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Enzymatic Baeyer–Villiger oxidation of steroids with cyclopentadecanone monooxygenase

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ABSTRACT

Recombinant cyclopentadecanone monooxygenase from *Pseudomonas* sp. catalyzed the preparative-scale Baeyer–Villiger oxidation of numerous 3 and 17-ketosteroids with a full control of the regiochemistry of the produced lactones. The recovered product yields were up to 42%.

1. Introduction

The Baeyer–Villiger (BV) reaction is among the most well-known and commonly applied reactions in organic synthesis. Its versatility is well established inasmuch (i) different types of carbonyl compounds such as aldehydes, ketones and cyclic ketones can be oxidized, (ii) the presence of other functional groups is well tolerated and (iii) the regiochemistry is usually predictable [1]. Commonly, the reaction is performed with expensive and/or hazardous peracids and a large amount of waste is produced. In order to run this reaction with "*greener*" procedures, new developments have been established using aqueous hydrogen peroxide or oxygen and catalysts [1]. On the other hand, the oxidation of unsaturated ketones may lead to a variety of products such as enol esters, epoxy esters, and epoxy ketones and the presence of two (or more) non-equivalent keto groups in the molecule makes the process even more challenging [2].

The biological BV reaction is an important type of bioconversion, utilized for numerous interesting synthetic applications because of its high regio and/or stereoselectivity [3]. The possibility to profitably use the enzymatic BV reaction also on diketones derivatives has been recently reported: the recombinant cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus*, selectively oxidized racemic bicyclic diketones such as the Wieland–Miescher and the Hajios–Parrish diketones and their derivatives [4]. CHMO belongs to the Baeyer–Villiger monooxygenases (BVMOs), a versatile group of enzymes able to catalyze a wide variety of oxidative reactions [3]. BVMOs are NADPH-dependent flavoenzymes that use atmospheric oxygen as "*green and free*" oxidant. These enzymes have been employed in purified form or directly as whole-cells; in the former case an ancillary enzymatic reaction is required for the regeneration of the nicotinamide cofactor.

Steroids represent an important class of natural products with a multitude of pharmacological properties. It is also recognized that minor changes in the structure of steroids can highly affect their biological activity, which has promoted countless studies on the modification of naturally occurring steroids in search of new and more active compounds. The catabolism of steroids is a viable way of producing new bioactive compounds and many examples based on the degradation (also by BV reaction) of steroidal A [5], B [6], and D rings [7] or of the side chain have been reported [7d–i,8]. In the majority of the cases, these modifications have been obtained by microorganisms with only few examples of isolated enzymes such as cytochrome P450 or BVMOs [6,8a].

So far the two reported BVMOs, they were isolated (and characterized) either from *Cylindrocarpon radicicola* [8b] or from *Rhodococcus rhodochrous* [8a,9]; only the latter was sequenced and produced as a recombinant protein in *Escherichia coli* (SMO). Furthermore, the biocatalytic properties of the two enzymes, studied

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with a small set of steroids demonstrated that they were able to oxidize 17- and 20-ketosteroids.

Recently, a new BVMO from *Pseudomonas* sp. strain HI-70, namely cyclopentadecanone monooxygenase (CPDMO), was cloned, sequenced and overexpressed in *E. coli* [10]. The amino acid sequence is quite different from those of CHMO and SMO, with whom it shows an identity of 31 and 33%, respectively.

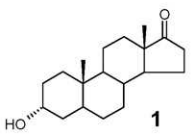
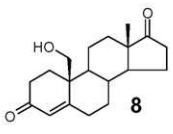
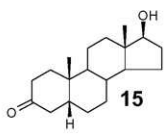
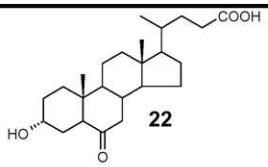
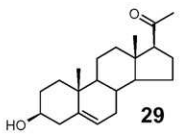
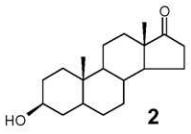
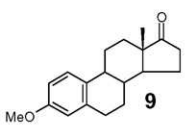
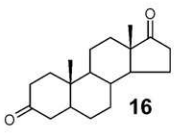
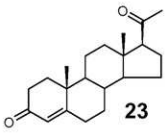
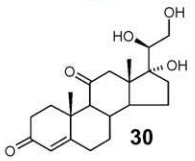
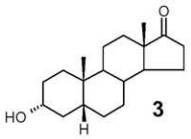
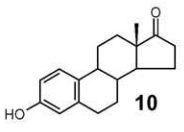
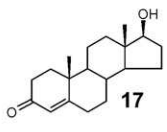
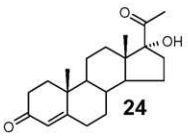
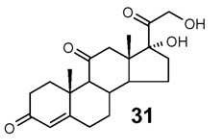
A preliminary characterization of this biocatalyst has shown that the enzyme prefers cyclic and bicyclic ketones with 7–16 carbons atoms [10]. To further explore the substrate-activity space that characterizes CPDMO, we herein report on our studies carried out with structurally demanding substrates such as steroids.

