Microbiological hydroxylation of some epoxy steroids by the fungus *Mucor plumbeus*

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The preparation of epoxy steroids derived from testosterone, dehydroisoandrosterone and epiandrosterone using *m*-chloroperbenzoic acid and their biotransformation by the fungus *Mucor plumbeus* is described. The results reveal an effect of the epoxide on the biotransformation.

Keywords: *Mucor plumbeus*, microbiological hydroxylation, epoxy steroids, *m*-chloroperbenzoic acid

The ability of enzymes to perform regio- and stereospecific reactions, under mild conditions, offers a valuable method for the production of chiral molecules with diverse applications in the synthesis of natural products, and medicines. The use of biotransformations was first reported when it helped to overcome a major problem in the synthesis of the cortical steroids by using the fungus *Rhizopus arrhizus*[1] (Fig. 1).

The factors that govern the microbiological hydroxylation of steroids involve a combination of effects based on the site, stereochemistry and nature of the existing functional groups in the substrate.[2] Predictive models for the microbiological hydroxylation of steroids envisage three active centres on the hydroxylase, two of which bind the substrate and one delivers the oxygen[3,4] (Fig. 2). The presence of the two binding oxygen groups (hydroxyl or carbonyl) in the steroid molecule increases the rate of reactivity for biotransformation and give mainly monohydroxylated products.[5]

Hydroxylation commonly takes place at least four or five atoms distant from the binding groups. The present work deals with the synthesis of steroids in which an epoxide replaces one of the hydroxyl or carbonyl groups and studies the effect of this new epoxy group on the biotransformation.

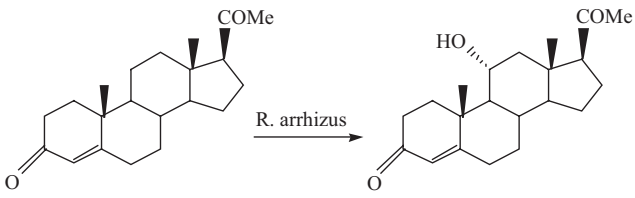


Fig. 1

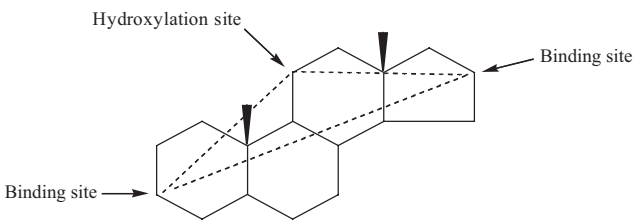


Fig. 2 The Jones model of enzyme-substrate interaction

Results and discussion

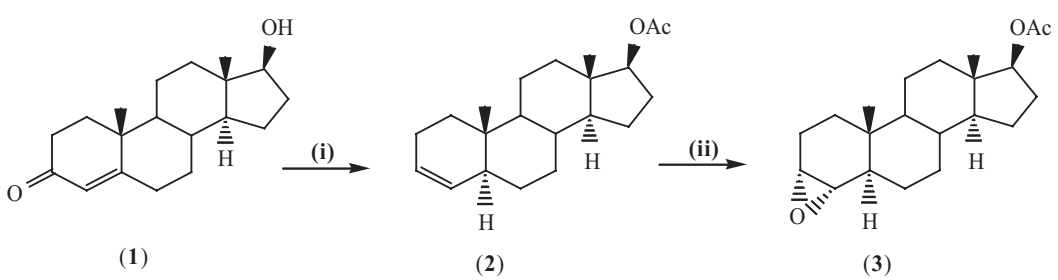
In order to examine the effect of the introduction of an epoxide on the biotransformation of a steroid three steroids with epoxides in ring A, B and D were prepared and biotransformed using the fungus *Mucor plumbeus*.

