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Regioselective Esterification of Polyhydroxylated Steroids by *Candida antarctica* Lipase B

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Abstract: The regioselectivity of *Candida antarctica* lipase B towards the acetylation of polyhydroxylated steroids has been systematically investigated. The enzyme showed a marked preference for the alcoholic moieties on the A ring and on the steroidal side chain, making it possible the selective acylation at the positions 3 or 21 of polyhydroxy steroids. Acylation with the synthetically useful esters chloroacetate and levulinate was also accomplished, whereas esterification with benzoate and pivaloate was unsuccessful.

Introduction

The innumerable successful applications of biocatalysis are spurring the search for new enzymes with increased stability and improved (or different) specificity.[1] Molecular biology has been greatly supporting this effort, allowing the overexpression of enzymes from different sources in easy-to-handle host microorganisms.[2] An example of this approach is given by the recent cloning of the two lipases from the yeast *Candida antarctica* into the host organism *Aspergillus oryzae*.[3] One of these two lipases (lipase B, presently commercially available in purified form and supported on a macroporous acrylic resin) has shown interesting enantioselective[4] and regioselective[5] properties.

In the framework of our research aimed to the selective enzymatic modification of steroids,[6] we decided to check the ability of *Candida antarctica* lipase B (*C.a.*B lipase) to discriminate between different OH's on the same steroid molecule, and in the following we will present the results of this study.

Results and Discussion

Biocatalysis has been playing a prominent role in the selective modification of the functional groups of the steroid skeleton.[7] In recent years, we have reported on the regioselective acylation of steroid hydroxyls catalyzed by lipases and proteases in organic solvents.[8] Specifically, lipases from *Chromobacterium viscosum* and *Candida cylindracea* have shown an exclusive action on the alcoholic functionalities of the A ring, while the protease subtilisin Carlsberg showed an overwhelming preference for the hydroxyl groups on the D ring of the steroid skeleton.

In order to get preliminary information on *C.a.*B lipase specificity, we applied the well-known protocol for irreversible enzymatic acetylation in organic solvents[9] to the series of monohydroxy steroids reported in **Chart 1**. The initial rates of acetylation of compounds **1-15**, determined by GC or HPLC, were quite indicative of the specificity of *C.a.*B lipase.

