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

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11

12 **Abstract**

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

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

32 **Keywords:** N-acylation; O-acylation; *Candida antarctica* lipase B; Organic solvent;
33 Chemoselectivity

34

35 **1. Introduction**

36 The selective acylation of amino-alcohols have applications in many areas, such as
37 pharmaceuticals and cosmetics, through the synthesis of ceramides [1, 2] as potential anti-viral
38 or anti-tumor drugs [3, 4] and anti-oxidant stabilizers [5], or for the environmental, food, and
39 agricultural industries, through the synthesis of glucamide or amino-acid based surfactants [6-
40 8].

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

60 Despite the attractive properties of lipases in organic solvents, few studies have been devoted
61 to the lipase-catalyzed acylation of bifunctional molecules exhibiting both amino and alcohol
62 groups such as ethanolamine, diethanolamine, 2-amino-1-butanol, 6-amino-1-hexanol, serine
63 and amino-alcohols with variable carbon chain length [9-14]. In such reactions, the lipase was
64 seen to catalyze O-acylation or N-acylation, with a chemoselectivity which is largely
65 dependent on amino-alcohol structure.

66 Among lipases used in organic synthesis, *Candida antarctica* lipase B is well known for its
67 ability to convert alcohols and amines into esters and amides in various organic solvents [8,
68 15] and seems to be the ideal enzyme for the acylation of compounds such as amino-alcohols.
69 In the present work, we thus investigated the *Candida antarctica* lipase B-catalyzed acylation
70 of various amines and amino-alcohols as acyl acceptors, using myristic acid as an acyl donor.
71 The results obtained under a kinetic approach were analyzed by comparing the apparent
72 kinetic parameters $V_{\max,app}$ and $K_{m,app}$ obtained for each acyl acceptor.

73 **2. Materials and Methods**

74 **2.1. Enzyme and chemicals**

75 Novozym[®] 435 (immobilized *Candida antarctica* lipase B), was kindly provided by
76 Novozymes A/S, Bagsvaerd, Denmark. (±)-alaninol **1** (98%), (R)-*sec*-butylamine **3** (99%),
77 (±)-1-methoxy-2-propylamine **5** (95%), (±)-1,2-diaminopropane **7** (≥98%) and 6-amino-1-
78 hexanol **11** (≥97%), as well as *tert*-amyl alcohol (99%) were purchased from Sigma-Aldrich
79 (St Louis, USA) while (±)-4-amino-1-pentanol **9** was from Santa Cruz Biotechnology (USA).
80 Myristic acid and acetic acid were from Fluka (St Quentin-Fallavier, Switzerland). All
81 chemicals were dried over molecular sieves. Pure water was obtained via a Milli-Q system
82 (Millipore, France). Acetonitrile and methanol were purchased from Carlo ERBA (Val-de-
83 Reuil, France).

84 2.2. Enzymatic reactions

85 In all cases, reactions were carried out in *tert*-amyl alcohol at 55°C in screw-caped tubes. 2 ml
86 reaction mixtures containing various amounts of acyl acceptor substrates (25-350 mM) and
87 175 mM of myristic acid as an acyl donor were incubated for 10 minutes prior to addition of
88 50 g l⁻¹ of *Candida antarctica* lipase B for the acylation of *sec*-butylamine **3** or 5 g l⁻¹ of
89 *Candida antarctica* lipase B for the acylation of another acyl acceptor. 100 µl samples were
90 taken at intervals and centrifuged at 14,000 rpm. The supernatants were then analyzed by LC-
91 MS, leading to the determination and quantification of remaining substrates and synthesized
92 products. Initial rate measurements were performed according to a previously established
93 procedure [16]. The initial rates were calculated from the linear relationship of the total
94 concentration of products against reaction time (0-2 h).

95 2.3. Evaluation of the chemoselectivity

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

$$101 C = (V_{max,app \text{ O-acylation}} / K_{m,app}) / (V_{max,app \text{ N-acylation}} / K_{m,app}) \quad (1)$$

$$102 C = V_{max,app \text{ O-acylation}} / V_{max,app \text{ N-acylation}} \quad (2)$$

103 2.4. HPLC and structural analysis

104 Structural and quantitative analysis of reaction products were conducted using a LC/MS-ES
105 system from Agilent (1100 LC/MSD Trap mass spectrometer VL) with a C18 Prontosil 120-
106 5-C18-AQ reversed-phase column (250×4 mm, 5 µm; Bischoff Chromatography). Products
107 were detected and quantified by differential refractometry and UV detection at 210 nm. An