2. Results and discussion

2.1. D-ring oxidation

The enzymatic BV oxidation of a set of 17-ketosteroids (**1**–**13**; Table 1) to the corresponding lactones was conducted with a purified preparation of recombinant mutant S216A of CPDMO from *E. coli* BL21(DE3) (pCpdB), which showed an apparent increase in affinity towards NADPH as indicated by a threefold-lower Km than that of the wild-type protein [10]. To *in situ* regenerate the NADPH consumed by the BV reaction, the ancillary enzymatic system glucose-6-phosphate/glucose-

Table 1
Range of steroids studied as possible substrates for Baeyer–Villiger oxidation catalyzed CPDMO[a].

 **1**	 **8**	 **15**	 **22**	 **29**
9%[b]	20%	14%	n.r.	n.r.
 **2**	 **9**	 **16**	 **23**	 **30**
10%	n.r	**16a**: 30%; **16b**:11%	n.r.	n.r.
 **3**	 **10**	 **17**	 **24**	 **31**
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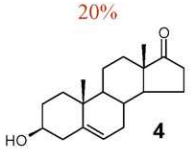
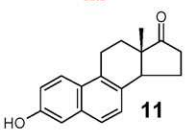
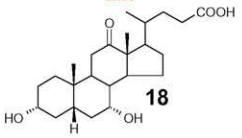
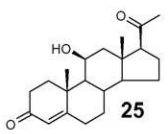
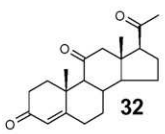
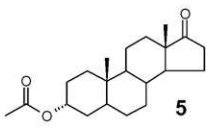
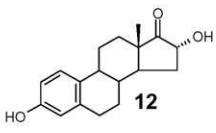
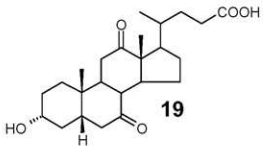
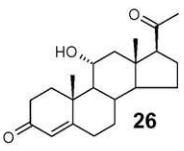
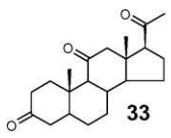
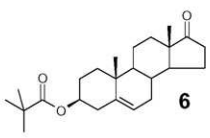
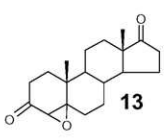
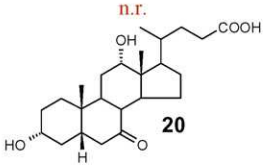
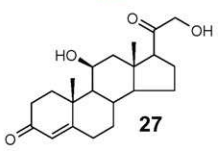
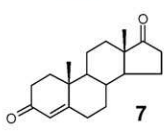
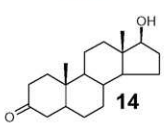
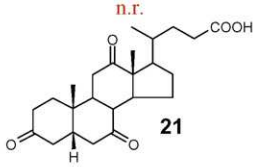
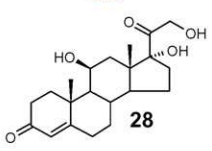
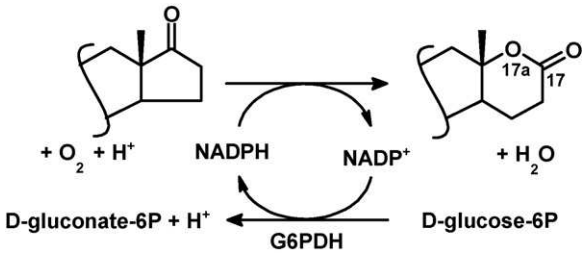
 **4**	 **11**	 **18**	 **25**	 **32**
28%; 42%[c]	n.r.	n.r.	n.r.	n.r.
 **5**	 **12**	 **19**	 **26**	 **33**
3%	20%	n.r.	n.r.	n.r.
 **6**	 **13**	 **20**	 **27**	
n.r.[d]	traces	n.r.	n.r.	
 **7**	 **14**	 **21**	 **28**	
7%	24%	n.r.	n.r.	

[a] For reaction details see Section 1.
[b] Isolated yield of product.
[c] Whole-cells.
[d] No reaction.



Scheme 1. General procedure for the enzymatic oxidation of steroids with *in situ* NADPH regeneration.

6-phosphate dehydrogenase (G6PDH) [11] was employed (Scheme 1).

Oxidation of androsterone (**1**) gave the 17a-oxa-17a-homo 17-one lactone (**1a**) in 9% yield. This is the lactone expected upon migration of the more substituted center, with retention of configuration [12]. Oxygen insertion at the C13–C17 carbon–carbon bond of **1** was inferred by NMR analysis, which revealed the absence the $-OCH_2-$ group (which rules out oxygen insertion at C16–C17 carbon–carbon bond), and showed the presence in the ^{13}C spectrum of a quaternary carbon linked to an oxygen atom. This type of oxygen insertion is in agreement with previous studies on chemical BV oxidation of **1** [13]. Inverting the stereochemistry of the 3-hydroxy group from α to β (**2**) did not affect the performance of the catalyst and lactone **2a** was obtained in 10% yield; instead, changing from the 5α to the 5β series as in **3**, the isolated yield of the lactone increased considerably (**3a**, 20% yield). A further increase in yield was obtained for steroid **4**, which has a double bond in position 5 (**4a**, 28% yield). The oxidation was also carried out using whole-cells, with a 42% yield for **4a**. These data suggest that CPDMO activity is quite insensitive to the stereochemistry of the 3-hydroxy group but is affected by the stereochemistry at position 5. It should be mentioned that steroids of the 5α series are in the *trans-trans-trans* conformation, while those of the 5β series are in the *cis-trans-trans* conformation, which gives them a "bent" structure. Replacing the 3-hydroxy group with the acetyl ester, as in **5**, causes a strong decrease in the recovered yield of product **5a**, which was only 3%. A further increase of the bulkiness of the molecule with the pivoyl ester (**6**), completely suppressed enzymatic activity.

