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Lipase-catalysed deacetylation of androstane and pregnane derivatives: influence of ring D substitution

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Abstract

A series of acetoxy derivatives of androstane and pregnane was deacetylated in organic solvents by microbial lipases. The best results were obtained with lipase from *Candida antarctica* (CAL B), *Candida rugosa* (CRL) and *Pseudomonas* sp. (PSL). In some derivatives, CAL B and CRL showed a regioselective behaviour towards the removal of the 3β- or 16α/16β-acetyl group. The results of the enzymatic deacetylation of pregnanes and androstanes substituted by various groups containing an sp^2-hybridised C-atom in ring D could suggest that CAL B activity seems to be conditioned by the occurrence of a polar carbon double bond in this part of the steroid skeleton. Ten new steroid derivatives were obtained through this approach.
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Keywords: Androstane; Pregnane; Lipase-catalysed deacetylation

1. Introduction

The pharmaceutical properties and high cost of steroids made the research on these natural compounds even more important. In the last years, highly selective synthetic procedures for the transformation of complex polyfunctional steroids were developed. Among them, the enzyme-catalysed approach was applied successfully in selective reactions producing new compounds with added value to the physiological properties [1].

The lipase-catalysed transesterification in organic solvents is very useful for steroids that are highly insoluble in water. The regioselective acylation and deacylation of some steroid compounds has been already described by several authors [2–6] and recently reviewed [7,8].

In earlier works we have reported the enzyme-catalysed alcoholysis of steroids 3β-acetates containing labile functional groups in the molecule [9] and the regioselective deacetylation of some androstane derivatives [10]. We have observed that lipases from two yeasts catalysed the alcoholysis of acetyl groups located at different positions of the steroid skeleton. *Candida rugosa* lipase (CRL) and *Candida antarctica* lipase (CAL B) have affinity for different regions of the rigid steroid molecule. While CRL removed acetyl groups situated in ring A, CAL B was preferentially active on substituents located in ring D. Considering the good performance of CAL B in alcoholysis of 16β-O-Ac with a carbonyl group at C(17) **1a** (Fig. 1) and the poor activity when the keto at C(17) is replaced by an acetoxy group [10], CAL B activity seems to be conditioned by the occurrence of an sp^2-hybridised C-atom in ring D.

To learn more about the regio- and stereoleselective behaviour of lipases in deacetylation of steroids we performed this reaction using several androstanes and pregnanes **2a**–**10a** as substrates (Figs. 1 and 2). We wish to report our results in this paper.

2. Experimental

2.1. Analysis and materials

The substrates were prepared in our laboratory. They have been purified by flash chromatography on silica

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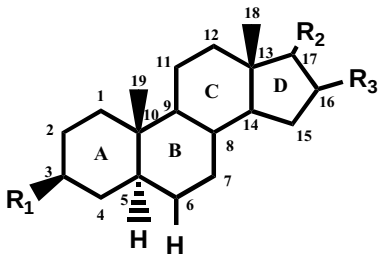


1a R_1 = AcO; R_2 = =O; R_3 = β-AcO
b R_1 = AcO; R_2 = =O; R_3 = β-OH
c R_1= OH; R_2 = =O; R_3 = β-AcO

2a R_1 = AcO; R_2 = =O; R_3 = α-AcO
b R_1 = AcO; R_2 = =O; R_3 = α-OH
c R_1= OH; R_2 = =O; R_3 = α-AcO
d R_1= OH; R_2 = =O; R_3 = α-OH

Fig. 1.

gel and identified by GC–MS and by ^{1}H and ^{13}C NMR spectroscopy.

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and $CDCl_3/D_3COD$ with TMS as internal standard using a Bruker-AC-200 spectrometer. Analytical GC was performed using a Hewlett-Packard-5890 gas chromatograph with an HP-5 capillary column (50 m × 0.32 mm) and HP-17 (10 m × 0.53 mm). For TLC, Merck silica gel 60F-254 aluminum sheets (0.2 mm thickness) was used. Gas chromatography–mass spectrometry (GC–MS) spectra were performed using a gas chromatograph coupled to a Varian Mat CH7-A spectrometer interfaced to a Varian-Mat Data System 166 and on a VG-TRIO-2-GC-MS instrument.

All solvents and reagents were of analytical grade. Lipase from *C. rugosa* (905 units/mg solid), was purchased from Sigma Chemical Co.; *C. antarctica* lipase B: Chirazyme L-2, c.-f.,C3, lyo (400 U/g) and *C. antarctica* lipase A: Chirazyme L-5, c.-f. lyo (400 U/g) were purchased to Roche Diagnostics GmbH; *Pseudomonas* lipase: Lipase PS-C Amano II (804 U/g) and Lipase PS Amano (33,200 U/g) were purchased to Amano Pharmaceutical Co. All enzymes were used "straight from the bottle".

