}}$ and $K_{\text{m},\text{app}}$ obtained for each acyl acceptor.

2. Materials and Methods

2.1. Enzyme and chemicals

Novozym® 435 (immobilized *Candida antarctica* lipase B), was kindly provided by Novozymes A/S, Bagsvaerd, Denmark. (±)-alaninol 1 (98%), (R)-sec-butylamine 3 (99%), (±)-1-methoxy-2-propylamine 5 (95%), (±)-1,2-diaminopropane 7 (≥98%) and 6-amino-1-hexanol 11 (≥97%), as well as tert-amyl alcohol (99%) were purchased from Sigma-Aldrich (St Louis, USA) while (±)-4-amino-1-pentanol 9 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 minutes prior to addition of 50 g l\(^{-1}\) of *Candida antarctica* lipase B for the acylation of sec-butylamine 3 or 5 g l\(^{-1}\) of *Candida 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 *Candida 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.

\[
C = \left( \frac{V_{\text{max,app O-acylation}}}{K_{m,app}} \right) / \left( \frac{V_{\text{max,app N-acylation}}}{K_{m,app}} \right) (1)
\]

\[
C = \frac{V_{\text{max,app O-acylation}}}{V_{\text{max,app N-acylation}}} (2)
\]

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 (250×4 mm, 5 µm; Bischoff Chromatography). Products were detected and quantified by differential refractometry and UV detection at 210 nm.
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 bars. 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 and 11, 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).

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

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A: acetonitrile/water/acetic acid (77/23/0.1, v/v/v) (%)</th>
<th>Solvent B: methanol/acetic acid (100/0.1, v/v) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
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<tr>
<td>20</td>
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<td>0</td>
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<td>90</td>
<td>100</td>
<td>0</td>
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</table>
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 *Candida antarctica* lipase B. Purified products were then characterized by \(^1\)H NMR and IR via preparative HPLC using a ProntoPrep C18 reversed-phase column (250×20 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 were 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).

**N-myristyl 2-amino-1-propanol 2a:** m/Z (LR-ESI\(^+\)) C\(_{17}\)H\(_{33}\)NO\(_2\) (M + H\(^+\)), found: 286.4, calculated for: 286.48. IR \(\nu_{\text{max}}\) (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\(_3\), \(\delta\) ppm): \(\delta\) 0.88 (t, 3H, \(J=6.06\)Hz, -CH\(_2\)-CH\(_3\)), 1.17 (d, 3H, \(J=6.06\)Hz, –CH-CH\(_3\)), 1.25 (m, 20H, -CH\(_2\)- of myristyl chain), 1.63 (m, 2H, -CH\(_2\)-CH\(_2\)-CO-NH- of myristyl chain), 2.19 (t, 2H, \(J=6.06\)Hz, -CH\(_2\)-CH\(_2\)-CO-NH- of myristyl chain), 3.46 (dd, 1H, \(J=3.7\)Hz, \(J=11\)Hz, \(J=3.7\)Hz, \(J=11\)Hz, –CH-CH\(_2\)-OH), 4.07 (m, 1H, –CH-), 5.7 (s, 1H, -NH-).

**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 \(\nu_{\text{max}}\) (cm\(^{-1}\)): 3301 (N-H, amide), 2800-3000 (CH of myristyl chain), 1737 (C=O, ester), 1542 (N-H, amide). \(^1\)H NMR (400 MHz, CDCl\(_3\), \(\delta\) ppm): \(\delta\) 0.88 (t, 6H, \(J=7.6\)Hz, 2x -CH\(_2\)-CH\(_3\)), 1.16 (d, 3H, \(J=7.6\)Hz, –CH-CH\(_3\)), 1.25 (m, 40H, -CH\(_2\)- of myristyl chain), 1.6 (m, 4H, 2x -CH\(_2\)-CH\(_2\)-CO- of myristyl chain), 2.14 (t, 2H, \(J=7.2\)Hz, -CH\(_2\)-CH\(_2\)-CO-O- of myristyl chain), 2.32 (t, 2H, \(J=7.2\)Hz, -CH\(_2\)-CH\(_2\)-CO-NH- of myristyl chain), 4 (dd, 1H, \(J=4.4\)Hz, \(J=10.7\)Hz, –CH-CH\(_2\)-O-), 4.13 (dd,
1H, \( J = 4.9 \text{Hz}, \ J = 10 \text{Hz}, -\text{CH-CH}_2-\text{O}- \), 4.29 (m, 1H, -\text{CH-}), 5.54 (d, 1H, \( J = 7.3 \text{Hz}, \text{C-NH-} \text{CH}_2\)).