108 external calibration was performed with pure myristic acid. Then calibrations for individual
109 acylation products were obtained after mass balance in reaction conditions enabling to obtain
110 only these products with myristic acid as an acyl donor. Low-resolution mass spectral
111 analyses were obtained by electrospray in the positive detection mode. Nitrogen was used as
112 the drying gas at 15 l min⁻¹ and 350 °C at a nebulizer pressure of 4 bars. The scan range was
113 50–1000 m/z using five averages and 13,000 m/z per second resolution. The capillary voltage
114 was 4000 V. Processing was done offline using HP Chemstation software.
115 Various eluent systems were used depending on the acyl acceptor used. Reaction samples
116 resulting from the acylation of (R)-*sec*-butylamine **3** were eluted with acetonitrile/water/acetic
117 acid (90/10/0.1, v/v/v) at room temperature and at a flow rate of 1 ml min⁻¹. Reaction samples
118 resulting from the acylation of 1-methoxy-2-propylamine **5** and 1,2-diaminopropane **7** were
119 eluted with methanol/water/acetic acid (95/5/0.1 and 93/7/0.1, v/v/v, respectively) at room
120 temperature and at a flow rate of 1 ml min⁻¹. The elution of reaction samples resulting from
121 the acylation of amino-alcohols **1**, **9** and **11**, was carried out at room temperature and at a flow
122 rate of 1 ml min⁻¹, using a gradient that was derived from two eluent mixtures (Table 1).

123

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

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

126

127 In order to perform the purification and characterization of acylated products, 20 ml reaction
128 mixtures containing 50 mM of the acyl acceptor and 175 mM of myristic acid in *tert*-amyl
129 alcohol were incubated for 24 h in presence of 15 g l⁻¹ of *Candida antarctica* lipase B.
130 Purified products were then characterized by ¹H NMR and IR via preparative HPLC using a
131 ProntoPrep C18 reversed-phase column (250×20 mm, 10 μm; Bischoff Chromatography)
132 eluted with the gradient given in Table 1, at room temperature and at a flow rate of 5 ml min⁻¹.
133 ¹H NMR were recorded on a JEOL-JNM LA400 spectrometer (400 MHz), with
134 tetramethylsilane as an internal reference. Samples were studied as solutions in CDCl₃.
135 Infrared (IR) spectra were recorded from 400 to 4000 cm⁻¹ with a resolution of 4 cm⁻¹ using a
136 100 ATR spectrometer (Perkin-Elmer, United States).

137 **N-myristyl 2-amino-1-propanol 2a:** m/Z (LR-ESI⁺) C₁₇H₃₅NO₂ (M + H⁺), found:
138 286.4, calculated for: 286.48. IR ν_{max} (cm⁻¹): 3100-3500 (O-H, alcohol and N-H, amide),
139 2800-3000 (CH of myristyl chain), 1638 (C=O, amide), 1543 (N-H, amide). ¹H NMR (400
140 MHz, CDCl₃, δ ppm): δ 0.88 (t, 3H, J= 6.06Hz, -CH₂-CH₃), 1.17 (d, 3H, J= 6.06Hz, -CH-
141 CH₃), 1.25 (m, 20H, -CH₂- of myristyl chain), 1.63 (m, 2H, -CH₂-CH₂-CO-NH- of myristyl
142 chain), 2.19 (t, 2H, J= 6,06Hz, -CH₂-CH₂-CO-NH- of myristyl chain), 3.04 (s, 1H, -OH), 3.27
143 (dd, 1H, J= 5Hz, J= 10Hz, -CH-CH₂-OH), 3.46 (dd, 1H, J= 3.7Hz, J= 11Hz, -CH-CH₂-
144 OH), 4.07 (m, 1H, -CH-), 5.7 (s, 1H, -NH-).

145 **N,O-dimyristyl 2-amino-1-propanol 2c:** m/Z (LR-ESI⁺) C₃₁H₆₂NO₃ (M + Na⁺), found:
146 518.6, calculated for: 518.85. IR ν_{max} (cm⁻¹): 3301 (N-H, amide), 2800-3000 (CH of myristyl
147 chain), 1737 (C=O, ester), 1643 (C=O, amide), 1542 (N-H, amide). ¹H NMR (400 MHz,
148 CDCl₃, δ ppm): δ 0.88 (t, 6H, J= 7.6Hz, 2x -CH₂-CH₃), 1.16 (d, 3H, J= 7.6Hz, -CH-CH₃),
149 1.25 (m, 40H, -CH₂- of myristyl chain), 1.6 (m, 4H, 2x -CH₂-CH₂-CO- of myristyl chain),
150 2.14 (t, 2H, J= 7.2Hz, -CH₂-CH₂-CO-O- of myristyl chain), 2.32 (t, 2H, J= 7.2Hz, -CH₂-
151 CH₂-CO-NH- of myristyl chain), 4 (dd, 1H, J= 4.4Hz, J= 10.7Hz, -CH-CH₂-O-), 4.13 (dd,

152 1H, $J = 4.9\text{Hz}$, $J = 10\text{Hz}$, $-\text{CH}-\underline{\text{CH}}_2-\text{O}-$), 4.29 (m, 1H, $-\text{CH}-$), 5.54 (d, 1H, $J = 7.3\text{Hz}$, C-NH-
153 CH_2-).