With androstenedione **7**, two keto groups are present in the molecule, but only the 17a-oxa-17a-homo 17-one lactone **7a** was obtained (7% yield). The formation of other compounds with a different structure was ruled out because of the lack of any NMR signal for $-OCH_2-$ or –OCHC– fragments. Oxygen insertion is in agreement with our previous studies on Wieland–Miescher ketone which have shown that another BVMO, i.e. CHMO, was not able to oxidize α,β-unsaturated ketones [4]. This lactone **7a** is a precursor of testolactone that is an aromatase inhibitor and anticancer drug [14]. Steroid **8** was easily transformed by CPDMO in the corresponding lactone **8a** (20% yield), while the BV oxidation with peracids of this 19-hydroxy steroid takes place through complex rearrangements in the A-ring [15]. CPDMO was unable to transform the three estrogens **9**, **10** and **11**, probably due to their very low solubility in aqueous buffer. Instead, estrogen **12**, bearing a 16α-hydroxy group, gave the usual lactone **12a** in 20% yield. Steroid **13** was synthesized as a mixture of β and α epoxide [16] and, when subjected to enzymatic BV oxidation, yielded only trace amounts of products, which prevented their complete characterization. The ESI mass spectrum revealed ions at m/z 319 $[M+H]^+$ and 341 $[M+Na]^+$, which is in agreement with the insertion of an oxygen atom in **13**. NMR spectra were rather complex since the starting steroid **13** was a mixture (7:3) of diastereoisomers because of the presence of α and β epoxide groups (which, in turn, can produce one or more isomers). The major isomer in the resulting mixture showed a singlet at 3.08 ppm in the proton spectrum, ascribed to the hydrogen in position 4, and the occurrence of a quaternary carbon at 82.7 ppm in the carbon spectrum, which is characteristic of the oxidized D-ring. These low conversions are depending on the low solubility of steroids in the reaction buffer and the little stability of the enzymes during the reaction.

2.2. A-ring oxidation

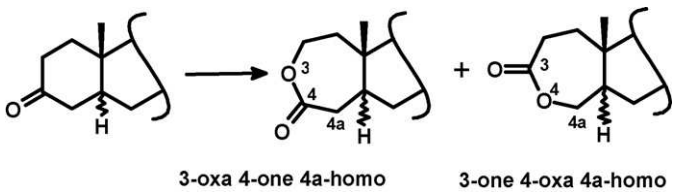
The regiochemistry of the 3-keto-5α and 5β steroid lactonization is markedly influenced by the stereochemistry at C5 and by the nature of the peracids used, e.g. *m*-chloroperbenzoic or trifluoroperacetic acid (Scheme 2) [17]. For the 3-keto-5α steroids studied, the only products obtained, with both peracids, were the 3-oxa 4-one-4a-homo steroids; this peculiar regioselectivity was explained with the preferential attack of the peracid, with a chair conformation of the Criegee intermediate, to the less hindered α-face [17]. For 3-keto-5β steroids, two regioisomers with different ratios depending on the nature of the oxidant, were obtained [17].

Table 1 depicts the structures of the 3-ketosteroids studied by us (**14**–**17**). With androstanolone (**14**) the enzymatic BV reaction gave the 3-one-4-oxa-4a-homo lactone **14a** as the only regioisomer (24% yield). The same was true for **15**, which is similar to **14** but possesses 5β configuration, where lactone **15a** was obtained in 14% yield. The occurrence and the regiochemistry of the $-OCH_2-$ group in products **14a** and **15a** were inferred from NMR spectra. ^{13}C spectra showed the oxymethylene signal at ca. 70 ppm for both compounds. In proton spectra, the signals of the $-OCH_2-$ hydrogens felt in the 3.5–4.6 ppm region and were coupled with a single vicinal proton. Such spin system is consistent only with the 3-one-4-oxa-4a-homo structure. These are the first examples of enzymatic BV oxidation with BVMOs at the A-ring of steroids.

More intriguing is **16**, where two keto groups in position 3 and 17 are present and, therefore, four mono-lactones and four di-lactones could be envisaged. The main product obtained **16a** (30% yield) was the 3-one-4-oxa-4a-homo lactone; another product **16b** (11% yield) was identified as the di-oxidized lactone 4,17a-dioxa-4a,17a-dihomo 5α-androstan 3,17-dione. This finding indicates that **16** is oxidized at the A-ring faster than at the D-ring, since the 17a-oxa-17a-homo 5α-androstan 3,17-dione was not detected: therefore CPDMO seems to prefer 3-keto to 17-ketosteroids. Furthermore, the enzymatic BV reaction seems to be independent of the stereochemistry at C5, since the oxidation was fully regiospecific for both 3-keto-5α- and 5β-steroid to give the 3-one-4-oxa-4a-homo lactones. Testosterone (**17**) was not a substrate for CPDMO, confirming once more that this enzyme is not able to oxidize α,β-unsaturated ketones.