**N-myristyl sec-butylamine 4a:** m/Z (LR-ESI\(^+\)) \( C_{18}H_{37}NO \) (M + H\(^+\)), found: 284.2, calculated for: 284.51.

\(^1\text{H NMR (400 MHz, CDCl}_3, \delta \text{ ppm):} \delta 0.89 (m, 6H, -\text{CH}_2-\text{CH}_3), 1.11 (d, 3H, \( J = 7 \text{Hz}, -\text{CH-CH}_3 \)), 1.25 (m, 20H, -\text{CH}_2- \text{of myristyl chain}), 1.43 (m, 2H, -\text{CH-CH}_2-\text{CH}_3), 1.6 (m, 2H, -\text{CH}_2-\text{CH}_2-\text{CO-NH- of myristyl chain}), 2.12 (st, 2H, \( J = 7 \text{Hz}, -\text{CH}_2-\text{CO-NH- of myristyl chain} \)), 2.19 (t, 1H, \( J = 6.8 \text{Hz}, -\text{CH-CH}_2-\text{OCH}_3 \)), 3.36 (m, 3H, -\text{OCH}_3), 4.06 (t, 1H, \( J = 6.45 \text{Hz}, -\text{CH-CH}_2-\text{OCH}_3 \)), 4.16 (m, 1H, -\text{CH-}), 5.62 (d, 1H, \( J = 5.84 \text{Hz}, -\text{NH-} \)).

**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 \( \nu_{\text{max}} \) (cm\(^{-1}\)): 3304 (N-H, amide), 2800-3000 (CH of myristyl chain), 1634 (C=O, amide), 1544 (N-H, amide).

\(^1\text{H NMR (400 MHz, CDCl}_3, \delta \text{ ppm):} \delta 0.88 (t, 3H, \( J = 6.79 \text{Hz}, -\text{CH}_2-\text{CH}_3 \)), 1.17 (d, 3H, \( J = 6.17 \text{Hz}, -\text{CH-CH}_3 \)), 1.26 (m, 20H, -\text{CH}_2- \text{of myristyl chain}), 1.62 (m, 2H, -\text{CH}_2-\text{CH}_2-\text{CO-NH- of myristyl chain}), 2.15 (t, 2H, \( J = 6.8 \text{Hz}, -\text{CH}_2-\text{CO-NH- of myristyl chain} \)), 2.19 (t, 1H, \( J = 6.8 \text{Hz}, -\text{CH-CH}_2-\text{OCH}_3 \)), 3.36 (m, 3H, -\text{OCH}_3), 4.06 (t, 1H, \( J = 6.45 \text{Hz}, -\text{CH-CH}_2-\text{OCH}_3 \)), 4.16 (m, 1H, -\text{CH-}), 5.62 (d, 1H, \( J = 5.84 \text{Hz}, -\text{NH-} \)).

**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.

IR \( \nu_{\text{max}} \) (cm\(^{-1}\)): 3200-3500 (O-H, alcohol and N-H, amide), 1750 (amide).

\(^1\text{H NMR (400 MHz, CDCl}_3, \delta \text{ ppm):} \delta 0.88 (t, 3H, \( J = 6.67 \text{Hz}, -\text{CH}_2-\text{CH}_3 \)), 1.22 (d, 3H, \( J = 6.67 \text{Hz}, -\text{CH-CH}_3 \)), 1.25 (m, 20H, -\text{CH}_2- \text{of myristyl chain}), 1.61 (q, 2H, \( J = 6.67 \text{Hz}, -\text{CH}_2-\text{CH}_2-\text{CO-NH- of myristyl chain} \)), 1.96 (s, 2H, -\text{NH}_2), 2.19 (t, 2H, \( J = 7.3 \text{Hz}, -\text{CH}_2-\text{CH}_2-\text{CO-NH- of myristyl chain} \)), 3.21(st, 1H, \( J = 5.9 \text{Hz}, -\text{CH-} \)), 3.27 (qd, 1H, \( J = 2.6 \text{Hz}, \ J = 11.8 \text{Hz}, -\text{CH-CH}_2-\text{NH-} \)), 3.46 (qd, 1H, \( J = 3.24 \text{Hz}, \ J = 13.6 \text{Hz}, -\text{CH-CH}_2-\text{NH-} \)), 7.22 (t, 1H, \( J = 4.42 \text{Hz}, -\text{NH-} \)).