154 **N-myristyl *sec*-butylamine 4a:** m/Z (LR-ESI⁺) $\text{C}_{18}\text{H}_{37}\text{NO}$ ($M + \text{H}^+$), found: 284.2,
155 calculated for: 284.51. ¹H NMR (400 MHz, CDCl_3 , δ ppm): δ 0.89 (m, 6H, 2x $-\text{CH}_2-\underline{\text{CH}}_3$), 1.1
156 (d, 3H, $J = 7\text{Hz}$, $-\text{CH}-\underline{\text{CH}}_3$), 1.25 (m, 20H, $-\text{CH}_2-$ of myristyl chain), 1.43 (m, 2H, $-\text{CH}-\underline{\text{CH}}_2-$
157 CH_3), 1.6 (m, 2H, $-\underline{\text{CH}}_2-\text{CH}_2-\text{CO}-\text{NH}-$ of myristyl chain), 2.12 (st, 2H, $J = 7\text{Hz}$, $-\text{CH}_2-\underline{\text{CH}}_2-$
158 $\text{CO}-\text{NH}-$ of myristyl chain), 3.9 (st, 1H, $J = 6.8\text{Hz}$, $-\text{CH}-$), 5.17 (s, 1H, $-\text{NH}-$).

159 **N-myristyl 1-methoxy-2-propylamine 6a:** m/Z (LR-ESI⁺) $\text{C}_{18}\text{H}_{37}\text{NO}_2$ ($M + \text{H}^+$),
160 found: 300.3, calculated for: 300.51. IR ν_{max} (cm^{-1}): 3304 (N-H, amide), 2800-3000 (CH of
161 myristyl chain), 1634 (C=O, amide), 1544 (N-H, amide). ¹H NMR (400 MHz, CDCl_3 , δ ppm):
162 δ 0.88 (t, 3H, $J = 6.79\text{Hz}$, $-\text{CH}_2-\underline{\text{CH}}_3$), 1.17 (d, 3H, $J = 6.17\text{Hz}$, $-\text{CH}-\underline{\text{CH}}_3$), 1.26 (m, 20H, -
163 CH_2- of myristyl chain), 1.62 (m, 2H, $-\underline{\text{CH}}_2-\text{CH}_2-\text{CO}-\text{NH}-$ of myristyl chain), 2.15 (t, 2H, $J =$
164 6.8Hz , $-\text{CH}_2-\underline{\text{CH}}_2-\text{CO}-\text{NH}-$ of myristyl chain), 2.19 (t, 1H, $J = 6.8\text{Hz}$, $-\text{CH}-\underline{\text{CH}}_2-\text{OCH}_3$), 3.36
165 (m, 3H, $-\text{OCH}_3$), 4.06 (t, 1H, $J = 6.45\text{Hz}$, $-\text{CH}-\underline{\text{CH}}_2-\text{OCH}_3$), 4.16 (m, 1H, $-\text{CH}-$), 5.62 (d, 1H,
166 $J = 5.84\text{Hz}$, $-\text{NH}-$).

167 **1-N-myristyl 1,2-diaminopropane 8a:** m/Z (LR-ESI⁺) $\text{C}_{17}\text{H}_{36}\text{N}_2\text{O}$ ($M + \text{H}^+$), found:
168 285.4, calculated for: 285.49. ¹H NMR (400 MHz, CDCl_3 , δ ppm): δ 0.88 (t, 3H, $J = 6.67\text{Hz}$, -
169 $\text{CH}_2-\underline{\text{CH}}_3$), 1.22 (d, 3H, $J = 6.67\text{Hz}$, $-\text{CH}-\underline{\text{CH}}_3$), 1.25 (m, 20H, $-\text{CH}_2-$ of myristyl chain), 1.61
170 (q, 2H, $J = 6.67\text{Hz}$, $-\underline{\text{CH}}_2-\text{CH}_2-\text{CO}-\text{NH}-$ of myristyl chain), 1.96 (s, 2H, $-\text{NH}_2$), 2.19 (t, 2H, $J =$
171 7.3Hz , $-\text{CH}_2-\underline{\text{CH}}_2-\text{CO}-\text{NH}-$ of myristyl chain), 3.21(st, 1H, $J = 5.9\text{Hz}$, $-\text{CH}-$), 3.27 (qd, 1H,
172 $J = 2.6\text{Hz}$, $J = 11.8\text{Hz}$, $-\text{CH}-\underline{\text{CH}}_2-\text{NH}-$), 3.46 (qd, 1H, $J = 3.24\text{Hz}$, $J = 13.6\text{Hz}$, $-\text{CH}-\underline{\text{CH}}_2-\text{NH}-$),
173 7.22 (t, 1H, $J = 4.42\text{Hz}$, $-\text{NH}-$).

174 **N-myristyl 4-amino-1-pentanol 10a:** m/Z (LR-ESI⁺) $\text{C}_{19}\text{H}_{39}\text{NO}_2$ ($M + \text{H}^+$), found:
175 314.2 calculated for: 314.53. IR ν_{max} (cm^{-1}): 3200-3500 (O-H, alcohol and N-H, amide),

176 2800-3000 (CH of myristyl chain), 1639 (C=O, amide), 1545 (N-H, amide). ¹H NMR (400
177 MHz, CDCl₃, δ ppm): δ 0.88 (t, 3H, *J*= 6.58Hz, -CH₂-CH₃), 1.14 (d, 3H, *J*= 6.23Hz, -CH-
178 CH₃), 1.25 (m, 20H, -CH₂- of myristyl chain), 1.53 (m, 4H, -CH-CH₂-CH₂-CH₂-OH), 1.63
179 (m, 2H, -CH₂-CH₂-CO-NH- of myristyl chain), 2.14 (t, 2H, *J*= 7.27Hz, -CH₂-CH₂-CO-NH-
180 of myristyl chain), 2.94 (s, 1H, -OH), 3.67 (m, 2H, -CH₂-CH₂-OH), 4.06 (m, 1H, -CH-), 5.28
181 (s, 1H, -NH-).