Thus, reduction of testosterone(**1**) with boron trifluoride diethyl etherate : sodium borohydride in diglyme followed by treatment with acetic anhydride gave 17β-acetoxy-5α-androst-3-ene (**2**).[6] The ^{1}H NMR spectrum of the product **2** exhibited new signals at δ 5.26 ppm (1H, dd, J = 10, 1.8 Hz, H-4) and δ 5.52 ppm (1H, dq, J = 10, 3.2 Hz, H-3) which revealed the presence of the 3-ene. The ^{1}H NMR also showed new signals at δ 2.04 ppm (3H, s, $COCH_3$) and δ 4.56 ppm (1H, t, J = 8.5 Hz, H-17) which were assigned to the presence of the 17β-acetoxy group.

17β-Acetoxy-5α-androst-3-ene(**2**) was treated with *m*-chloroperbenzoic acid in chloroform to obtain 17β-acetoxy-3α,4α-epoxy-5α-androstane (**3**) (Scheme 1). The ^{1}H NMR spectrum of the product **3** possessed new signals at δ 2.61 ppm (1H, d, J = 4.1 Hz, H-4) and δ 3.08 ppm (1H, m, H-3) which were assigned to the presence of an epoxide. Further confirmation of the structure for compound **3** came from the presence of two new signals at δ 55.87 ppm (C-4) and δ 52.77 ppm (C-3) in the ^{13}C NMR spectrum of the product. The stereochemistry of the epoxide was assigned by comparison of the ^{1}H NMR of a similar epoxide.[7]

Dehydroisoandrosterone(DHA)(**4**) when treated with *m*-chloroperbenzoic acid in chloroform yielded the known 3β-hydroxy-5α,6α-epoxyandrostan-17-one (**5**) (Scheme 2) which was identified by comparing that spectroscopic data with the literature values.[8]

3β-Hydroxy-5α-androstan-17-one(**6**) was refluxed with hydrazine hydrate in ethanol with few drops of triethylamine.



(**1**) (**2**) (**3**)

Scheme 1 (i) $NaBH_4$/Diglyme/$BF_3.OEt_2$/Ac_2O; (ii) m-CPBA/$CHCl_3$.

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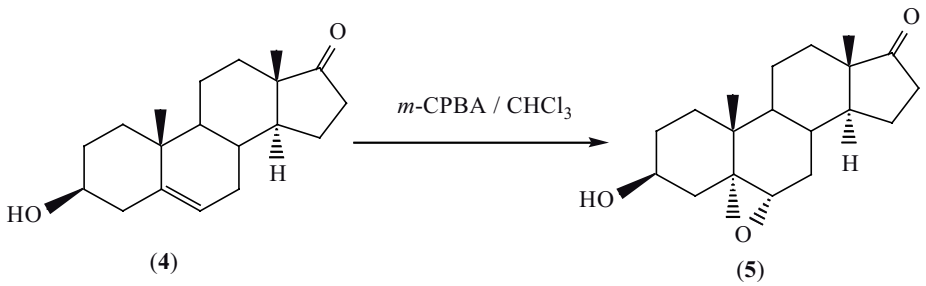