**N-myristyl 4-amino-1-pentanol 10a:** m/Z (LR-ESI\(^+\)) \( C_{19}H_{39}NO_2 \) (M + H\(^+\)), found: 314.2 calculated for: 314.53. IR \( \nu_{\text{max}} \) (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= 6.58Hz, -CH$_2$-CH$_3$), 1.14 (d, 3H, J= 6.23Hz, -CH-CH$_3$), 1.25 (m, 20H, -CH$_2$- of myristyl chain), 1.53 (m, 4H, -CH-CH$_2$-CH$_2$-CH$_2$-OH), 1.63 (m, 2H, -CH$_2$-CH$_2$-CO-NH- of myristyl chain), 2.14 (t, 2H, J= 7.27Hz, -CH$_2$-CH$_2$-CO-NH- of myristyl chain), 2.94 (s, 1H, -OH), 3.67 (m, 2H, –CH$_2$-CH$_2$-OH), 4.06 (m, 1H, -CH-), 5.28 (s, 1H, -NH-).

O-myristyl 4-amino-1-pentanol 10a: m/Z (LR-ESI$^+$) C$_{19}$H$_{39}$NO$_2$ (M + H$^+$), found: 314.2 calculated for: 314.53. IR $\nu$ 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= 6.99Hz, -CH$_2$-CH$_3$), 1.14 (d, 3H, J= 8Hz, –CH-CH$_3$), 1.25 (m, 20H, -CH$_2$- of myristyl chain), 1.52 (m, 4H, -CH-CH$_2$-CH$_2$-CH$_2$-O-), 1.62 (m, 2H, -CH$_2$-CH$_2$-CO-O- of myristyl chain), 2.22 (t, 1H, J= 7.16Hz, -CH$_2$-CH$_2$-CO-O- of myristyl chain), 2.29 (t, 1H, J= 7.5Hz, -CH$_2$-CH$_2$-CO-O- of myristyl chain), 3.43 (m, 1H, -CH$_2$-CH$_2$-O-), 3.69 (m, 1H, -CH$_2$-CH$_2$-O-), 4.1 (m, 1H, -CH-).

N,O-dimyristyl 4-amino-1-pentanol 10c: m/Z (LR-ESI$^+$) C$_{33}$H$_{66}$NO$_3$ (M + Na$^+$), found: 546.2 calculated for: 546.9. IR $\nu$ 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= 7.43Hz, 2x -CH$_2$-CH$_3$), 1.14 (d, 3H, J= 6.83Hz, –CH-CH$_3$), 1.25 (m, 40H, -CH$_2$- of myristyl chain), 1.53 (m, 4H, -CH-CH$_2$-CH$_2$-CH$_2$-O-C), 1.6 (m, 4H, 2x -CH$_2$-CH$_2$-CO- of myristyl chain), 2.14 (t, 4H, J= 7.08Hz, -CH$_2$-CH$_2$-CO- of myristyl chain), 3.68 (m, 2H, -CH$_2$-CH$_2$-O-C), 4.07 (m, 1H, -CH-), 5.27 (d, 1H, J= 6.86Hz, -NH-).

N-myristyl aminohexanol 12a: m/Z (LR-ESI$^+$) C$_{20}$H$_{41}$NO$_2$ (M + H$^+$), found: 329.5 calculated for: 328.56. IR $\nu$ 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\(_3\), \(\delta\) ppm): \(\delta\) 0.88 (t, 3H, J= 7.5Hz, -CH\(_2\)-CH\(_3\)), 1.25 (m, 20H, -CH\(_2\)- of myristyl chain), 1.51 (m, 2H, -CH\(_2\)-CH\(_2\)-CO-O- of myristyl chain), 1.59 (m, 4H, -CH\(_2\)-CH\(_2\)-CH\(_2\)-CH\(_2\)-OH), 2.26 (t, 2H, J= 7.65Hz, -CH\(_2\)-CH\(_2\)-CO-OH of myristyl chain), 2.72 (s, 1H, -OH), 3.25 (t, 2H, J= 7.07Hz, –CH\(_2\)-CH\(_2\)-O-C), 3.63 (t, 2H, J= 7.29Hz, –CH\(_2\)-CH\(_2\)-NH-CO-CH\(_2\)), 5.41 (s, 1H, -NH-).