Chart 1

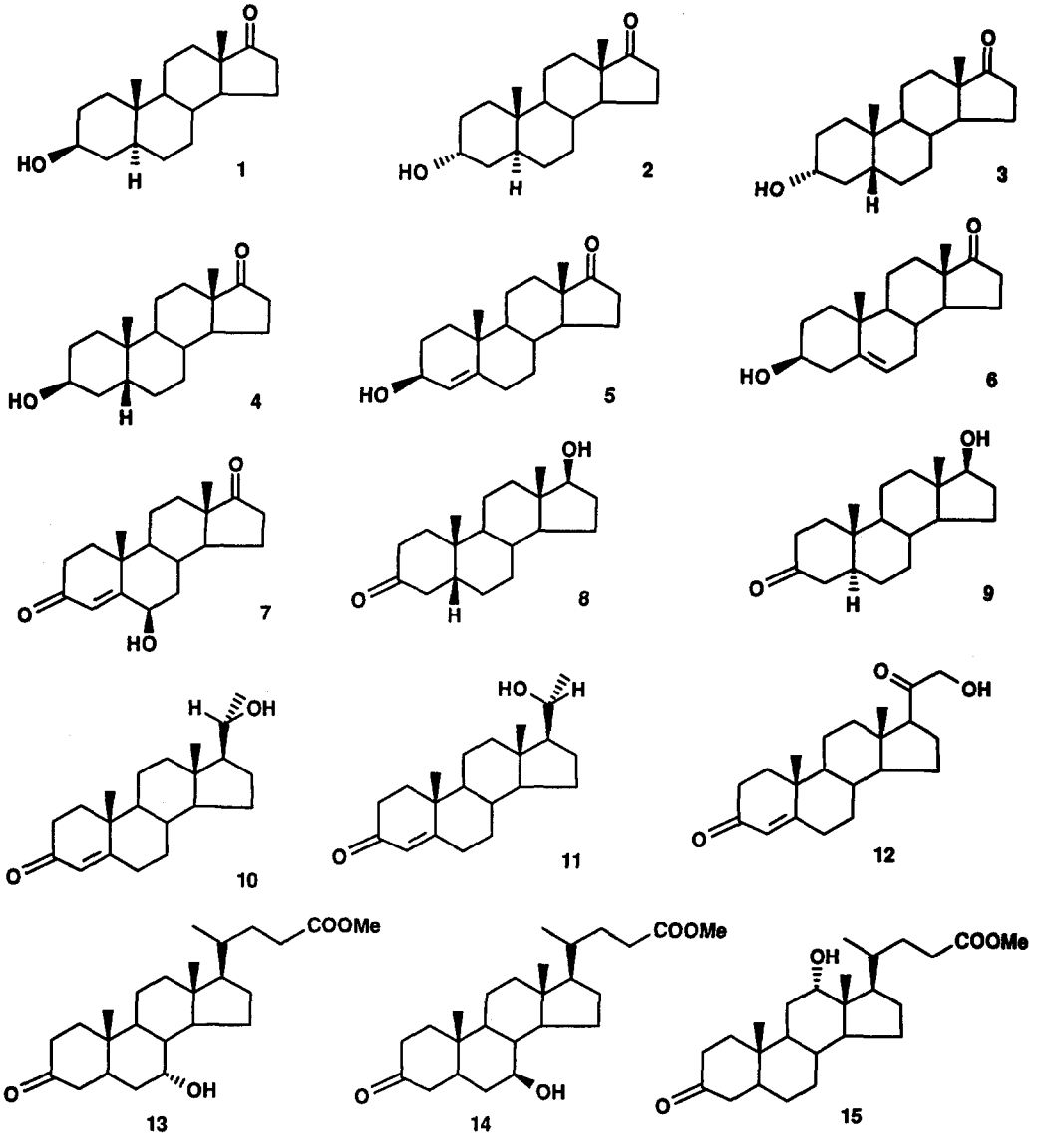


Table 1.Initial Rates of Acetylation of Various Hydroxysteroids Catalyzed by *Candida antarctica* Lipase

steroid[a)]		initial rate[b)] (μmol/h)	relative rate
3β-hydroxy-5α-androstane-17-one	(1)	1.12	17
3α-hydroxy-5α-androstane-17-one	(2)	0.13	2
3α-hydroxy-5β-androstane-17-one	(3)	12.06	172
3β-hydroxy-5β-androstane-17-one	(4)	0.07	1
3β-hydroxy-4-androstene-17-one	(5)	0.41	6
3β-hydroxy-5-androstene-17-one	(6)	1.11	16
6β-hydroxy-4-androstene-3,17-dione	(7)	0	—
17β-hydroxy-5β-androstane-3-one	(8)	0	—
17β-hydroxy-5α-androstane-3-one	(9)	0	—
20α-hydroxy-4-pregnene-3-one	(10)	0	—
20β-hydroxy-4-pregnene-3-one	(11)	0.44	6
21-hydroxy-4-pregnene-3,20-dione	(12)	37.06	529
7α-hydroxy-3-oxo-5β-cholan-24-oic acid methyl ester	(13)	0	—
7β-hydroxy-3-oxo-5β-cholan-24-oic acid methyl ester	(14)	0	—
12α-hydroxy-3-oxo-5β-cholan-24-oic acid methyl ester	(15)	0	—

a) Conditions: 15 mM steroid, 0.5 ml THF, 0.5 ml vinyl acetate, 20 mg *C.a.*B lipase. Suspensions were shaken at 45°C. At regular times aliquots were withdrawn and assayed for product formation.

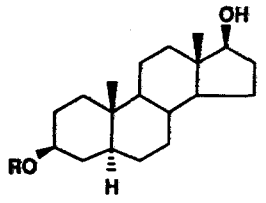
b) Determined by capillary GC (compounds 1-12) or HPLC (compounds 13-15)

As shown in **Table 1**, OH's at the C-3 position were always esterified even though with different rates: the best substrates (3α,5β, 3) was acylated about 170 times faster than the worst one (3β,5β, 4). On the other hand, the enzyme was inactive with the alcholic moieties on the internal B and C rings.[10]

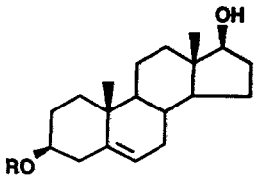
Finally, the data relative to the other limb of the tetracyclic molecule were quite variegated. While C-17 OH's were not substrates, the enzyme showed an interesting stereoselectivity for the C-20 OH: the pregnene **11**

(20β-OH) was acylated while compound **10** (20α-OH) was not, a selectivity opposite to the one previously observed with subtilisin.[8] Besides, the primary C-21 OH was easily acetylated, being the best substrate of the series. Therefore, contrary to the behaviours of the other hydrolases studied so far,[8] *C.a.*B lipase does not have an exclusive preference for a portion of the steroid skeleton, but it accomodates these molecules in its active site in different ways, even if with distintive preferences towards the positional substitutions.