2.3. B- and C-rings and exocyclic C20 keto groups

To check the feasibility of enzymatic oxidation at B- and C-rings, cholic acids **18**–**20**, which have keto groups in position 7 (B-ring) and/or 12 (C-ring), were assayed. None of these steroids was oxidized by CPDMO, included the triketo derivative **21**, which has also a keto group in position 3, and the 5α-cholanoic acid **22**, which has the only keto group in position 6. This behavior is probably due to



Scheme 2. Lactones expected by a BV oxidation of 3-keto-steroids.

the presence of the side chain, that prevents the access or the correct placement of the substrate inside the enzyme active side. The enzymatic BV oxidation of the exocyclic ketone of pregnene steroids was attempted with various substrates (**23**–**32**), but without success; this supports the hypothesis that the keto group in position 20 is not accepted by the enzyme. Similarly, the keto group present in the C-ring in position 11 of **30**–**33** was not accepted by CPDMO.

3. Conclusions

The exploration of the substrate-activity space of CPDMO was conducted with 33 ketosteroids. The study showed that CPDMO in not only able to direct and accommodate in the active site flexible rings such as cyclopentadecanone, but also more structurally demanding compounds such as steroids. The enzyme was able to catalyze the BV oxidation of 3-keto and 17-ketosteroids with full control of the regiochemistry of the produced lactones. The enzymatic oxidations were carried out under non-optimized conditions and, in order to have information on the relative reactivities of the various steroids, low amounts of catalyst were employed. Substantial improvements in conversion and yield are expected by optimization of reaction conditions and by increasing the amount of catalyst used. The products obtained widen the repertory of functionalized compounds of potential interest in the area of medicinal chemistry. The chance that BVMOs can take part in the catabolism of steroidal hormones may be envisaged.

4. Experimental

General: Recombinant CPDMO was overexpressed and purified according to previously described methods [10]. Oxidation reactions were conducted using the purified enzyme. One unit of CPDMO oxidizes 1.0 μmol of 1-decalone per minute at pH 9 and 25 °C in the presence of NADPH. Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* was obtained from Fluka-BioChemika. Glucose-6-phosphate and NADPH were purchased from Sigma–Aldrich. Fermentations were carried out with the Labfors Infors fermenter. Flash chromatography: silica gel 60 (70–230 mesh, Merck). Melting points (uncorrected) were determined with a Reichert–Kofler apparatus. IR spectra were recorded on a Jasco FTIR 610. NMR spectra were recorded either on a Bruker ARX 400 or a Bruker Avance 500 spectrometer, operating at proton resonance frequencies of 400 and 500 MHz (100 and 125 MHz for ^{13}C NMR, respectively) using $CDCl_3$ as solvent and TMS as internal reference. Partial signal assignment, where necessary, was achieved performing proton–proton homocorrelation experiments (COSY) for the hydrogen spectra and DEPT or proton–carbon heterocorrelation experiments for the carbon spectra.

The ESI-MS spectra were recorded on a Bruker Esquire 3000+ instrument with an electrospray source and a quadrupole ion trap detector. The samples were dissolved in CH_3OH, added with a trace of a solution of H_2O containing 1% formic acid and infused into the ESI source via a microsyringe pump at a rate of 4 μL/min.

All other compounds and steroids were purchased from Aldrich-Fluka-Sigma and Steraloids.

4.1. Typical procedure for steroid oxidation with isolated enzymes

Substrates (**1**: 50; **2**: 50; **3**: 25; **4**: 25; **5**: 140; **6**: 25; **7**: 60; **8**: 25; **9**: 90; **10**: 100; **11**: 25; **12**: 25; **13**: 35; **14**: 100; **15**: 25; **16**: 77; **17**–**33**: 25 mg) were dissolved in a Tris/HCl buffer (50 mM, pH 9.0, 10 mL), containing glucose-6-phosphate (1.5 equiv.), glucose-6-phosphate dehydrogenase (0.6 units/mg$_{steroid}$), NADP (0.08 mg/mg$_{steroid}$) and 0.1 units/mg$_{steroid}$ of CPDMO. The mixture was shaken at 250 rpm and 25 °C in a rotatory shaker for 1 day. The reactions were then stopped, worked up by extraction with $CHCl_3$ (3× 5 mL), dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residues were purified by flash chromatography on silica gel with petroleum ether/ethyl acetate to afford the corresponding lactones.

4.2. Steroid oxidation with whole-cells

Fresh LB$_{amp}$ medium (1 l) was inoculated with 0.2% (2 ml) of an 8 h preculture of *E. coli* BL21(DE3) (pCpdB) in a fermenter. The culture was incubated at 450 rpm at 30 °C overnight, and then IPTG was added to a final concentration of 1 mM. Steroid **4** (100 mg) was added along with β-cyclodextrin (0.5 equiv.) and incubated overnight, then the biomass was separated by centrifugation (20 min, 5000 rpm). The supernatant was filtered through a bed of Celite, which was subsequently washed with extraction solvent. The aqueous layer was extracted with $CHCl_3$ (4× 200 ml) and the combined organic layers were dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residues were purified by flash chromatography on silica gel with petroleum ether/ethyl acetate to afford the corresponding lactone **4a** (yield 49%).