**O-myristyl aminohexanol 12b:** m/z (LR-ESI\(^+\)) C\(_{20}\)H\(_{41}\)NO\(_2\) (M + H\(^+\)), found: 329.5 calculated for: 328.56. IR \(\nu\) \(_{max}\) (cm\(^{-1}\)): 3400 (N-H, amine), 2800-3000 (CH of myristyl chain), 2061736 (C=O, ester), 1544 (N-H, amine). \(^1\)H NMR (400 MHz, CDCl\(_3\), \(\delta\) ppm): \(\delta\) 0.88 (t, 3H, J= 7.28Hz, -CH\(_2\)-CH\(_3\)), 1.25 (m, 20H, -CH\(_2\)- of myristyl chain), 1.55 (m, 2H, -CH\(_2\)-CH\(_2\)-CO-O- of myristyl chain), 1.62 (m, 4H, -CH\(_2\)-CH\(_2\)-CH\(_2\)-CH\(_2\)-NH\(_2\)), 2.28 (t, 2H, J= 7.65Hz, -CH\(_2\)-CH\(_2\)-CO-O- of myristyl chain), 2.81 (s, 2H, -NH\(_2\)), 3.64 (t, 2H, J= 6.47Hz, -CH\(_2\)-CH\(_2\)-NH\(_2\)), 4.04 (t, 2H, J= 6.47Hz, -CH\(_2\)-CH\(_2\)-O-CO-CH\(_2\)).

**N,O-dimyristyl aminohexanol 12c:** m/z (LR-ESI\(^+\)) C\(_{34}\)H\(_{67}\)NO\(_3\) (M + Na\(^+\)), found: 560.7, calculated for: 560.93. IR \(\nu\) \(_{max}\) (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\(_3\), \(\delta\) ppm): \(\delta\) 0.88 (t, 6H, J= 6.48Hz, 2x -CH\(_2\)-CH\(_3\)), 1.25 (m, 40H, -CH\(_2\)- of myristyl chain), 1.5 (m, 4H, -CH\(_2\)-CH\(_2\)-CO- of myristyl chain), 1.6 (m, 4H, -CH\(_2\)-CH\(_2\)-CH\(_2\)-CH\(_2\)-O-C), 2.15 (t, 2H, J= 7.8Hz, -CH\(_2\)-CH\(_2\)-CO-NH-), 2.29 (t, 2H, J= 7.8Hz, -CH\(_2\)-CH\(_2\)-CO-NH-), 3.24 (q, 2H, J= 6.5Hz, -CH\(_2\)-CH\(_2\)-NH-), 4.06 (t, 2H, J= 5.9Hz, -CH\(_2\)-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 *Candida antarctica* lipase B using myristic acid (175 mM) as an acyl donor and tert-amyl alcohol as 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.
Table 2. Structure of substrates and products resulting from the acylation of various acyl acceptors catalysed by *Candida antarctica* lipase B using myristic acid as an acyl donor in *tert*-amyl alcohol.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Acyl acceptor</th>
<th>Products</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Amide</td>
</tr>
<tr>
<td>1</td>
<td>(±)-alaninol (1)</td>
<td>N-myristyl 2-amino-1-propanol (2a)</td>
</tr>
<tr>
<td>2</td>
<td>(R)-sec-butylamine (3)</td>
<td>N-myristyl sec-butylamine (4a)</td>
</tr>
<tr>
<td>3</td>
<td>(±)-1-methoxy-2-propylamine (5)</td>
<td>N-myristyl 1-methoxy-2-propylamine (6a)</td>
</tr>
<tr>
<td>4</td>
<td>(±)-1,2-diamino propane (7)</td>
<td>1-N-myristyl 1,2-diaminopropane (8a)</td>
</tr>
<tr>
<td>5</td>
<td>(±)-4-amino-1-pentanol (9)</td>
<td>N-myristyl 4-amino-1-pentanol (10a)</td>
</tr>
<tr>
<td>6</td>
<td>6-amino-1-hexanol (11)</td>
<td>N-myristyl aminohexanol (12a)</td>
</tr>
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</table>
The acylation of alaninol (amino-alcohol 1, Table 2, entry 1) was chosen as the model reaction to study the kinetic behaviour of *Candida 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 on Fig. 1. The intercepts of the y-axis and the x-axis gave the values of $V_{\text{max,app}}$ and $K_{\text{m,app}}$. The $V_{\text{max,app}}$ 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 $V_{\text{max,app}}$ 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 $K_{\text{m,app}}$ value of alaninol was found to be 182 mM.

**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 *Candida 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%.
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]. 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.
Fig. 2: IR analysis of mixtures containing 175 mM of myristic acid and various concentrations of alaninol in tert-amyl alcohol.