Scheme 2

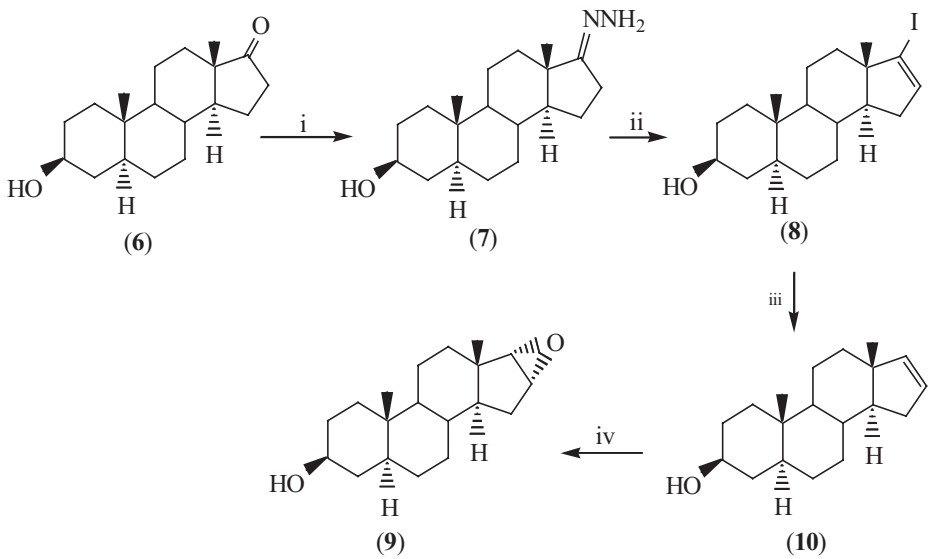
The product was 3β-hydroxy-5α-androstan-17-hydrazone(**7**) which was isolated and identified by its spectroscopic data. Treatment of the hydrazone with triethylamine and iodine in THF afforded the known 17-iodo-5α-androst-16-en-3β-ol(**8**). Refluxing 17-Iodo-5α-androst-16-en-3β-ol(**8**) in ethanol with sodium metal produced 5α-androst-16-ene-3β-ol(**10**). Compound **10** in chloroform was treated with *m*-chloroperbenzoic acid afforded a known compound 16α,17α–epoxy-5α-androstan-3β-ol(**9**) (Scheme 3). The structure and stereochemistry of this epoxy compound **9** was confirmed by comparing the spectroscopic data with the literature.[9]

Biotransformations results

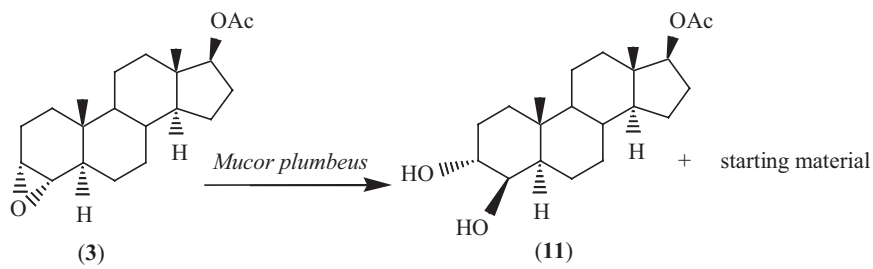
Metabolism of the epoxy steroids by Mucor plumbeus

Incubation of 17β-acetoxy-3α,4α-epoxy-5α-androstane (**3**): The incubation of 17β-acetoxy3α,4α-epoxy-5α-androstane (**3**) with *Mucor plumbeus* for 7 days gave one metabolite and 20% of the starting material was recovered. The crude product was purified by column chromatography of silica gel. The only metabolite that was eluted with 45% ethyl acetate in light petroleum was identified as the 3α,4β-dihydroxy-17β-acetoxy-5α-androstane (**11**)(35%). The ^{1}H NMR spectrum of the product **11** showed signals at 3.62 ppm (1H, br.s, 3β-H) and 3.91 ppm (1H, br.s, 4α-H) instead of signals at δ 3.08 ppm (1H, m, 3β-H) and 2.61 ppm (1H, d, J = 4.4 Hz, 4β-H) in compound **3**. The ^{13}C NMR of the product **11** showed new signals at δ 70.19 (C-3) and 76.3 (C-4) instead of signals at δ 52.77 ppm (C-3) and 55.87 ppm (C-4) of starting material (2). The stereochemistry of the product was assigned by comparison with the ^{1}H NMR spectra of a similar 3α,4β– dihydroxy steroid[8] (Scheme 4).

Incubation of 17β-acetoxy-3α,4α-epoxy-5α-androstane (**3**) *in the medium without the fungus*: 17β-Acetoxy-3α,4α-epoxy-5α-androstane (**3**) was dissolved in ethanol and under the same conditions. The solution was left in the medium for 7 days on orbital shaker without the fungus *Mucor plumbeus*.



Scheme 3 (i) H_2NNH_2/TEA/EtOH; (ii) I_2/TEA/THF; (iii) Na/EtOH; (iv) m-CPBA/$CHCl_3$.