4.2.1. 3α-Hydroxy 17a-oxa-17a-homo 5α-androstan 17-one (*1a*)

Yield 9%. White powder; m.p. 233 °C; IR (nujol) 1715 cm^{-1}; ESI-MS *m*/*z* 307 [M+H]$^+$, 329 [M+Na]$^+$; ^{1}H NMR: δ = 4.05 (1H, quint., *J* = 2.8 Hz), 2.66 (1H, ddd, *J* = 19.1, 8.8, 2.6 Hz), 2.55 (1H, ddd, *J* = 19.1, 9.5, 8.5 Hz), 2.00–1.93 (2H, m), 1.86 (1H, dtd, *J* = 12.7, 6.6, 2.7 Hz), 1.80 (1H, dq, *J* = 13.6, 3.7 Hz), 1.70–1.35 (11H, m), 1.31 (3H, d, *J* = 0.9 Hz), 1.30–1.11 (4H, m), 1.02–0.94 (2H, m), 0.76 (3H, d, *J* = 0.7 Hz); ^{13}C NMR: δ = 171.57, 83.44, 66.31, 53.15, 46.41, 39.38, 38.53, 37.96, 36.12, 35.62, 31.99, 30.57, 28.97, 28.72, 28.17, 21.63, 20.16, 19.81, 11.06.

4.2.2. 3β-Hydroxy 17a-oxa-17a-homo 5α-androstan 17-one (*2a*)

Yield 10%. White powder; m.p. 145 °C; IR (nujol) 1714 cm^{-1}; ESI-MS: *m*/*z* 307 [M+H]$^+$, 329 [M+Na]$^+$; ^{1}H NMR: δ = 3.61 (1H, tt, *J* = 11.1, 4.8 Hz), 2.66 (1H, ddd, *J* = 19.0, 8.5, 2.6 Hz), 2.55 (1H, ddd, *J* = 19.0, 9.4, 8.2 Hz), 2.00–1.71 (6H, m), 1.65–1.31 (9H, m), 1.30 (3H, d, *J* = 0.7 Hz), 1.27–1.09 (4H, m), 1.04–0.82 (2H, m), 0.789 (3H, s); ^{13}C NMR: δ = 171.47, 83.34, 71.10, 53.19, 46.40, 44.29, 39.38, 37.99, 37.89, 36.84, 35.54, 31.39, 30.66, 28.69, 28.33, 22.07, 20.13, 19.87, 12.17.

4.2.3. 3α-Hydroxy 17a-oxa-17a-homo 5β-androstan 17-one (*3a*)

Yield 20%. White powder; m.p. 175 °C; IR (nujol) 1715 cm^{-1}; ESI-MS: *m*/*z* 307 [M+H]$^+$, 329 [M+Na]$^+$; ^{1}H NMR: δ = 3.65 (1H, tt, *J* = 11.1, 4.6 Hz), 2.69–2.52 (2H, m), 2.00–1.79 (4H, m), 1.75–1.31 (13H, m), 1.29 (3H, s), 1.26–1.09 (3H, m), 1.03 (1H, td, *J* = 14.2, 3.5 Hz), 0.89 (3H, s); ^{13}C NMR δ = 171.35, 83.32, 71.58, 46.45, 41.57, 39.74, 39.53, 38.38, 36.26, 35.09, 34.78, 30.62, 28.73, 26.95, 25.21, 23.14, 21.83, 20.13, 19.95.

4.2.4. 3β-Hydroxy 17a-oxa-17a-homo-androst-5-ene-17-one (*4a*)

Yield 28% or 42%. White powder; m.p. 210 °C; IR (nujol) 1683 cm^{-1}; ESI-MS: *m*/*z* 305 [M+H]$^+$, 327 [M+Na]$^+$; ^{1}H NMR: δ = 5.36 (1H, dt, *J* = 5.4, 2.0 Hz), 3.54 (1H, tt, *J* = 11.5, 4.4 Hz), 2.69 (1H, ddd, *J* = 18.9, 8.5, 2.2 Hz), 2.58 (dt, *J* = 18.9, 9.0 Hz), 2.34 (1H, ddd, *J* = 13.0, 4.8, 2.4), 2.26–2.15 (2H, m), 2.00–1.78 (5H, m), 1.71–1.35 (8H, m), 1.33 (3H, s), 1.19–1.09 (2H, m), 0.99 (3H, s); ^{13}C NMR: δ = 171.37, 140.73, 120.57, 83.11, 71.50, 49.06, 46.79, 41.94, 38.97, 36.96, 36.63, 34.51, 31.52, 31.11, 28.83, 21.99, 20.07, 19.95, 19.30.