2.2. Standard deacetylation procedure

To a solution of the acetoxy steroid (0.75 mmol) in 15 ml of the indicated solvent containing 5 mol equiv of 1-octanol, the corresponding amount of lipase was added (1.5 g of CAL A, CAL B or PSL; 0.8 g of CRL or PSL-C). The suspension was shaken (200 rpm) at 30 °C and the progress of the reaction was monitored by GC. After indicated time, the enzyme was filtered off, the solvent was evaporated, and the crude residue purified by flash chromatography on silica gel and hexane: ethyl acetate 95:5, and identified by GC–MS and by ^{1}H and ^{13}C NMR spectroscopy.

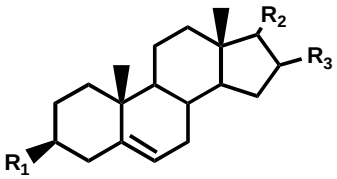
3. Results and discussion

3.1. Optimisation of the reaction conditions

Four commercial lipases were tested in the deacetylation of diacetate **2a**: *C. rugosa* lipase, *C. antarctica* lipase B, lipase PS and PS-C from *Pseudomonas* sp.

As it can be seen from Table 1, the lipases from *C. rugosa* and *C. antarctica* gave the most satisfactory results in terms of conversion and selectivity. PSL-C was also selective and active but showing a lower performance.

As in a previous work [10], both enzymes acted in a regioselective and complementary manner, catalysing the alcoholysis of acetyl groups located at different rings of the steroid skeleton. CRL only produced deacetylation



3a R_1 = AcO; R_2 = =CH_2; R_3 = β-AcO
b R_1 = AcO; R_2 = =CH_2; R_3 = β-OH
c R_1= OH; R_2 = =CH_2; R_3 = β-AcO

4a R_1 = AcO; R_2 = =CH_2; R_3 = α-AcO
b R_1 = AcO; R_2 = =CH_2; R_3 = α-OH
c R_1 = OH; R_2 = =CH_2; R_3 = α-AcO

5a R_1 = TBDMSO; R_2 = =CH_2; R_3 = α-AcO
b R_1 = TBDMSO; R_2 = =CH_2; R_3 = α-OH
c R_1 = TBDMSO; R_2 = =CH_2; R_3 = α-OH

6a R_1 = AcO; R_2 = (E) =$CHCH_3$; R_3 = β-AcO
b R_1 = AcO, R_2 = (E) =$CHCH_3$; R_3 = β-OH
c R_1 = OH; R_2 = (E) =$CHCH_3$; R_3 = β-AcO

7a R_1 = AcO, R_2 = (E) =$CHCH_3$; R_3 = α-AcO
b R_1 = AcO; R_2 = (E) =$CHCH_3$; R_3 = α-OH
c R_1 = AcO, R_2 = (E) =$CHCH_3$; R_3 = α-OH

8a R_1 = AcO; R_2 = (E) =$CHCH_2OAc$; R_3 = H
b R_1 = AcO; R_2 = (E) =$CHCH_2OH$; R_3 = H
d R_1 = OH; R_2 = (E) =$CHCH_2OH$; R_3 = H

9a R_1 = TBDMSO; R_2 = (Z) =$CHCH_2OAc$, R_3 = α-AcO
b R_1 = TBDMSO; R_2 = (Z) =$CHCH_2OH$; R_3 = α-AcO

10a R_1 = AcO; R_2 = (Z) =$CHCH_2OAc$; R_3 = α-AcO
b R_1 = AcO; R_2 = (Z) =$CHCH_2OH$; R_3 = α-AcO
c R_1 = OH, R_2 = (Z) =$CHCH_2OH$, R_3 = α-AcO

Fig. 2.

Table 1
Enzyme catalysed alcoholysis of 17-oxo-5α-androstan-3β,16α-diyl diacetate (**2a**)[a]

Lipase	Conversion[b] (%)		
	2b	**2c**	**2d**
None	n.d	n.d.	n.d.
C. rugosa lipase (CRL)	n.d.	66	32
C. antarctica lipase (CAL B)	76	n.d.	24
Pseudomonas lipase PS (PSL)	n.d	n.d.	n.d.
Pseudomonas lipase PS-C (PSL-C)	n.d.	50	n.d.