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

<table>
<thead>
<tr>
<th>Entry</th>
<th>Acyl acceptor</th>
<th>$K_{m,app}$ (mM)</th>
<th>$V_{max,app}$ of N-acylation (mmol.h$^{-1}$.g$^{-1}$)</th>
<th>$V_{max,app}$ of O-acylation (mmol.h$^{-1}$.g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(±)-alaninol 1</td>
<td>182</td>
<td>4.3</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>(R)-sec-butylamine 3</td>
<td>619</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>(±)-1-methoxy-2-propylamine 5</td>
<td>185</td>
<td>1.6</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>(±)-1,2-diaminopropane 7</td>
<td>252</td>
<td>7.6</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>(±)-4-amino-1-pentanol 9</td>
<td>75</td>
<td>1.1</td>
<td>7.3</td>
</tr>
<tr>
<td>6</td>
<td>6-amino-1-hexanol 11</td>
<td>63</td>
<td>1</td>
<td>10.1</td>
</tr>
</tbody>
</table>

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 (scheme 1 and Table 2, entries 1 to 4).

![Scheme 1: Alaninol (1) and structurally related amines (3,5,7).](image)

The $K_{m,app}$ of amine 3 ((R)-sec-butylamine) (619 mM; Table 3, entry 2) was 3-fold higher than the $K_{m,app}$ of amino-alcohol 1 (alaninol) (182 mM; Table 3, entry 1). This showed a better affinity of *Candida antarctica* lipase B toward a bifunctionnal 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 ($K_{m,app}$ respectively equal to 182 mM, 185 mM and 252 mM; Table 3, entries 1, 3 and 4). 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 Candida 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 alaninol, for which no mono-O-acylation product was detected and a value of $V_{max,app}$ equal to 4.3 mmol h$^{-1}$ g$^{-1}$ was obtained for N-acylation. To better understand the influence of substrate structure on the chemoselectivity of Candida antarctica lipase B, we compared the $V_{max,app}$ of mono-N-acylation of structurally related amines (scheme 1, compounds 1, 3, 5 and 7, Table 3, entries 1, 2, 3 and 4). The order of $V_{max,app}$ values was found to be: diamine 7 (7.6 mmol h$^{-1}$ g$^{-1}$) > amino-alcohol 1 (alaninol) (4.3 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.
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), 9 (four carbons) and 11 (six carbons) (Table 2, entries 1, 5 and 6). In terms of $K_{m,app}$ values, the $K_{m,app}$ of amino-alcohol 9 (75 mM; Table 3, entry 5) was in the same order than the $K_{m,app}$ of amino-alcohol 11 (63 mM; Table 3, entry 6), whereas the $K_{m,app}$ of amino-alcohol 1 (182 mM; Table 3, entry 1) was higher. This pointed out a better affinity of *Candida antarctica* lipase B toward long chain amino-alcohols 9 and 11 than for short amino-alcohols 1. On the other hand, the acylation of long chain amino-alcohols 9 and 11 by *Candida antarctica* lipase B gave $V_{max,app}$ of N-acylation of 1.1 and 1 mmol h$^{-1}$ g$^{-1}$ (Table 3, entries 5 and 6), 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 9 and 10.1 mmol h$^{-1}$ g$^{-1}$ for amino-alcohol 11 (Table 3, entries 5 and 6) 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 9 and 11, giving either N- or O-acylated products 10a, 10b, 12a and 12b (Table 2, entries 5 and 6), 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 *Candida antarctica* lipase B-catalyzed acylation of long chain amino-alcohols 9 and 11, 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 *Candida antarctica* lipase B for the O-acylation of these substrates.
To interpret all these data resulting from the acylation of structurally related amines (1, 3, 5 and 7) and amino-alcohols with various carbon chain length (1, 9 and 11), we formulated the following postulate: the presence of a nucleophilic group in β-position of the amino group of the acyl acceptor amine resulted in the enhancement of the $V_{\text{max,app}}$ of N-acylation of this amino group. This may 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 9 and 11, considering the longer distance between both functional groups, giving as a result a decrease in the $V_{\text{max,app}}$ of N-acylation.

**Conclusion**

In this work, we investigated the *Candida 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 $V_{\text{max,app}}$ 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 *Candida antarctica* lipase B-catalyzed acylation of amino-alcohols. The $V_{\text{max,app}}$ of N-acylation was indeed improved when the carbon chain included two carbons (alaninol 1) whereas the $V_{\text{max,app}}$ of O-acylation was improved when the carbon chain included four carbons or more (4-amino-1-pentanol 9 and 6-amino-1-hexanol 11). The present investigation demonstrated the great influence of substrate structure on the chemoselectivity of *Candida antarctica* lipase B, providing new insights for the selective synthesis of amides or esters produced from the acylation of bifunctional substrates. Molecular modelling studies are currently in progress to study in details the acylation mechanism of amino-alcohols.
Finally, the ability to understand and control the chemoselectivity of *Candida 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).
Acknowledgments

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References