4.2.5. 3α-Acetoxy 17a-oxa-17a-homo 5β-androstan 17-one (*5a*)

Yield 3%. White powder; m.p. 150 °C; IR (nujol) 1731 cm^{-1}; ESI-MS: *m*/*z* 349 [M+H]$^+$, 371 [M+Na]$^+$; ^{1}H NMR: δ = 5.02 (1H, quint., *J* = 2.6 Hz), 2.67 (1H, ddd, *J* = 18.9, 8.5, 2.4 Hz), 2.56 (1H, ddd, *J* = 18.9,

9.3, 8.0 Hz), 2.12–2.06 (1H, m), 2.05 (3H, s), 2.02–1.93 (3H, m), 1.87 (1H, dtd, J = 12.8, 3.8, 2.6 Hz), 1.82–1.73 (2H, m), 1.67–1.38 (7H, m), 1.30 (3H, d, J = 0.8 Hz), 1.25–1.11 (4H, m), 1.02–0.93 (2H, m), 0.77 (3H, s); ^{13}C NMR: δ = 171.53, 170.62, 83.38, 73.38, 53.04, 46.44, 39.48, 39.37, 37.93, 35.83, 32.70, 32.65, 30.50, 28.69, 27.99, 26.02, 21.64, 21.52, 20.14, 19.82, 11.20.

*4.2.6. 17a-Oxa-17a-homo-androst-4-ene 3,17-dione (**7a**)*

Yield 7%. White powder; m.p. 195 °C; IR (nujol) 1716, 1666 cm^{-1}; ESI-MS: m/z 303 [M+H]$^+$, 325 [M+Na]$^+$; ^{1}H NMR: δ = 5.76 (1H, dt, J = 1.8, 0.8 Hz), 2.70 (1H, ddd, J = 18.9, 8.8, 2.4 Hz), 2.58 (1H, ddd, J = 18.9, 9.3, 8.4 Hz), 2.46–2.31 (4H, m), 2.08–1.98 (5H, m), 1.81 (1H, dq, J = 13.7, 3.6 Hz), 1.74 (1H, td, J = 13.7, 5.6 Hz), 1.67 (1H, tdd, J = 12.8, 4.2, 0.9 Hz), 1.61–1.39 (3H, m), 1.36 (3H, d, J = 0.9 Hz), 1.35–1.29 (1H, m), 1.17 (3H, d, J = 0.7 Hz), 1.17–1.06 (1H, m); ^{13}C NMR: δ = 198.90, 170.98, 169.04, 124.18, 82.63, 52.58, 45.80, 39.05, 38.47, 38.06, 35.58, 33.83, 32.35, 30.48, 28.56, 21.88, 20.06, 19.94, 17.43.

*4.2.7. 19-Hydroxy 17a-oxa-homo-androst-4ene 3,17-dione (**8a**)*

Yield 20%. White powder; m.p. 230 °C; IR (nujol) 1700, 1656 cm^{-1}; ESI-MS: m/z 319 [M+H]$^+$, 341 [M+Na]$^+$; ^{1}H NMR: δ = 5.95 (1H, dt, J = 1.7, 0.8 Hz), 3.99 (1H, d, J = 10.6 Hz), 3.89 (1H, d, J = 10.6 Hz), 2.72–2.54 (3H, m), 2.46–2.34 (4H, m), 2.12–1.88 (5H, m), 1.80 (1H, tdd, J = 13.4, 5.1, 1.1 Hz), 1.67–1.38 (6H, m), 1.35 (3H, d, J = 0.9 Hz), 1.13 (1H, qd, J = 12.4, 5.3 Hz); ^{13}C NMR: δ = 199.23, 170.97, 165.30, 126,87, 82,61, 65, 81, 52.78, 46.21, 43.49, 39.52, 38.75, 34.77, 33.35, 33.17, 30.58, 28.52, 22.46, 20.17, 19.89.

*4.2.8. 3,16α-Dihydroxy 17a-oxa-17a-homo 1,3,5(10)-estratrien 17-one (**12a**)*

Yield 20%. White powder; m.p. 248 °C; IR (nujol) 1715 cm^{-1}; ESI-MS: m/z 303 [M+H]$^+$, 325 [M+Na]$^+$; ^{1}H NMR: δ = 7.12 (1H, d, J = 8.6 Hz), 7.16 (1H, s br), 6.64 (1H, dd, J = 8.6, 2.8 Hz), 6.57 (1H, d, J = 2.8 Hz), 4.48 (1H, dd, J = 10.4, 4.5 Hz), 2.86–2.82 (2H, m), 2.48 (1H, dq, J = 13.5, 3.7 Hz), 2.41 (1H, td, J = 11.0, 4.2 Hz), 2.24–2.00 (3H, m), 1.88 (1H, td, J = 13.8, 4.4 Hz), 1.79 (1H, ddd, J = 12.4, 11.0, 7.4 Hz), 1.45–1.38 (1H, m), 1.39 (3H, s), 1.38–1.18 (4H, m); ^{13}C NMR: δ = 175.14, 153.74, 137.71, 131.10, 126.51, 115,16, 113.10, 85.29, 64.62, 45.30, 42.45, 42.07, 39.40, 30.53, 29.72, 27.47, 26.04, 19.43.