182 **O-myristyl 4-amino-1-pentanol 10a:** m/Z (LR-ESI⁺) C₁₉H₃₉NO₂ (M + H⁺), found:
183 314.2 calculated for: 314.53. IR ν_{max} (cm⁻¹): 3291 (N-H, amine), 2800-3000 (CH of myristyl
184 chain), 1736 (C=O, ester), 1557 (N-H, amine). ¹H NMR (400 MHz, CDCl₃, δ ppm): δ 0.88 (t,
185 3H, *J*= 6.99Hz, -CH₂-CH₃), 1.14 (d, 3H, *J*= 8Hz, -CH-CH₃), 1.25 (m, 20H, -CH₂- of myristyl
186 chain), 1.52 (m, 4H, -CH-CH₂-CH₂-CH₂-O-), 1.62 (m, 2H, -CH₂-CH₂-CO-O- of myristyl
187 chain), 2.22 (t, 1H, *J*= 7.16Hz, -CH₂-CH₂-CO-O- of myristyl chain), 2.29 (t, 1H, *J*= 7.5Hz, -
188 CH₂-CH₂-CO-O- of myristyl chain), 3.43 (m, 1H, -CH₂-CH₂-O-), 3.69 (m, 1H, -CH₂-CH₂-O-
189), 4.1 (m, 1H, -CH-).

190 **N,O-dimyristyl 4-amino-1-pentanol 10c:** m/Z (LR-ESI⁺) C₃₃H₆₆NO₃ (M + Na⁺),
191 found: 546.2 calculated for: 546.9. IR ν_{max} (cm⁻¹): 3304 (N-H, amide), 2800-3000 (CH of
192 myristyl chain), 1732 (C=O, ester), 1640 (C=O, amide), 1546 (N-H, amide). ¹H NMR (400
193 MHz, CDCl₃, δ ppm): δ 0.88 (t, 6H, *J*= 7.43Hz, 2x -CH₂-CH₃), 1.14 (d, 3H, *J*= 6.83Hz, -CH-
194 CH₃), 1.25 (m, 40H, -CH₂- of myristyl chain), 1.53 (m, 4H, -CH-CH₂-CH₂-CH₂-O-C), 1.6
195 (m, 4H, 2x -CH₂-CH₂-CO- of myristyl chain), 2.14 (t, 4H, *J*= 7.08Hz, -CH₂-CH₂-CO- of
196 myristyl chain), 3.68 (m, 2H, -CH₂-CH₂-O-C), 4.07 (m, 1H, -CH-), 5.27 (d, 1H, *J*= 6.86Hz, -
197 NH-).

198 **N-myristyl aminohexanol 12a:** m/Z (LR-ESI⁺) C₂₀H₄₁NO₂ (M + H⁺), found: 329.5
199 calculated for: 328.56. IR ν_{max} (cm⁻¹): 3385 (O-H, alcohol), 3314 (N-H, amide), 2800-3000

200 (CH of myristyl chain), 1634 (C=O, amide), 1534 (N-H, amide). ^1H NMR (400 MHz, CDCl_3 ,
201 δ ppm): δ 0.88 (t, 3H, $J= 7.5\text{Hz}$, $-\text{CH}_2-\underline{\text{CH}}_3$), 1.25 (m, 20H, $-\text{CH}_2-$ of myristyl chain), 1.51 (m,
202 2H, $-\underline{\text{CH}}_2-\text{CH}_2-\text{CO}-\text{O}-$ of myristyl chain), 1.59 (m, 4H, $-\text{CH}_2-\underline{\text{CH}}_2-\underline{\text{CH}}_2-\text{CH}_2-\text{OH}$), 2.26 (t, 2H,
203 $J= 7.65\text{Hz}$, $-\text{CH}_2-\underline{\text{CH}}_2-\text{CO}-\text{OH}$ of myristyl chain), 2.72 (s, 1H, $-\text{OH}$), 3.25 (t, 2H, $J= 7.07\text{Hz}$,
204 $-\text{CH}_2-\underline{\text{CH}}_2-\text{OH}$), 3.63 (t, 2H, $J= 7.29\text{Hz}$, $-\text{CH}_2-\underline{\text{CH}}_2-\text{NH}-\text{CO}-\text{CH}_2$), 5.41 (s, 1H, $-\text{NH}-$).

