86. Lipase-Catalysed Regioselective Deacetylation of Androstane Derivatives

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A series of acetoxy derivatives of androstane was deacetylated in organic solvents by several lipases. The most satisfactory results were obtained with lipase from *Candida cylindracea* (CCL) and *Candida antarctica* (CAL). In some derivatives, CCL and CAL showed an overwhelming regioselectivity towards the removal of the 3β- or the 17β-acetyl group (see *Table 2*). Three new steroid derivatives were obtained through this approach. A hypothetical rationale for the behaviour of these enzymes is given.

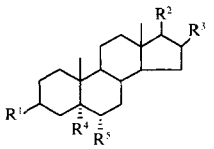
Introduction. – The application of steroids as hormonal and pharmacological agents, recognized and implemented in clinical practice, stimulated the research on these compounds. As a result of this effort, highly selective synthetic procedures for the transformation of complex polyfunctional steroid compounds were developed in the last years, and almost every new synthetic method was tested in this area.

In the course of the synthesis of steroid compounds performed in our laboratory [1], we required a mild regioselective procedure for the deprotection of specific ester moieties present in labile molecules such as androstane derivatives. This objective was almost impossible to achieve following classical chemical procedures. *E.g.*, it has been reported [2] that the chemical deacetylation of 17-oxoandrost-5-ene-3β,16β-diyl diacetate afforded a complex mixture of the two isomeric monoacetates, the diol, and rearranged products.

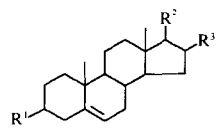
The enzyme-catalysed approach offers a viable alternative to these methods [3], and it is well known that the regio- and chemoselective mode of action of enzymes may be applied for the manipulation of protecting groups [4]. A previous work has reported that the enzymatic deacetylation of the above mentioned substrate in aqueous medium using hog pancreatic α-amylase produced the diol, but it was not possible to achieve a regioselective deprotection to obtain some of the monoacetates [5].

Our interest in the chemoselective preparation of esters by lipase-catalysed transesterification reactions [6–8] prompted us to apply the enzymatic methodology on steroid derivatives. We have recently studied [9] the enzyme-catalysed alcoholysis of steroid 3β-acetates containing labile functional groups in the molecule. The successful results persuaded us to implement this methodology to the more challenging alcoholysis of different acetoxy groups in some androstane derivatives. We report in this paper the regioselective removal of the esters groups from several diacetylated steroid derivatives.

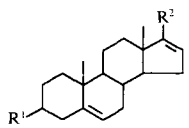
Results and Discussion. – The general methodology developed by *Klibanov* [10] for enzymatic transesterification in organic solvents was applied to compounds **1a** to **6a**.



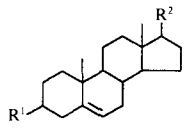
1a $R^1 = R^3 = AcO$, $R^2 = =O$, $R^4 = R^5 = H$
b $R^1 = AcO$, $R^2 = =O$, $R^3 = OH$, $R^4 = R^5 = H$
c $R^1 = OH$, $R^2 = =O$, $R^3 = AcO$, $R^4 = R^5 = H$
3a $R^1 = R^5 = AcO$, $R^2 = =O$, $R^3 = R^4 = H$
b $R^1 = OH$, $R^2 = =O$, $R^3 = R^4 = H$, $R^5 = AcO$
5a $R^1 = R^2 = AcO$, $R^3 = R^4 = R^5 = H$
b $R^1 = OH$, $R^2 = AcO$, $R^3 = R^4 = R^5 = H$
6a $R^1 = R^2 = R^3 = AcO$, $R^4 = R^5 = H$
b $R^1 = OH$, $R^2 = R^3 = AcO$, $R^4 = R^5 = H$



2a' $R^1 = R^3 = AcO$, $R^2 = =O$
b $R^1 = AcO$, $R^2 = =O$, $R^3 = OH$
c $R^1 = OH$, $R^2 = =O$, $R^3 = AcO$



4a $R^1 = R^2 = AcO$



4b $R^1 = AcO$, $R^2 = =O$

Table 1. *Enzyme-Catalysed Alcoholysis of 17-Oxo-5α-androstane-3β,16β-diyl Diacetate* (**1a**)[a])

Enzyme	% Conversion to[b])	
	1b	**1c**
None	n.d.	n.d.
Pancreatic porcine lipase	n.d.	18.9
Lipozyme	n.d.	12.3
Candida cylindracea lipase (CCL)	n.d.	69.8
Candida antarctica lipase (CAL)	78.3	n.d.
Bacterial protease	1.3	2.5
Fungal protease	n.d.	1.8
Chymotrypsin	1.2	1.3
Papain	n.d.	n.d.
Trypsin	2.8	n.d.