Scheme 4

The aqueous layer was extracted with ethyl acetate. The solvent was removed *in vacuo* to afford 71% of the starting material 17β-acetoxy-3α,4α-epoxy-5α-androstane (**3**) and no hydrolysed product.

Incubation of 3β-hydroxy-5α, 6α-epoxyandrostan-17-one (**5**): Under similar conditions incubation of 3β-hydroxy-5α,6α-epoxyandrostan-17-one (**5**) afforded one hydroxylated product and 25% of the starting material. The only metabolite was purified by column chromatography on silica gel and eluted by 30% ethyl acetate in petroleum ether. The product was identified as 3β,7α-dihydroxy-5α,6α-epoxyandrostan-17-one (**12**) (21%) (Scheme 5). ^{1}H NMR spectrum of the product **12** possessed a signal at δ 3.3 ppm (1H, d, J = 4.6 Hz, 6βH) in place of δ 2.93 ppm (1H, d, J = 3.76 Hz, H-6) of compound **3**. The ^{1}H NMR spectrum of **12** contained a new signal at δ 4.05 ppm (1H, brs, 7βH) which was not present in the the starting material and in the ^{13}C NMR there was a new signal at δ 76.7 ppm (C-7). The ^{13}C NMR of product **12** showed two signals at δ 67.32 ppm (C-5) and δ 62.21 ppm (C-6) which were consistent with the presence of the epoxide. This suggested that hydroxylation had taken place at C-7. The site of the newly introduced hydroxyl group at C-7 in **12** was established by the 2D-COSY NMR spectrum which revealed a correlation between δ 3.3 and 4.05 ppm. This correlation peak confirmed that there was a vicinal spin coupling between 6β-H and 7β. The stereochemistry of the new hydroxyl group at C-7 in compound **12** was established by the 2D-NOESY NMR spectrum which showed three cross peaks at δ 4.05 and 1.12 ppm for the 7β-H with the methyl –19 protons and 4.05 and 3.3 ppm for the 7β-H with the 6β-H. These cross peaks prove that 7β-H is close in space to the methyl –19 protons and 6β-H and confirm that these is a 7β-H and C-7α-hydroxyl group.

Incubation of 16α,17α-epoxy-5α-androstan-3β-ol (**9**): Under same condition incubation of 16α,17α-epoxy-5α-androstan-3β-ol (**9**) gave two biotransformation products and 24% of the starting material. The crude product was purified by column chromatography on silica gel. The first metabolite **13** was eluted with 10% ethyl acetate in light petroleum and second **14** was eluted with 25% ethyl acetate in light petroleum. The product **13** was identified as 16α,17α-epoxy-5α-androstan-3-one (12%). The IR spectrum of the product **13** showed a band at 1705 cm^{-1} which was attributed to the presence of carbonyl group at C-3. This oxidation was revealed by the ^{1}H NMR in the absence of the signal at δ 3.57 ppm (1H, tt, J = 11.3, 5.2 Hz, 3α-H) present in the starting material **9**. Finally, ^{13}C NMR of the product **13** showed a new signal at δ 210.63 ppm for the carbonyl group at C-3. The second metabolite **14** was identified as the known 3β,6β-dihydroxy-16α,17α-epoxy-5α-androstane (37%) (Scheme 6). The site and the stereochemistry of the hydroxyl group were confirmed by comparing its ^{1}H NMR data with the ^{1}H NMR of an authentic sample which had been prepared by incubation of this compound with another fungus.[9]

Conclusion

The transformation of the epoxy steroids suggests that the presence of epoxy group did not prevent the enzyme from binding the substrate and consequently from biotransforming these steroids. However, they seem to behave like the mono-oxygenated steroids in giving poor yields and few hydroxylation products compared with dioxygenated steroids. One epoxide was hydrolysed during these biotransformations but further investigations showed that these results were not just hydrolysis in the slightly acidic medium but that fungus was involved. Repetition of biotransformation experiment of the epoxy steroid **3** in the absence of the fungus led to no hydrolysed product.