205 **O-myristyl aminohexanol 12b:** m/Z (LR-ESI $^+$) $\text{C}_{20}\text{H}_{41}\text{NO}_2$ ($\text{M} + \text{H}^+$), found: 329.5
206 calculated for: 328.56. IR ν_{max} (cm^{-1}): 3400 (N-H, amine), 2800-3000 (CH of myristyl chain),
207 1736 (C=O, ester), 1544 (N-H, amine). ^1H NMR (400 MHz, CDCl_3 , δ ppm): δ 0.88 (t, 3H, $J=$
208 7.28Hz , $-\text{CH}_2-\underline{\text{CH}}_3$), 1.25 (m, 20H, $-\text{CH}_2-$ of myristyl chain), 1.55 (m, 2H, $-\underline{\text{CH}}_2-\text{CH}_2-\text{CO}-\text{O}-$
209 of myristyl chain), 1.62 (m, 4H, $-\text{CH}_2-\underline{\text{CH}}_2-\underline{\text{CH}}_2-\text{CH}_2-\text{NH}_2$), 2.28 (t, 2H, $J= 7.65\text{Hz}$, $-\text{CH}_2-$
210 $\underline{\text{CH}}_2-\text{CO}-\text{O}-$ of myristyl chain), 2.81 (s, 2H, $-\text{NH}_2$), 3.64 (t, 2H, $J= 6.47\text{Hz}$, $-\text{CH}_2-\underline{\text{CH}}_2-\text{NH}_2$),
211 4.04 (t, 2H, $J= 6.47\text{Hz}$, $-\text{CH}_2-\underline{\text{CH}}_2-\text{O}-\text{CO}-\text{CH}_2$).

212 **N,O-dimyristyl aminohexanol 12c:** m/Z (LR-ESI $^+$) $\text{C}_{34}\text{H}_{67}\text{NO}_3$ ($\text{M} + \text{Na}^+$), found:
213 560.7, calculated for: 560.93. IR ν_{max} (cm^{-1}): 3298 (N-H, amide), 2800-3000 (CH of myristyl
214 chain), 1726 (C=O, ester), 1635 (C=O, amide), 1547 (N-H, amide). ^1H NMR (400 MHz,
215 CDCl_3 , δ ppm): δ 0.88 (t, 6H, $J= 6.48\text{Hz}$, $2x -\text{CH}_2-\underline{\text{CH}}_3$), 1.25 (m, 40H, $-\text{CH}_2-$ of myristyl
216 chain), 1.5 (m, 4H, $-\underline{\text{CH}}_2-\text{CH}_2-\text{CO}-$ of myristyl chain), 1.6 (m, 4H, $-\text{CH}_2-\underline{\text{CH}}_2-\underline{\text{CH}}_2-\text{CH}_2-\text{O}-\text{C}$),
217 2.15 (t, 2H, $J= 7.8\text{Hz}$, $-\text{CH}_2-\underline{\text{CH}}_2-\text{CO}-\text{NH}-$), 2.29 (t, 2H, $J= 7.8\text{Hz}$, $-\text{CH}_2-\underline{\text{CH}}_2-\text{CO}-\text{NH}-$), 3.24
218 (q, 2H, $J= 6.5\text{Hz}$, $-\text{CH}_2-\underline{\text{CH}}_2-\text{NH}-$), 4.06 (t, 2H, $J= 5.9\text{Hz}$, $-\text{CH}_2-\underline{\text{CH}}_2-\text{O}-\text{CO}-\text{CH}_2$), 5.4 (s,
219 1H, $-\text{NH}-$).

220 **2.5. Analysis of the ionization state of substrates**

221 The ionization state of myristic acid in *tert*-amyl alcohol was investigated using infrared
222 spectroscopy analysis. Infrared (IR) spectra of samples containing 175 mM myristic acid and
223 from 0 to 250 mM alaninol were recorded from 1500 to 1800 cm^{-1} with a resolution of 4

224 cm⁻¹ using a 100 ATR spectrometer (Perkin-Elmer, United States). Before the interpretation
225 of data, a treatment (base line correction, smoothing and normalization min–max) was applied
226 to spectra.

227 **3. Results and discussion**

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

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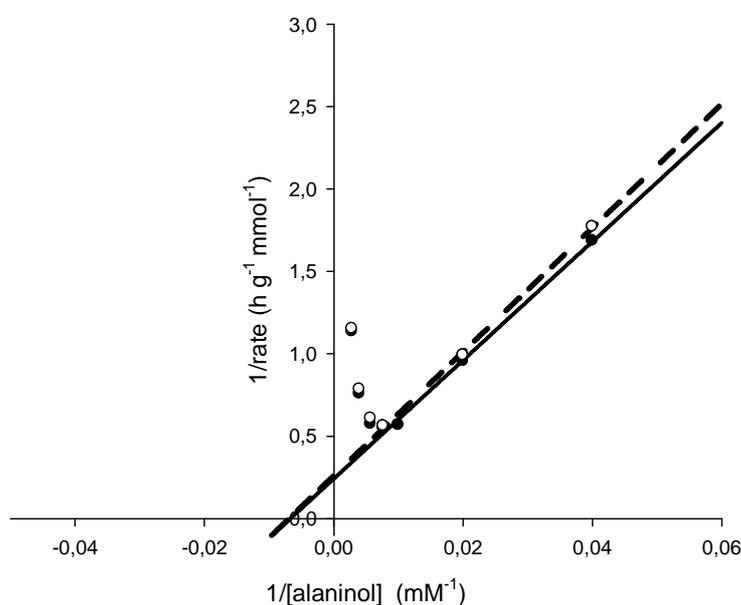
Table 2. Structure of substrates and products resulting from the acylation of various acyl acceptors catalysed by *Candida antarctica* lipase B using myristic acid as an acyl donor in *tert*-amyl alcohol.