[a] Reactions were performed under standard conditions. Time: 144 h, solvent: CRL: toluene, CAL B: acetonitrile, PSL and PSL-C: dioxane.
[b] Determined by GC and GC/MS analysis; n.d.: not detected.

at O–C(3) while CAL B only reacted with the acetyl at O–C(16). All products were determined by GC–MS analysis. The reaction was monitored at different periods of time; **2b** and **2c** were obtained at its maximum concentration at 48 h. At longer times the diol **2d** appeared.

The enzymatic deacetylation of **2a** was tested with absolute ethanol, 1-butanol and 1-octanol, using CAL B and CRL as biocatalysts. Results are shown in Table 2.

Ethanol acted both as solvent and nucleophilic agent. All three alcohols afforded the corresponding hydroxy steroid according to the enzyme but in different yield, 1-octanol being the best nucleophile with both lipases. CAL B was efficient in all three alcohols and yield in **2b** increased slightly from ethanol to octanol. Only in octanol it showed an excellent regioselectivity. On the other hand, CRL was moderately active only in octanol (66%) and less regioselective than in the previously studied diacetate **1a** (70%) [10]; 18% of **2b** was observed by GC analysis.

The lipase-catalysed deacetylation of **2a** with 1-octanol and CAL B was carried out at different alcohol concentrations. The best yield was obtained at an alcohol/steroid molar ratio equal to 5 and at 50 mM steroid concentration. The amount of CAL B in the reaction system was varied from 100 to 5000 mg at 50 mM steroid concentration. Yields of 20% of **2b** were observed just using 300 mg of lipase, affording the best results (76%) with 1500 mg.

We tested the activity of each lipase in different solvents. In previous works [9,10] we have found that activity is related to the nature of the solvent. So CAL B showed the best performance in acetonitrile, CRL in toluene and PSL and PSL-C in dioxane.

Concerning to the enzyme re-use possibilities, we observed a loss of only 13% in yield when recovered enzyme was used in six consecutive reactions with fresh substrate and octanol.

Table 2
Enzyme catalysed alcoholysis of 17-oxo-5α-androstan-3β-16α-diyl diacetate (**2a**)[a]

Alcohol	Conversion with CAL B[b] (%)		Conversion with CRL[b] (%)	
	2b	**2c**	**2b**	**2c**
None	n.d.	n.d.	n.d.	n.d.
Ethanol	70	11	11	10
1-Butanol	74	9	12	12
1-Octanol	76	n.d.	18	66

[a] Reactions were performed under standard conditions. Time: 144 h, solvent: CAL B: acetonitrile, CRL: toluene.
[b] Determined by GC analysis; n.d.: not detected.

Table 3
Lipase-catalysed alcoholysis of androstane and pregnanes derivatives (**2a**–**10a**)[a]

	CAL B			CRL		
	Product	Yield (%)	*t* (h)	Product	Yield (%)	*t* (h)
2a	**2b**	72	48	**2c**	61	48
				2b	12	
3a	**3b**	n.d.[b]	168	**3c**	65	48
4a	**4b**	n.d.	168	**4c**	68	48
5a	**5b**	n.d.	168	**5b**	8[c]	168
6a	**6b**	9	168	**6c**	70	48
7a	**7b**	n.d.	168	**7c**	69	48
8a	**8b**	82	48	**8b**	32	48
				8d	36	
9a	**9b**	75[d]	48	**9b**	30	168
10a	**10b**	81	48	**10c**	79	48

[a] Reactions were performed under standard conditions. Solvent: CAL B: acetonitrile, CRL: toluene.
[b] Not detected by GC.
[c] Temperature: 50 °C.
[d] The 68% with CAL A and 56% with PSL-C.

3.2. Lipase-catalysed deacetylation reaction on different D-ring substituted steroids

The deacetylation reaction was scaled up to 300 mg to isolate and characterise the products. Table 3 shows the results of the preparative transesterification reactions using the steroids **2a**–**10a** as substrates. The site of alcoholysis was unambiguously established by ^{1}H NMR spectroscopic analysis and by comparison with data for the parent **2a**.

Then, on treatment of **2a** with CAL B and 1-octanol, the until now unknown 16α-hydroxy-17-oxo-5α-androstan-3β-yl acetate **2b** was obtained in 72% yield, and using CRL, the also novel 3β-hydroxy-16α-acetate **2c**, was produced in 61%.

CAL B proved to be more regioselective than CRL, because a little amount of **2b** (12%) was obtained together with **2c** in enzymatic deacetylation with CRL. On the other hand, the α configuration of the 16 acetate in **2a** did not inhibit CAL B activity and 3β-acetate-16α-hydroxy derivative **2b** was obtained, though in lower yield than the previously reported 3β-acetate-16β-hydroxy **1b** (78%) [10]. This result is showing a poor stereoselectivity in CAL B, being active with both stereoisomers 16-α and 16-β acetates. As CRL displays its activity in ring A, it seems that its performance is not influenced by the stereochemistry of the acetate in ring D.