*4.2.9. 17β-Hydroxy 4-oxa-4a-homo 5α-androstan 3-one (**14a**)*

Yield 24%. White powder; m.p. 240 °C; IR (nujol) 1732 cm^{-1}; ESI-MS: m/z 307 [M+H]$^+$, 329 [M+Na]$^+$; ^{1}H NMR: δ = 4.28 (1H, dd, J = 13.0, 8.9 Hz), 3.69 (1H, dd, J = 13.0, 1.2 Hz), 3.63 (1H, t, J = 8.6 Hz), 3.62 (1H, s br), 2.69 (1H, ddd, J = 14.5, 13.7, 1.8 Hz), 2.50 (1H, ddd, J = 14.5, 7.4, 1.5 Hz), 2.10–2.01 (1H, m), 1.93 (1H, ddd, J = 14.4, 7.6, 1.9 Hz), 1.82 (1H, ddd, J = 12.6, 4.2, 2.8 Hz), 1.74 (1H, ddd, J = 12.8, 4.1, 2.5 Hz), 1.65–1.20 (11H, m), 1.06 (1H, td, J = 12.8, 3.8 Hz), 0.98–0.92 (1H, m), 0.94 (3H, s), 0.78–0.73 (1H, m), 0.74 (3H, s); ^{13}C NMR: δ = 176.03, 81.78, 70.02, 53.82, 50.93, 48.75, 42.83, 37.79, 36.66, 35.67, 35.08, 31.20, 30.58, 29.74, 26.29, 23.35, 20.76, 12.38, 11.12.

*4.2.10. 17β-Hydroxy 4-oxa-4a-homo 5β-androstan 3-one (**15a**)*

Yield 14%. White powder; m.p. 130 °C; IR (nujol) 1733 cm^{-1}; ESI-MS: m/z 307 [M+H]$^+$, 329 [M+Na]$^+$; ^{1}H NMR: δ = 4.63 (1H, dd, J = 13.0, 10.1 Hz), 3.90 (1H, dd, J = 13.0, 1.6 Hz), 3.66 (1H, t, J = 8.6 Hz), 2.70 (1H, ddd, J = 14.7, 12.9, 1.3 Hz), 2.38 (1H, ddd, J = 14.7, 8.1, 1.1 Hz), 2.11–1.84 (4H, m), 1.77 (1H, ddt, J = 10.2, 5.0, 2.2 Hz), 1.61–1.22 (11H, m), 1.13 (1H, td, J = 12.6, 4.3 Hz), 1.07–1.01 (1H, m), 1.04 (3H, s), 0,75 (3H, d, J = 0.6 Hz), 0.75–0.77 (1H, m); ^{13}C NMR: δ = 176.00, 81.81, 70.40, 51.02, 45.59, 43.14, 41.77, 36.86, 36.73, 35.94, 33.39, 30.64, 28.35, 27.01, 26.89, 23.88, 23.37, 20.67, 11.12.

*4.2.11. 4-Oxa-4a-homo 5α-androstan 3,17-dione (**16a**)*

Yield 30%. White powder; m.p. 233 °C; IR (nujol) 1733 cm^{-1}; ESI-MS: m/z 305 [M+H]$^+$, 327 [M+Na]$^+$; ^{1}H NMR: δ = 4.29 (1H, dd, J = 13.1, 9.0 Hz), 3.71 (1H, dd, 13.1, 1.3 Hz), 2.70 (1H, ddd, J = 14.6, 13.2, 1.8 Hz), 2.52 (1H, ddd, J = 14.6, 7.4, 1.5 Hz), 2.45 (1H, ddd, J = 19.4, 9.1, 1.3 Hz), 2.08 (1H, dt, J = 19.4, 8.9 Hz), 1.97–1.70 (5H, m) 1.61–1.23 (9H, m), 1.06 (1H, qd, J = 13.2, 4.5 Hz), 0.96 (3H, s), 0.86 (3H, s), 0.83 (1H, ddd, J = 11.8, 10.7, 4.1 Hz); ^{13}C NMR: δ = 220.46, 175.84, 69.84, 53.72, 51.23, 48.64, 47.48, 37.83, 35.78, 35.53, 34.57, 31.45, 30.43, 29.65, 26.10, 21.71, 20.41, 13.78, 12.34.

*4.2.12. 4,17a-Dioxa-4a,17a-dihomo 5α-androstan 3,17-dione (**16b**)*

Yield 11%. White powder; m.p. 260 °C; IR (nujol) 1733 cm^{-1}; ESI-MS: m/z 321 [M+H]$^+$, 343 [M+Na]$^+$; ^{1}H NMR: δ = 4.27 (1H, dd, J = 13.2, 8.9 Hz), 3.72 (1H, dd, J = 13.2, 1.3 Hz), 2.72–2.51 (4H, m), 2.00–1.91 (4H, m), 1.84 (1H, dq, J = 13.8, 3.8 Hz), 1.68–1.37 (7H, m), 1.30 (3H, d, J = 0.7 Hz), 1.29–1.14 (2H, m), 1.07–0.96 (2H, m), 0.91 (3H, s); ^{13}C NMR: δ = 175.66, 171.17, 82.76, 69.54, 52.35, 48.02, 46.08, 39.09, 37.67, 37.43, 35.30, 30.13, 29.54, 28.56, 25.99, 21.92, 20.08, 19.79, 12.24.