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)- <i>sec</i> -butylamine (3)	 N-myristyl <i>sec</i> -butylamine (4a)	-	-
3	 (±)-1-methoxy-2-propylamine (5)	 N-myristyl 1-méthoxy-2-propylamine (6a)	-	-
4	 (±)-1,2-diaminopropane (7)	 1-N-myristyl 1,2-diaminopropane (8a)	-	-
5	 (±)-4-amino-1-pentanol (9)	 N-myristyl 4-amino-1-pentanol (10a)	 O-myristyl 4-amino-1-pentanol (10b)	 N,O-dimyristyl 4-amino-1-pentanol (10c)
6	 6-amino-1-hexanol (11)	 N-myristyl amino-1-hexanol (12a)	 O-myristyl amino-1-hexanol (12b)	 N,O-dimyristyl amino-1-hexanol (12c)

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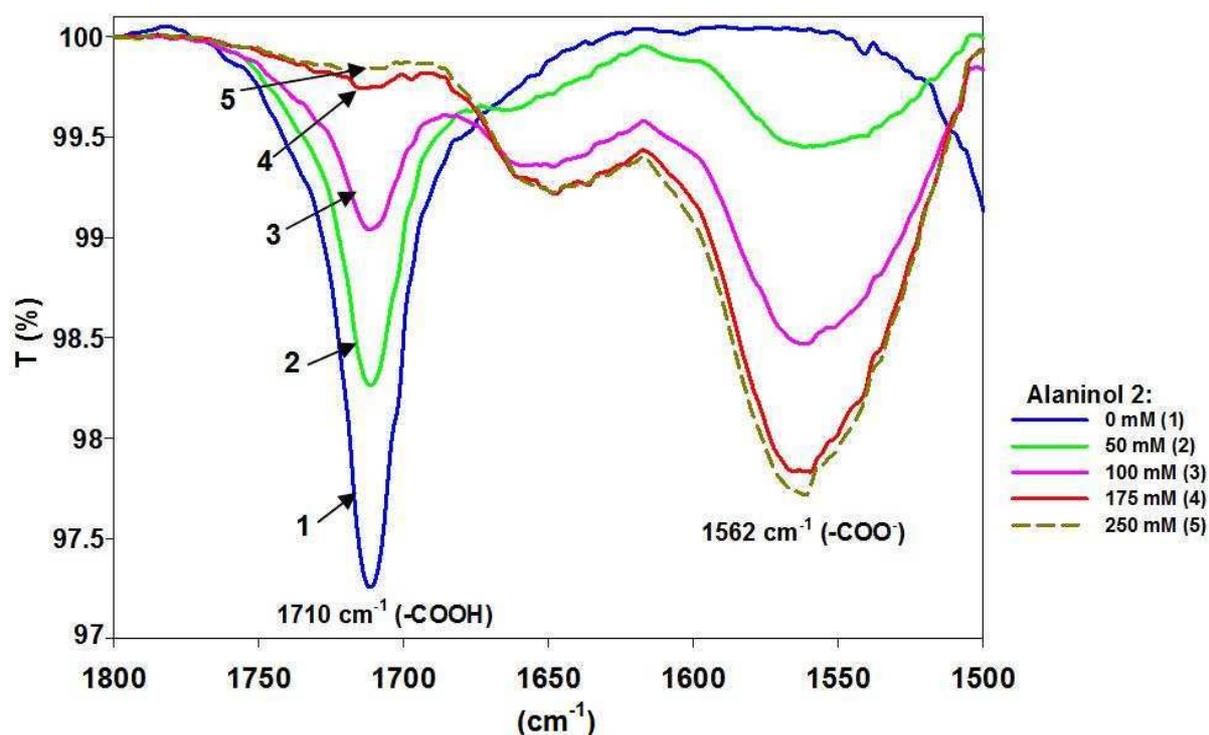
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242 The acylation of alaninol (amino-alcohol **1**, Table 2, entry 1) was chosen as the model
243 reaction to study the kinetic behaviour of *Candida antarctica* lipase B when catalyzing the
244 acylation of amino-alcohols. To achieve this, we determined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



255
256 **Fig. 1:** Reciprocal initial rates of myristic acid conversion (●) and alaninol N-acylation (○)
257 versus reciprocal alaninol concentrations. Reactions were carried out at 55°C using a fixed
258 concentration of myristic acid (175 mM) and 5 g l^{-1} of *Candida antarctica* lipase B in 2 ml of
259 *tert*-amyl alcohol. The data represent the averages of triplicate runs whose standard deviations
260 were always lower than 15%.