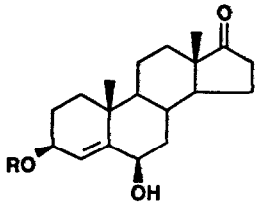
Chart 2



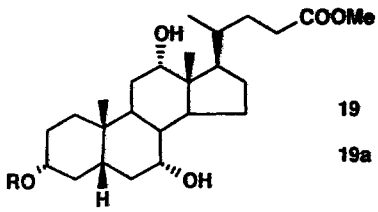
16 R = H
16a R = Ac
16b R = Lev



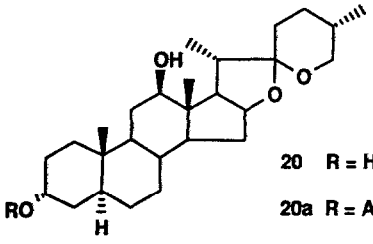
17 R = H
17a R = Ac
17b R = Lev
17c R = ClAc



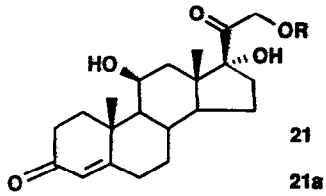
18 R = H
18a R = Ac



19 R = H
19a R = Ac



20 R = H
20a R = Ac



21 R = H
21a R = Ac

The analytical data were confirmed in the preparative-scale experiments on polyhydroxy steroids. The representative compounds **16-20** (**Chart 2**) were all quantitatively esterified, and the corresponding 3-O-acetyl derivatives **16a-20a** were isolated in pure form without chromatographic purifications (see Experimental for details). Moreover, as expected, hydrocortison **21** was quantitatively converted to the corresponding 21-O-acetate (**21a**), while its C-11β and C-17α were unaffected. As expected, reactions did not occur in the absence of the enzyme.

Subsequently, we studied the ability of *C.a.*B lipase to introduce different acyl moieties. It is well-known that some esters are either more resistant (i.e., benzoate and pivaloate) or more labile (i.e.,

chloroacetate) than acetate toward hydrolysis, while other esters, such as levulinate, can be removed under chemoselective conditions.[11] These properties can be particularly useful when esterification is used as a protective step in a synthetic sequence. We have already found that the lipase from *Pseudomonas cepacia* can introduce these acyl moieties in carbohydrate derivatives[12] and therefore we analyzed the behaviour of *C.a.*B lipase with different trifluoroethyl and vinyl esters. We chose compound **6** as a model, and the results are reported in **Table 2.**

Table 2.Initial Rates of Acylation of 6 with Different Trifluoroetyhyl (TFE) or Vinyl Esters Catalyzed by *Candida antarctica* Lipase[a)]

Ester	initial rate[b)] (μmol/h)	relative rate
Vinyl acetate	2.08	1
Vinyl chloroacetate	22.36	10.8
Vinyl benzoate	0	—
Vinyl pivaloate	0	—
TFE-chloroacetate	1.12	0.5
TFE-benzoate	0	—
TFE-pivaloate	0	—
TFE-levulinate	0.25	0.1

a) Conditions: 17 mM of 6; 0.8 ml of toluene; 0.2 ml activated ester; 20 mg *C.a.*B lipase. Suspension were shaken at 45°C. At regular time aliquots of solution were withdrawn and assayed for product formation.

b) Determined by capillary GC

Again, analysis of the initial rates of transesterification gave a clear indication of lipase's properties. The steroid was not acylated by the bulky benzoate and pivaloate, while the chloroacetyl and levulinyl moieties were accepted by the enzyme, even if with neat different efficiency. Reactions were scaled up on compound **16** and **17**, and the corresponding 3-O-chloroacetyl (**17c**) and 3-O-levuloinyl (**16b** and **17b**) derivatives were isolated in good yields.