[a]) Reactions were performed under standard conditions.
[b]) Determined by GC and GC/MS analysis; n.d. = not detected.

Diacetate **1a** was subjected to the action of several lipases and proteases under different conditions. As it can be seen from *Table 1*, none of the proteases removed acetyl groups, and among lipases, those from *Candida cylindracea* (CCL) and *Candida antarctica* (CAL) gave the most satisfactory results in terms of conversion and selectivity. It is remarkable that both enzymes acted in a regioselective and complementary manner. They catalysed the alcoholysis of acetyl groups located at different positions of the steroid skeleton, *i.e.*, CCL only produced deacetylation at O–C(3) whilst CAL only reacted with the acetyl at O–C(16) as determined by GC/MS analysis. Considering these results, the reaction was scaled up to isolate and characterize the products. Thus, on treatment of **1a** with CAL, the hitherto unknown 3β-acetate **1b** was obtained in 75% yield, and using CCL, 16β-acetate **1c** was produced in 66% yield. Compound **1c** had been previously prepared in five steps from 3β-hydroxy-5α-androstan-17-one *via* 16-diazo-3β-hydroxy-5α-androstan-17-one with an overall yield of *ca.* 20% [11].

Table 2 shows the results of the preparative transesterification reactions using androstanes **1a**–**6a** as substrates. The site of alcoholysis was unambiguously established by ^{1}H-NMR spectroscopic analysis and by comparison with data for the parent **1a** (see *Table 3*). In case of **2a**, CCL and CAL showed the same pattern of results as with **1a**,

Table 2. *Lipase-Catalysed Alcoholysis of Androstane Derivatives* **1a**–**6a**[a])

	CCL			CAL		
	Product	Yield [%]	*t* [h]	Product	Yield [%]	*t* [h]
1a	**1c**	66	96	**1b**	75	120
2a	**2c**	86	120	**2b**	76	120
3a	**3b**	53	240	**3b**	9	240
4a	**4b**	54	3	**4b**	98	3
5a	**5b**	79	216	**5b**	18	216
6a	**6b**	87	120	**6b**	19	168

[a]) Reactions were performed under standard conditions.

Table 3. *Selected ^{1}H-NMR Data for Compounds* **1**–**6**[a])

	H–C(3)	H–C(6)	H–C(16)	H–C(17)	AcO_β–C(3)	AcO_α–C(6)	AcO_β–C(16)	AcO_β–C(17)
1a	4.68 (*m*)	–	4.98 (*t*, *J* = 8)	–	2.02 (*s*)	–	2.11 (*s*)	–
b	4.65 (*m*)	–	3.92 (*t*, *J* = 8)	–	2.00 (*s*)	–	–	–
c	3.47 (*m*, 1H)	–	4.95 (*t*, *J* = 8)	–	–	–	2.10 (*s*)	–
2a	4.65 (br.)	5.42 (*m*)	5.01 (*t*, *J* = 8)	–	2.04 (*s*)	–	2.12 (*s*)	–
b	4.60 (br.)	5.40 (*m*)	3.95 (*t*, *J* = 8)	–	2.02 (*s*)	–	–	–
c	3.50 (*m*)	5.40 (*m*)	5.00 (*t*, *J* = 8)	–	–	–	2.10 (*s*)	–
3a	4.69 (br., 2 H)		–	–	2.03 (*s*)	2.04 (*s*)	–	–
b	3.55 (*m*)	4.70 (*dt*, *J* = 5, 11)	–	–	–	2.04 (*s*)	–	–
4a	4.55 (*m*)	5.40 (*m*)	5.50 (*m*)	–	2.04 (*s*)	–	–	2.16 (*s*)
b	4.55 (*m*)	5.40 (*m*)	–	–	2.04 (*s*)	–	–	–
5a	4.68 (*m*)	–	–	4.58 (*dd*, *J* = 9, 7)	2.02 (*s*)	–	–	2.03 (*s*)
b	3.50 (*m*)	–	–	4.54 (*dd*, *J* = 9, 7)	–	–	–	2.03 (*s*)
6a	4.68 (*m*)	–	5.24 (*m*)	4.52 (*d*, *J* = 7)	2.02 (*s*)	–	2.04 (*s*)	2.03 (*s*)
b	3.59 (*m*)	–	5.23 (*m*)	4.52 (*d*, *J* = 7)	–	–	2.02 (*s*)	2.01 (*s*)