261
262 For high concentrations of amino-alcohol (superior to 100 mM), a decrease in initial rates was
263 observed. This phenomenon was most likely due to an inhibitor effect similar to an excess
264 substrate inhibition probably due to an interaction between myristic acid and the amino group
265 of alaninol. Indeed, the presence of an amino substrate and a fatty acid in an organic solvent
266 generally leads to the formation of an ion-pair complex between both substrates, depending
267 on the acido-basic conditions of the medium [16, 18, 19]. This salt complex makes the ion
268 forms of both substrates non reactive (NH_3^+ amine form and COO^- fatty acid form) and
269 therefore leads to the overestimation of the substrate concentrations that are really available
270 for the enzyme in the reaction medium. This ion-pair complex was already described by
271 Maugard et al. [16] as a limiting factor of the lipase-catalyzed acylation under conditions
272 where it was less soluble than free substrates. To ascertain this hypothesis, the composition of
273 the medium, especially the carbonyl species, was analyzed by infrared spectroscopy at the
274 start of the reaction, for four concentrations of alaninol within the range 50-250 mM (Fig. 2).
275 When only myristic acid was solubilized in *tert*-amyl alcohol, only one carbonyl band was
276 observed at 1710 cm^{-1} , corresponding to the acid form. When the concentration of alaninol
277 was increased, the carbonyl acid band disappeared in favor of a band at 1562 cm^{-1}
278 corresponding to a carboxylate ion. This additional band demonstrated the formation of an
279 ion-pair complex between myristic acid and alaninol when using an alaninol concentration
280 superior or equal to 100 mM.



281
 282 **Fig. 2:** IR analysis of mixtures containing 175 mM of myristic acid and various
 283 concentrations of alaninol in *tert*-amyl alcohol.
 284

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

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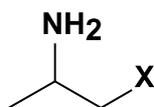
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298 **Table 3.** Apparent kinetics parameters for the N-acylation and O-acylation of various acyl
 299 acceptors using *Candida antarctica* lipase B in *tert*-amyl alcohol at 55°C. Myristic acid (175
 300 mM) was used as an acyl donor.

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)- <i>sec</i> -butylamine 3	619	0.2	-
3	(±)-1-methoxy- 2-propylamine 5	185	1.6	-
4	(±)-1,2-diaminopropane 7	252	7.6	-
5	(±)-4-amino-1-pentanol 9	75	1.1	7.3
6	6-amino-1-hexanol 11	63	1	10.1

301

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



305 $X = \text{OH}$ (**1**), CH_3 (**3**), OCH_3 (**5**) or NH_2 (**7**)

306 **Scheme 1:** Alaninol (**1**) and structurally related amines (**3,5,7**).

307 The $K_{m,app}$ of amine **3** ((R)-*sec*-butylamine) (619 mM; Table 3, entry 2) was 3-fold higher
 308 than the $K_{m,app}$ of amino-alcohol **1** (alaninol) (182 mM; Table 3, entry 1). This showed a better
 309 affinity of *Candida antarctica* lipase B toward a bifonctionnal amino-alcohol rather than a
 310 monofunctional amine. On the other hand, the $K_{m,app}$ values of bifunctional compounds **1**

311 (alaninol), **5** (1-methoxy-2-propylamine) and **7** (1,2-diaminopropane), structurally related
312 substrates with two carbons between the two functional groups, were of the same order ($K_{m,app}$
313 respectively equal to 182 mM, 185 mM and 252 mM ; Table 3, entries 1, 3 and 4). On the
314 contrary, $V_{max,app}$ values of N-acylation for compounds **1**, **5** and **7** were variable (from 1.6 to
315 7.6 mmol h⁻¹ g⁻¹), which showed that *Candida antarctica* lipase B catalytic efficiency was
316 mainly depending on its differential catalytic activity for these substrates. Furthermore, in a
317 previous work we have compared the acylation of mono-amine **3** ((R)-*sec*-butylamine) and a
318 structurally similar secondary alcohol: (R)-2-butanol. We noticed that the $V_{max,app}$ of O-
319 acylation of (R)-2-butanol was 23-fold higher than the $V_{max,app}$ of N-acylation of amine **3** [20].
320 These results were in contrast with those obtained for the acylation of alaninol, for which no
321 mono-O-acylation product was detected and a value of $V_{max,app}$ equal to 4.3 mmol h⁻¹ g⁻¹ was
322 obtained for N-acylation. To better understand the influence of substrate structure on the
323 chemoselectivity of *Candida antarctica* lipase B, we compared the $V_{max,app}$ of mono-N-
324 acylation of structurally related amines (scheme 1, compounds **1**, **3**, **5** and **7**, Table 3, entries
325 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) >
326 amino-alcohol **1** (alaninol) (4.3 mmol h⁻¹ g⁻¹) > methoxyamine **5** (1.6 mmol h⁻¹ g⁻¹) >> amine **3**
327 (0.2 mmole h⁻¹ g⁻¹). The first substrate diamine **7**, which exhibits the highest $V_{max,app}$ values
328 was mono-N-acylated only in position 1. Indeed, only mono-amide **8a** was detected (Table 2,
329 entry 4). The last substrate amine **3** is a monofunctional amine with no nucleophilic group in
330 β -position of an amino group. From these results, we could thus conclude that the presence of
331 a nucleophilic group (alcohol group, methoxy group or a second amino group) in β -position
332 of the acyl-acceptor amino group was responsible for the enhancement of $V_{max,app}$ of N-
333 acylation of this amino group and that the more nucleophilic group (-NH₂ > -OH > -OCH₃) in
334 β -position, the higher the reactivity for the amino group.