In conclusion, we have shown that *C.a.*B lipase can be efficiently used for the regioselective esterification of polyhydroxy steroids. Compared to other lipases, which are often commercialized in very

crude form containing different proteins (i.e., porcine pancreatic lipase), this enzyme has the advantage of being available in a purified and definite form. As soon as crystallographic data of the *C.a.*B lipase's active site will be available, they might help in rationalizing the specificity displayed by the enzyme towards the various steroids investigated.

Experimental Part

Materials and methods.

Candida antarctica lipase B (Novozym 435) was a generous gift of Novo-Nordisk. Steroids were purchased from Steraloids. ^{1}H-NMR spectra were recorded on a Bruker AC-300. Melting points were measured on a Kofler melting point microscope. Trifluoroethyl and vinyl esters were either commercially available or prepared with conventional procedures.[12]

Enzymatic transesterifications of compounds **1-12** were followed by gas chromatography with a 5 m HP1 capillary column coated with methylsilicone gum (Hewlett-Packard) using H_2 as carrier gas. Enzymatic transesterifications of compounds **13-15** were followed by HPLC using a silica gel column (250 mm x 4.6 mm internal diameter, Whatman) eluted with the appropriate mixture of 2-propanol and hexane. The flow rate was 1 ml/min and readings were made at 220 nm.

The positions of acylation in all the enzymatically prepared steroid monoesters were established by ^{1}H-NMR. Acylation of a given OH group resulted in a downfield shift of the signal due the proton(s) linked to the same carbon (compared to the starting steroid).

5α-Androstane-3β,17β-diol- 3-acetate (**16a**).

5α-Androstane-3β,17β-diol (**16,** 100 mg) was dissolved in 3 ml of THF. Vinyl acetate (7 ml) and *C.a.*B lipase were added and the suspension shaken at 45°C for 40 hours. After this time, GC analysis showed a 97 % conversion to a single product. The enzyme was filtered off, the solvent evaporated and the residue crystallized from hexane (m.p. 123-4°C, lit. (Steraloids catalogue) 117-8°C). Its ^{1}H-NMR was identical to the one obtained from an authentic sample of **16a** purchased from Steraloids.

5α-Androstane-3β,17β-diol-3-levulinate (**16b**)

5α-Androstane-3β,17β-diol (**16,** 100 mg, 0.34 mmol) was dissolved in 10 ml of THF- trifluoroethyl levulinate 1:1. The enzyme was added (500 mg) and the suspension was shaken at 45°C for 60 hours. GC and TLC analysis showed a complete conversion of the starting **16**. Usual work-up followed by purification with flash-chromatography (eluent $CHCl_3$-MeOH 95:5) gave 95 mg (0.24 mmol, 71 %) of **16b**, which was crystallized from diethyl ether (m.p. 108-9°C).

^{1}H-NMR ($CDCl_3$, 300 MHz), δ = 4.70 (sept, J_1= 5.5 Hz, J_2=11 Hz, 1 H, H-3α); 3.63 (t, J=8 Hz, 1 H, H-17α)); 0.84 (s, 3H, 19-CH_3); 0.74 (s, 3H, 18-CH_3); levulinyl moiety : 2.70 (t, J = 7 Hz, 2H); 2.53 (t, J=7 Hz, 2 H); 2.17 (s, 3 H). Elemental analysis $C_{24}H_{38}O_4$ (390): calc. C 73.85, H 9.74; found C 73.79, H 9.61.

5-Androstene-3β,17β-diol-3-acetate (**17a**).

This compound was prepared similarly to **16a** and crystallized from hexane (m.p. 144-6°C. Lit. (Steraloids catalogue): 146-8°C). Its ^{1}H-NMR was identical to the one obtained from an authentic sample of **16a** purchased from Steraloids.

5-Androstene-3β,17β-diol-3-levulinate (**17b**).

50 mg of **17** in 5 ml of THF-trifluoroethyl levulinate (1:1) were shaken at 45°C for 4 days in the presence of 200 mg of *C.a.*B. lipase. Usual work-up and purification by flash chromatography (eluent $CHCl_3$-MeOH 95:5) gave 47 mg (0.127 mmol, 74 %) of pure **17b**, which was crystallized from diethyl ether (m.p. 114-5°C).
^{1}H-NMR ($CDCl_3$, 300 MHz), δ = 5.37 (d, J = 4.5 Hz, 1H, H-6); 4.59 (m, 1H, H-3α); 3.65 (t, J = 7.5 Hz, 1H, H-17α); 1.05 (s, 3H, 18-CH_3); 0.78 (s, 3H, 19-CH_3); levulinyl moiety : 2.72 (t, J = 7 Hz, 2H); 2.54 (t, J=7 Hz, 2 H); 2.18 (s, 3 H). Elemental analysis $C_{24}H_{36}O_4$ (388): calc. C 74.23, H 9.28; found C 74.35, H 9.34.