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References

[1] G.-J. ten Brink, I.W.C.E. Arends, R.A. Sheldon, Chem. Rev. 104 (2004) 4105–4123.
[2] (a) E. Butkus, S. Stončius, J. Chem. Soc. Perkin Trans. 1 (2001) 1885–1888;
(b) A. Corma, L.T. Nemeth, M. Renz, S. Valencia, Nature 412 (2001) 423–425.
[3] (a) M.W. Fraaije, D.B. Janssen, in: R.D. Schmid, V.B. Urlacher (Eds.), Modern Biooxidation, Wiley-VCH, Weinheim, 2007, pp. 77–97;
(b) M.D. Mihovilovic, Curr. Org. Chem. 10 (2006) 1265–1287;
(c) V. Alphand, G. Carrea, R. Wohlgemuth, R. Furstoss, J.M. Woodley, Trends Biotechnol. 21 (2003) 318–323;
(d) F. Zambianchi, P. Pasta, G. Carrea, S. Colonna, N. Gaggero, J.M. Woodley, Biotechnol. Bioeng. 78 (2002) 489–496.
[4] G. Ottolina, G. de Gonzalo, G. Carrea, B. Danieli, Adv. Synth. Catal. 347 (2005) 1035–1040.
[5] (a) A.I. Laskin, P. Grabowich, C. Meyers, J. Fried, J. Med. Chem. 7 (1964) 406–409;
(b) G.E. Turfitt, Biochem. J. 42 (1948) 376–383.
[6] (a) T.-W. Kim, J.-Y. Hwang, Y.-S. Kim, S.-H. Joo, S.C. Chang, J.S. Lee, S. Takatsuto, S.-K. Kim, Plant Cell 17 (2005) 2397–2412;
(b) J. Winter, B. Schneider, S. Meyenburg, D. Stranck, G. Adam, Phytochemistry 51 (1999) 237–242.
[7] (a) H.-M. Liu, H. Li, L. Shan, J. Wu, Steroids 71 (2006) 931–934;
(b) A. Bartmańska, J. Dmochowska-Gładysz, E. Huszcza, Steroids 70 (2005) 193–198;
(c) E. Itagaki, J. Biochem. 99 (1986) 825–832;
(d) M.A. Rahim, C.J. Sih, J. Biol. Chem. 241 (1966) 3615–3623;
(e) O. El-Tayeb, S.G. Knight, C.J. Sih, Biochim. Biophys. Acta 93 (1964) 411–417;
(f) R.L. Praire, P. Talalay, Biochemistry 2 (1963) 203–208;
(g) D.H. Peterson, S.H. Eppstein, P.D. Meister, H.C. Murray, H.M. Leigh, A. Weintraub, L.M. Reineke, J. Am. Chem. Soc. 75 (1953) 5768–5769;
(h) J. Friend, R.W. Thoma, A. Klingsberg, J. Am. Chem. Soc. 75 (1953) 5764–5765;
(i) T. Kołek, A. Szpineter, A. Świzdor, Steroids (2008), doi:10.1016/j.steroids.2008.07.008.
[8] (a) M. Miyamoto, J. Matsumoto, T. Iwaya, E. Itagaki, Biochim. Biophys. Acta 1251 (1995) 115–124;
(b) E. Itagaki, J. Biochem. 99 (1986) 815–824;
(c) G.S. Fonken, H.C. Murray, L.M. Reineke, J. Am. Chem. Soc. 82 (1960) 5507–5508.
[9] S. Morii, S. Sawamoto, Y. Yamauchi, M. Miyamoto, M. Iwami, E. Itagaki, J. Biochem. 126 (1999) 624–631.
[10] H. Iwaki, S. Wang, S. Grosse, H. Bergeron, A. Nagahashi, J. Lertvorachon, J. Yang, Y. Konishi, Y. Hasegawa, P.C.K. Lau, Appl. Environ. Microbiol. 72 (2006) 2707–2720.
[11] D.R. Light, D.J. Waxman, C. Walsh, Biochemistry 21 (1982) 2490–2498.
[12] (a) J.M. Schwab, J. Am. Chem. Soc. 103 (1981) 1876–1878;
(b) J.M. Schwab, W.-B. Li, L.P. Thomas, J. Am. Chem. Soc. 105 (1983) 4800–4808.
[13] P. Catsoulacos, L. Boutis, K. Dimitropoulos, Eur. J. Med. Chem. 11 (1976) 189–191.
[14] E.J. Tavares da Silva, M.L. Sá e Melo, A.S. Campos Neves, J.A. Paixão, L.C.R. Andrade, M.-J.M. Almeida, M.M.R. Costa, J. Chem. Soc. Perkins Trans. I (1997) 3487–3489.
[15] S. Hrycko, P. Morand, F.L. Lee, E.J. Gabe, J. Chem. Soc. Perkins Trans. I (1989) 1311–1317.
[16] D. Lesuisse, J.F. Gourvest, C. Hartmann, B. Tric, O. Benslimane, D. Philibert, J.P. Vevert, J. Med. Chem. 35 (1992) 1588–1597.
[17] D.G. Rivera, O. Pando, R. Suardiaz, F. Coll, Steroids 72 (2007) 466–473.