[a]) Chemical shifts δ in ppm, coupling constants *J* in Hz. All assignments agree with literature data or expected values.

affording **2b** with CAL and **2c** with CCL. These two isomeric monoacetates **2b** and **2c** had been obtained before through hydrolysis of the diacetate **2a** with H_2SO_4 in dioxane/H_2O in *ca.* 10% yield each, besides starting material and diol [2]. The similar results obtained with substrates **1a** and **2a** indicate that the C(5)=C(6) bond does not affect the enzymatic behaviour either in activity or selectivity.

To know more about the influence on the enzymatic activity of substrates having different functions at rings A and B, we studied the alcoholysis of diacetate **3a**, which contains an AcO group at C(6). By enzymatic catalysis with CCL, the 3β-*O*-Ac group was removed while the 6α-AcO group remained unaltered. The reaction was rather slow giving **3b** in moderate yield. Performing the alcoholysis through CAL catalysis, only 9% of **3b** could be obtained. However, this enzymatic approach allowed the synthesis and characterization of **3b** for the first time. The site of alcoholysis was established by direct comparison of the ^{1}H-NMR data of **3b** and **3a**.

The ^{1}H-NMR signals of H–C(3) and H–C(6) of **3a** could not be well resolved. In **3b**, the removal of 3β-*O*-Ac shifted H–C(3) to δ 3.55. This is in accordance with the values observed for H–C(3) in the ^{1}H-NMR spectra of the 3β-hydroxy derivatives **1c** and **2c** (δ 3.47 and 3.50, resp.). Moreover, the H–C(6) signal of **3b**, a *dt*, remains at δ 4.70. This pattern is consistent with the coupling of H_β–C(6) to H_α–C(5) and H_α–C(7) with each J = 11 Hz and to H_β–C(7) with J = 5 Hz. Application of *Altona*'s equation on molecular-mechanics geometry to structure **3b** afforded the calculated values $J(5\alpha,6\beta) = 10.58$, $J(7\alpha,6\beta) = 11.08$ and $J(7\beta,6\beta) = 4.78$ Hz, in accordance with the experimental ones.

The fast and very effective removal of the 17-*O*-Ac group in **4a** by CAL catalysis producing the keto group at C(17) *via* the corresponding enol was the only example in which the reaction with CAL was faster than with CCL. This result is in agreement with the behaviour of CAL in the catalysis of the preceding substrates (**1a** and **2a**) in which the Ac groups situated on the cyclopentane ring were selectively removed without producing changes in the AcO group at ring A of the steroid. Though **4b** can be chemically obtained with the same degree of selectivity, the enzymatic method may be considered more satisfactory because it is cleaner, and better yields are produced [12].

The enzymatic alcoholysis of the diacetylated androstane **5a** with both enzymes only afforded the 3β-alcohol **5b**. After nine days of reaction, no 3β-acetoxy-17β-hydroxy derivative could be detected, and much longer reaction times produced the diol. Similar results were obtained with substrate **6a** containing three β-oriented AcO groups at C(3), C(16) and C(17). Only the 3β-*O*-Ac group was removed, faster and in better yield with CCL, leading to compound **6b** not previously described in the literature.

Considering the results of the alcoholysis reactions, it could be postulated that each enzyme has affinity for different regions of the rigid steroid molecule. While CCL is preferentially active in catalysing the removal of the Ac groups situated in ring A of the steroid skeleton, CAL is preferentially active on substituents located in ring D. CAL Activity seems to be conditioned by the occurrence of an sp^2-hybridized C-atom in ring D (C=O in **1a** and **2a** and C=C in **4a**).

A hypothetical explanation for the somewhat different behaviour of the two enzymes can be given when considering their three dimensional structures. Both are yeast lipases and belong to the α/β hydrolase family, sharing a catalytic triad with the same sequential order of the catalytic residues: Ser–His–Asp/Glu (aspartic acid in CAL and glutamic acid in CCL, serine being the nucleophile in both). Two fractions A and B have been isolated from CAL [13]. The present work was done with form B (CALB), which contains 317 amino acid residues. Though the sequence around serine is usually a conserved region found in lipases, this consensus is broken in CALB [14]. Instead of the sequence Gly-*x*-Ser-*x*-Gly, there is a threonine substituting one glycine residue which modifies the relative orientation of the helix and the strand of this region compared with other lipases. Moreover, the region around Ser^{105} is remarkably polar in CAL with amino acids that have a polar side chain, such as threonine, glutamine and aspartic acid. These form a H-bond network that is fully accessible to the solvent. In a previous work [9], we have found that MeCN is the best solvent in CAL-catalysed alcoholysis of steroid acetates. The polar character of this solvent would be in coincidence with the polar environment described around the active site. In contrast, the best solvent for CCL is toluene coinciding with the fact that, in this enzyme, the hydrophilic areas surrounding the active site are almost entirely uncharged polar residues [15].