5-Androstene-3β,17β-diol-3-chloroacetate (**17c**).
100 mg of **17** were dissolved in 5 ml THF. Trifluoroethyl chloroacetate (5 ml) and *C.a.*B lipase (200 mg) were added and the suspension shaken at 45°C for 1 hour (100 % conversion by GC and TLC). Usual work-up and crystallization from AcOEt-diethyl ether gave pure **17c** (m.p. 212-3°C).
^{1}H-NMR ($CDCl_3$, 300 MHz), δ = 5.39 (d, J = 4.4 Hz, 1H, H-6); 4.70 (m, 1H, H-3α); 4.03 (s, 2H, CH_2Cl); 3.63 (t, J = 7.5 Hz, 1H, H-C17α); 1.02 (s, 3H, 18-CH_3); 0.75 (s, 3H, 19-CH_3). Elemental analysis: $C_{20}H_{31}O_3Cl$ (354.5): calc. C 67.70, H 8.98; found C 67.65, H 8.86.

4-Androstene-3β,6β-diol-17-one-3-acetate (**18a**).
Substrate **18** (40 mg, 0.13 mmol) was dissolved in 8 ml THF-vinyl acetate (1:1). *C.a.*B lipase (160 mg) was added and the suspension shaken for a week at 45°C. Usual work-up and purification by flash chromatography (eluent $CHCl_3$-MeOH 97:3) gave 37 mg (0.11 mmol, 82 %) of **18a**, which was crystallized from MeOH (m.p. 152-3°C; lit.[13] 148-150).
^{1}H-NMR ($CDCl_3$, 300 MHz), δ = 5.51 (br s, 1H, H-4); 5.23 (m, 1H, H-3α); 4.27 (t, J = 3 Hz, H-6α); 2.06 (s, 3H, CH_3CO); 1.29 (s, 3H, 19-CH_3); 0.91 (s, 3H, 18-CH_3).

3α,7α,12α-Trihydroxy- 5β-cholan-24-oic acid -3-acetate methyl ester (**19a**).
Cholic acid methyl ester (**19**, 100 mg) was dissolved in 10 ml of vinyl acetate. *C.a.* B lipase (200 mg) was added and the suspension shaken for 1 hour at 45°C. After this time, HPLC and TLC showed a complete conversion to a single product which, after usual work-up, was crystallized from hexane-diethyl ether (m.p. 153°C; lit.[14] 153-4°C).
^{1}H-NMR ($CDCl_3$, 300 MHz), δ = 4.57 (m, 1H, H-3α); 3.98 (t, J = 2.2 Hz, 1H, H-12α); 3.84 (q, J = 2 Hz, 1H, H-7α); 3.67 (s, 3H, $COOCH_3$); 2.00 (s, 3H, CH_3CO); 0.98 (d, J = 7 Hz, 3H, CH_3-21); 0.90 (s, 3H, CH_3-19); 0.70 (s, 3H, CH_3-18).

3β,12β-Dihydroxy-5α,20α,22α,25D-spirostane-3-acetate (**20a**).
Rockogenin (**20**, 100 mg) was dissolved in 10 ml of vinyl acetate and shaken at 45°C for 4 days in the presence of 200 mg of *C.a.* B lipase. After this time, a complete conversion to a single product was observed by TLC. Usual work-up and crystallization from methanol gave pure 20a (m.p. 226-7°C; lit.[15] 214-9°C from methanol).
^{1}H-NMR ($CDCl_3$, 300 MHz), δ = 4.67 (sept, J_1= 5.5 Hz, J_2=11 Hz, 1 H, H-3α); 2.03 (s, 3H, CH_3CO).

11β,17α,21-Trihydroxy-4-pregnene-3,20-dione-21-acetate (**21a**).
Hydrocortison (**21**, 100 mg) was dissolved in THF-vinyl acetate 1:1 and *C.a.*B lipase (200 mg) was added. The suspension was shaken at 45°C untill complete conversion (about 4 hours). Usual work-up and crystallization from acetone gave pure **21a** (m.p. 224-5°C. Lit. (Steraloids catalogue): 220-4°C), identical to an autenthic sample purchased from Steraloids.

Acknowledgments

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