Experimental

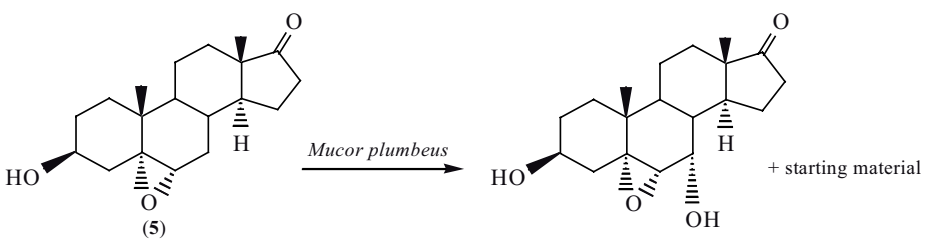
Melting points were determined by Thomas–Hoover capillary melting point apparatus and are uncorrected. IR spectra were recorded using KBr disks on a Nicolet Magna 520 Fourier transform spectrometer. ^{1}H NMR spectra were determined in deuteriochloroform with TMS as an internal standard reference at 400 MHz on a Bruker Avance DPX 400 spectrometer while ^{13}C NMR spectra were recorded in deuteriochloroform at 100 MHz with a Bruker DPX 400 spectrometer. Mass spectra were recorded on a VG Autospec. Micro-analysis were carried out using Perkin Elmer analyser.

The compounds **5** and **9** were prepared by the literature methods reported previously:[8,9]

3β-hydroxy-5α,6α-epoxyandrostan-17-one(**5**): M.p. 180–183°C (lit.,[8] m.p. 182–185°C).

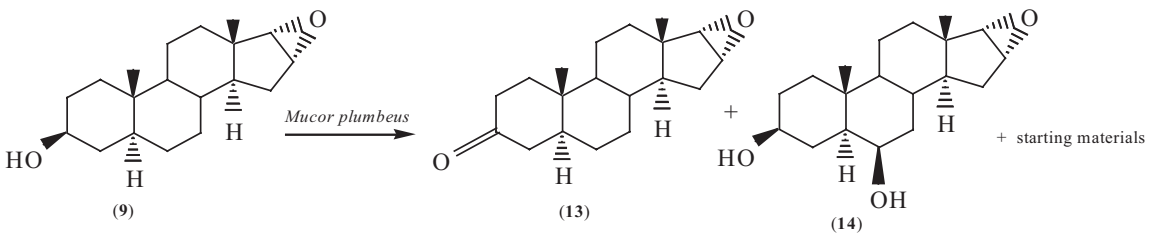
16α,17α-epoxy-5α-androstan-3β-ol (**9**): M.p. 149–150°C (lit. [9], 151.5–152.5°C).

Preparation of 17β-acetoxy-3α,4α-epoxy-5α-androstane (**3**): 17β-Acetoxy-5α-androst-3-ene (**2**) (1.0 g, 3.29 mmol)) in chloroform (100 ml) was treated with *m*-chloroperbenzoic acid (1.0 g, 5.78 mmol) at 0°C. The mixture was allowed to attain room temperature and



(**5**) (**12**)

Scheme 5



(**9**) (**13**) (**14**)

Scheme 6

Table 1 ^{13}C NMR data determined in $CDCl_3$ at 100 MHz of new compounds **3, 11, 12** and **13**