In conclusion, we have shown that lipases from *Candida cylindracea* and *Candida antarctica* easily remove acetyl groups in a regioselective fashion from di- and triacety-

lated androstane derivatives. Some of these compounds were proved to be useful intermediates in the synthesis of biologically active steroids.

Experimental Part

General. Steroid substrates were obtained following literature procedures (^{1}H- and ^{13}C-NMR and MS in accordance with structures): 17-oxo-5α-androstan-3β,16β-diyl diacetate (**1a**) [16]; 17-oxoandrost-5-en-3β,16β-diyl diacetate (**2a**) [16]; 17-oxo-5α-androstan-3β,6α-diyl diacetate (**3a**) by acetylation with Ac_2O/pyridine of the commercial 3β,6α-dihydroxy-5α-androstan-17-one; androsta-5,16-diene-3β,17-diyl diacetate (**4a**) [17]; 5α-androstane-3β,17β-diyl diacetate (**5a**) [18]; 5α-androstane-3β,16β,17β-triyl triacetate (**6a**) [17]. Lipase from *Candida cylindracea* (905 units/mg solid) and lipase (type II, crude) from porcine pancreas (190 units/mg protein) were purchased from *Sigma Chemical Co.* Lipozyme (lipase IM-60 from *Mucor miehei* in the immobilized form on a microporous anion exchange resin) and *Candida antarctica* lipase (novozym 435 (7400 PLU/g) acrylic resin supported lipase produced by a host organism *Aspergillus oryzae*, after transfer of the genetic coding for lipase B from *Candida antarctica*) were generous gifts of *Novo Nordisk Bioindustrial Group.* Papain (2468 units/mg solid), trypsin (3495 units/mg solid), and chymotrypsin (1311 units/mg solid) were a gift of *Biobras S.A.* Fungal protease (31 000 HU/g) and bacterial protease (200 NU/g) were provided by *Solvay Enzimas S.A.* All enzymes were used 'straight from the bottle'. All solvents were of anal. grade. Octanol was purchased from *Riedel-de Haën.* Gas-liquid chromatography (GC): *Hewlett-Packard-5890* gas chromatograph; *HP-5* capillary column (50 m × 0.32 mm) and *HP-17* (10 m × 0.53 mm). IR Spectra: *Nicolet-Magna-550-FT/IR* spectrophotometer; in cm^{-1}. ^{1}H- and ^{13}C-NMR Spectra: *Bruker-AC-200* spectrometer (200 and 50.2 MHz, resp.); $CDCl_3$ solns. with $SiMe_4$ as internal standard; δ in ppm, *J* in Hz. GC/MS: 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; in *m/z* (%). HR-MS: *VG-ZAB-BEQ* instrument. Molecular-mechanics calculations were performed with PCMODEL 4.0 from *Serena Software.*

16β-Hydroxy-17-oxo-5α-androstan-3β-yl Acetate (**1b**). To a soln. of **1a** (292 mg, 0.75 mmol) in MeCN (15 ml) containing octan-1-ol (0.8 ml, 5 mol-equiv.), 1.5 g of CAL were added. The suspension was shaken (200 rpm) at 30°, and the progress of the reaction was monitored by GC. After 5 days, the enzyme was filtered off, the solvent evaporated, and the crude residue purified by flash chromatography (CH_2Cl_2/MeOH 95:5): 196 mg (75%) of **1b**. M.p. 141–142°. IR (KBr): 3422 (OH), 1747 (C=O), 1723 (O–C=O). ^{1}H-NMR: *Table 3.* ^{13}C-NMR: 220.5 (C(17)); 170.7 (Me*C*OO); 75.4 (C(16)); 73.4 (C(3)); 54.3 (C(9)); 46.9 (C(13)); 45.6 (C(14)); 44.7 (C(5)); 36.5 (C(1)); 35.8 (C(10)); 34.4 (C(8)); 33.9 (C(4)); 31.9 (C(15)); 31.8 (C(12)); 30.5 (C(7)); 28.2 (C(6)); 27.4 (C(2)); 20.5 (*Me*COO); 20.3 (C(11)); 14.9 (C(18)); 12.2 (C(19)). EI-MS: 348 (18, M^+), 274 (10), 215 (100), 107 (32), 43 (38). HR-MS: 348.230331 ($C_{21}H_{32}O_4^+$; calc. 348.230060).