To know more about the influence of different functional groups in ring D of steroid substrates on the

enzymatic activity, we performed the enzymatic deacetylation with various androstanes and pregnanes (see Figs. 1 and 2).

When carbonyl group in 17 position of ring D was replaced by a carbon–carbon double bond C(17) = C(20) as in substrates **3a**, **4a** and **5a**, no deacetylation product was obtained with CAL B as biocatalyst. Moreover, when the susbstituent in 17 position of ring D was a $=CH–CH_3$ of (*E*) configuration in pregnanes **6a** and **7a**, only conversion of **6a** was observed and the 16-hydroxy compound was obtained in very low yield (about 6% of **6b** after 7 days of reaction). This poor reactivity was observed in both pairs of stereoisomers **3a**–**4a** and **6a**–**7a** containing the carbon 16 acetate group in configuration α (**4a** and **7a**) or β (**3a** and **6a**). These results would indicate that the best substrate for CAL B activity in the lipase-catalysed deacetylation of carbon 16 could involve the presence of a polar double bond such a carbonyl group. A carbon–carbon double bond in position 17, substituted (**6a** and **7a**) or not (**3a** and **4a**), does not seem a good environment for the enzyme active moiety.

Enzymatic alcoholysis of **3a**, **4a**, **6a** and **7a** with CRL as biocatalyst afforded the corresponding 3β-hydroxy derivatives in good yield (65–70%) and remarkable regioselectivity. As we have observed in a previous work [10] the presence of the Δ5 double bond does not influence CRL activity and the yields in **3c**, **4c**, **6c** and **7c** showed that the enzyme performance was comparable to the saturated substrates. These last four compounds had not been previously reported in literature.

As it can be expected the 3β-*t*-butyldimethylsilyloxy group in **5a** kept unaltered in presence of both CAL B and CRL and only 8% of the 16α-hydroxy compound **5b** was obtained by performing the alcoholysis reaction at 50 °C during 7 days using CRL as biocatalyst.

The primary acetyl group in **8a** showed to be very reactive and the alcoholysis product with hydroxyl group in carbon 21 **8b** was obtained in high yield with CAL B. A mixture of the same product (**8b**) and the diol **8d** was obtained when CRL was used as biocatalyst suggesting that the higher reactivity of the primary group prevented the regioselective behaviour of the enzyme. Both **8b** and **8d** had not been prepared previously.

With the aim to compare the enzymatic behaviour of both primary and secondary acetyl groups in ring D we performed the enzymatic alcoholysis of **9a** with CAL A, PSL-C and CAL B. CAL A and PSL-C have been used as biocatalysts in acetylation of primary alcohols, and it is known that CAL B is reactive on secondary ones [8,9,11]. In this case all three enzymes showed comparable results in the alcoholysis reaction, affording only the 21 hydroxy derivative **9b**. A small amount of **9b** was also obtained by using CRL, suggesting the high reactivity of the primary group. We tested that no alcoholysis reaction was produced without enzymes, so the spontaneous deacetylation did not occur.

By enzymatic alcoholysis of the triacetylated derivative **10a** with CAL B the 21-AcO group was removed while 3β-AcO and 16α-AcO remained unaltered. The reaction afforded compound **10b** in very good yield. Performing the alcoholysis through CRL catalysis, only the 3β-16α-dihydroxy compound **10d** could be obtained. It was not possible to obtain the 3β-monohydroxy derivative despite that several reaction conditions were tested. Both **10b** and **10d** were not previously reported in literature.

4. Conclusion

An efficient procedure for the enzymatic selective deacetylation of pregnanes and androstanes by using microbial lipases in organic medium has been described. Both *C. rugosa* and *C. antarctica* B lipases showed a remarkable regioselectivity in the reaction with several substrates. Acetate groups in carbon 16 could not be removed to give the corresponding alcohols by action of CAL B when there were no polar substituents in carbon 14. These results confirm that the presence of a polar group in carbon 14 is necessary for the enzyme to develop its catalytic activity.

On the other hand CRL activity was almost exclusively developed in acetates located in carbon 3 of the steroid structure. The presence of a double bond Δ5 did not affect lipases activity and selectivity.

Ten new steroid compounds were obtained by using the regioselective enzymatic deacetylation reaction, which are useful synthetic intermediates in the synthesis of biological active molecules.

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