335 Secondly, we compared the acylation of three amino-alcohols that exhibited a variable carbon
336 chain length between the amino and alcohol groups: amino-alcohols **1** (two carbons), **9** (four
337 carbons) and **11** (six carbons) (Table 2, entries 1, 5 and 6). In terms of $K_{m,app}$ values, the $K_{m,app}$
338 of amino-alcohol **9** (75 mM; Table 3, entry 5) was in the same order than the $K_{m,app}$ of amino-
339 alcohol **11** (63 mM; Table 3, entry 6), whereas the $K_{m,app}$ of amino-alcohol **1** (182 mM; Table
340 3, entry 1) was higher. This pointed out a better affinity of *Candida antarctica* lipase B
341 toward long chain amino-alcohols **9** and **11** than for short amino-alcohols **1**. On the other
342 hand, the acylation of long chain amino-alcohols **9** and **11** by *Candida antarctica* lipase B
343 gave $V_{max,app}$ of N-acylation of 1.1 and 1 mmol h⁻¹ g⁻¹ (Table 3, entries 5 and 6), respectively,
344 as short chain amino-alcohol **1** was N-acylated 4-fold faster ($V_{max,app}$ of N-acylation: 4.3 mmol
345 h⁻¹ g⁻¹; Table 3, entry 1) than long chain amino-alcohols. In contrast, $V_{max,app}$ of O-acylation of
346 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,
347 entries 5 and 6) were obtained, whereas no mono-O-acylated product and only trace amounts
348 of amido-ester **2c** were detected during the acylation of short chain amino-alcohol **1**. This was
349 attributed to the fact that the reaction could take place at the amino group (N-acylation) and/or
350 alcohol group (O-acylation) of long chain amino-alcohols **9** and **11**, giving either N- or O-
351 acylated products **10a**, **10b** **12a** and **12b** (Table 2, entries 5 and 6), while the mono-O-
352 acylation of short chain amino-alcohol **1** did not occur. Starting from these results, we could
353 calculate the chemoselectivity ratio (Eq. (2)) of the *Candida antarctica* lipase B-catalyzed
354 acylation of long chain amino-alcohols **9** and **11**, which was close to 6.6 and 10.1,
355 respectively. From these results, we could conclude that the increase in the carbon chain
356 length between the alcohol and amino groups of long chain amino-alcohols was concomitant
357 with the increase in the chemoselectivity of *Candida antarctica* lipase B for the O-acylation
358 of these substrates.

359 To interpret all these data resulting from the acylation of structurally related amines (**1**, **3**, **5**
360 and **7**) and amino-alcohols with various carbon chain length (**1**, **9** and **11**), we formulated the
361 following postulate: the presence of a nucleophilic group in β -position of the amino group of
362 the acyl acceptor amine resulted in the enhancement of the $V_{\max,app}$ of N-acylation of this
363 amino group. This may be due to the formation of an intramolecular interaction between the
364 amino group and the nucleophilic group located in β -position, which is strengthened by the
365 fact that this interaction could not occur for long chain amino-alcohols **9** and **11**, considering
366 the longer distance between both functional groups, giving as a result a decrease in the $V_{\max,app}$
367 of N-acylation.

368 **Conclusion**

369 In this work, we investigated the *Candida antarctica* lipase B-catalyzed acylation of various
370 amines and amino-alcohols as acyl acceptors, using myristic acid as an acyl donor, and
371 showed that the presence of a nucleophilic group ($-\text{NH}_2$ or $-\text{OH}$ or $-\text{OCH}_3$) in β -position of the
372 amino group of the acyl acceptor enhances the $V_{\max,app}$ of N-acylation and thus the enzyme
373 activity. Moreover, the crucial role of the carbon chain length between the alcohol and amino
374 groups was highlighted in the *Candida antarctica* lipase B-catalyzed acylation of amino-
375 alcohols. The $V_{\max,app}$ of N-acylation was indeed improved when the carbon chain included
376 two carbons (alaninol **1**) whereas the $V_{\max,app}$ of O-acylation was improved when the carbon
377 chain included four carbons or more (4-amino-1-pentanol **9** and 6-amino-1-hexanol **11**). The
378 present investigation demonstrated the great influence of substrate structure on the
379 chemoselectivity of *Candida antarctica* lipase B, providing new insights for the selective
380 synthesis of amides or esters produced from the acylation of bifunctional substrates.
381 Molecular modelling studies are currently in progress to study in details the acylation
382 mechanism of amino-alcohols.

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

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