3β-Hydroxy-17-oxo-5α-androstan-16β-yl Acetate (**1c**). As described for **1b**, but using 3 g of CCL and toluene as solvent: 172 mg (66%). M.p. 219–220° ([11]: 219–220°). ^{1}H-NMR: *Table 3.* EI-MS: 348 (8, M^+), 288 (18), 216 (30), 201 (19), 108 (100), 43 (57).

16β-Hydroxy-17-oxoandrost-5-en-3β-yl Acetate (**2b**). As described for **1b**, with **2a**: 197 mg (76%). M.p. 163–164°. ^{1}H-NMR: *Table 3.* EI-MS: 286 (90, $[M - 60]^+$), 214 (48), 43 (100). ^{13}C-NMR: data in accordance with [1].

3β-Hydroxy-17-oxoandrost-5-en-16β-yl Acetate (**2c**). As described for **2b**, but using CCL and toluene as solvent: 223 mg (86%). M.p. 160–162°. ^{1}H-NMR: *Table 3.* EI-MS: 346 (19, M^+), 328 (10), 286 (9), 199 (38), 43 (100).

3β-Hydroxy-17-oxo-5α-androstan-6α-yl Acetate (**3b**). As described for **1b**, with **3a**: 138 mg (53%). M.p. 197–198°. IR (KBr): 3498 (OH), 1739 (C=O). ^{1}H-NMR: *Table 3.* ^{13}C-NMR: 220.2 (C(17)); 170.7 (Me*C*OO); 72.1 (C(6)); 70.8 (C(3)); 53.8 (C(9)); 51.2 (C(14)); 48.7 (C(5)); 47.8 (C(13)); 37.1 (C(1)); 36.7 (C(10)); 36.5 (C(7)); 35.7 (C(16)); 33.8 (C(8)); 32.2 (C(2)); 31.4 (C(4)); 31.1 (C(12)); 21.7 (C(15)); 21.2 (*Me*COO); 20.3 (C(11)); 13.7 (C(18)); 13.3 (C(19)). EI-MS: 348 (12, M^+), 288 (44), 270 (87), 43 (100). HR-MS: 348.230205 ($C_{21}H_{32}O_4^+$; calc. 348.230060).

17-Oxoandrost-5-en-3β-yl Acetate (**4b**). As described for **1b**, with **4a**: 242 mg (98%). M.p. 169–171° ([19]: 168–170°). ^{1}H-NMR: *Table 3.* EI-MS: 270 (100, $[M - 60]^+$), 255 (27), 121 (78), 43 (94).

3β-Hydroxyandrostane-17β-yl Acetate (**5b**). As described for **1c**, with **5a**: 198 mg (79%). M.p. 148–149°. ^{1}H-NMR: *Table 3.* EI-MS: 334 (5, M^+), 274 (39), 259 (28), 43 (100).

3β-Hydroxy-5α-androstane-16β,17β-diyl Diacetate (**6b**). As described for **1c**, with **6a**: 245 mg (83%). M.p. 172–173°. IR (KBr): 3508 (OH), 1745 (C=O), 1716 (C=O). ^{1}H-NMR: *Table 3.* ^{13}C-NMR: 170.4 (Me*C*OO); 170.2

(MeCOO); 80.5 (C(17)); 71.2 (C(16)); 70.9 (C(3)); 54.5 (C(9)); 47.7 (C(13)); 44.9 (C(5)); 42.2 (C(14)); 38.2 (C(4)); 37.0 (C(1)); 35.7 (C(10)); 34.8 (C(8)); 32.9 (C(12)); 31.6 (C(2)); 31.5 (C(7)); 29.7 (C(15)); 28.5 (C(6)); 20.9 (*Me*COO); 20.5 (*Me*COO); 19.4 (C(11)); 12.6 (C(18)); 12.4 (C(19)). EI-MS: 392 (4), 332 (8), 290 (28), 272 (35), 43 (100). HR-MS: 392.256417 ($C_{23}H_{36}O_5^+$; calc. 392.256275).

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