	3	11	12	13
C-1	36.8	30.9	31.5	37.2
C-2	30.9	21.7	24.8	38.1
C-3	68.3	51.9	71.0	211.6
C-4	41.7	55.8	76.1	43.6
C-5	67.3	45.9	43.6	45.3
C-6	62.2	26.7	24.8	27.1
C-7	76.4	31.7	30.9	30.3
C-8	37.5	35.4	35.2	34.2
C-9	45.1	52.6	51.2	49.9
C-10	34.5	34.8	35.9	35.3
C-11	21.6	20.3	20.3	20.9
C-12	31.4	36.9	37.1	35.9
C-13	49.4	42.3	42.7	47.1
C-14	50.9	50.4	51.1	51.6
C-15	21.8	23.7	23.2	35.0
C-16	35.8	27.9	27.5	53.1
C-17	220.2	82.4	81.6	64.1
C-18	14.1	12.2	12.2	13.8
C-19	17.1	13.6	14.3	16.9
17β-OCO<u>C</u>H$_3$	—	21.0	22.1	—
17β-O<u>C</u>OCH$_3$	—	171.2	170.8	

stirred for 1 h. The TLC showed that all starting materials had reacted. The solution was then washed with aqueous sodium sulfite, aqueous sodium hydrogen carbonate, water and brine, and dried over anhydrous sodium sulfate. The solvent was evaporated *in vacuo* to give 17β-acetoxy-3α,4α-epoxy-5α-androstane (**3**) (0.82 g, 78%). The product was crystallised from ethyl acetate as plates; m.p. 105–107°C; (Found: C,75.8; H, 9.6, $C_{21}H_{30}O_3$ requires C,75.9; H, 9.7%); FTIR 1739 (C=O) cm^{-1}.

^1H NMR ($CDCl_3$, 400 MHz) 0.81 (3H, s, 18-H), 0.88 (3H, s, 19-H), 2.05 (3H, s, 17β-OCOCH$_3$), 2.61 (1H, d, J = 4.4 Hz, 4β-H), 3.08 (1H, m, 3β-H), 4.56 (1H, t, J = 8.5 Hz, 17α-H).

Biotransformation experiments

General fermentation details

The fungus *Mucor plumbeus* (IMI 116688) was grown on shake culture in 250 ml conical flasks on a medium (100ml) comprising (per litre), Glucose (30g), potassium dihydrophosphate (1g), magnesium sulfate (1g), ammonium tartrate (2g), yeast extract (1g), calcium chloride (0.25g), sodium chloride (1g), ferrous ammonium sulfate (1g), trace element solution 2 ml, distilled water to 1l, neutralisation to pH 7 by adding NaOH.

Trace element solution contained (per litre) zinc sulfate (1.6g), ferrous sulfate (1g), cobalt nitrate (1g), ammonium molybdate (1g), copper sulfate (0.1g) and magnesium sulfate (0.1g). The fungus was grown for 2 days before the substrate (0.5g) in ethanol (30ml) was distributed over 50 flasks. The fermentation was then continued for further 7 days. The broth was filtered and the mycelium was washed and the water layer extracted with ethyl acetate. The extracts were washed with water and dried. The solvent was evaporated and the residue was chromatographed on silica and eluted with an increasing gradient of ethyl acetate in light petroleum.

17β-Acetoxy-3α,4α-epoxy-5α-androstane (**3**) (0.5g) gave 3α,4β-dihydroxy-17β-acetoxy-5α-androstane (V)(35%) as white cubes m.p.180–184°C. (Found: C,71.4; H, 9.6, $C_{21}H_{34}O_4$ requires C,72.0; H, 9.8%). FTIR: ν_{max}/cm^{-1} 3396, 1739. δ_H ($CDCl_3$, 400 MHz) 0.81 (3H, s, 18-H), 0.88 (3H, s, 19H), 2.05 (3H, s, 17β-OCOCH$_3$), 3.62 (1H, br.s, 3β-H), 3.91 (1H, br.s, 4α-H).

3β-Hydroxy-5α,6α-epoxyandrostan-17-one (**5**) (0.5g) gave 3β,7α-dihydroxy-5α,6α-epoxyandrostan-17-one (**12**) (21%) as white needles. m.p.241–243°C. (Found C; 70.8, H; 8.3 $C_{19}H_{28}O_4$ requires C; 71.2, H; 8.8%). FTIR: ν_{max}/cm^{-1} 3390 and 1747. δ_H ($CDCl_3$, 400 MHz) 0.81 (3H, s, 18-H), 1.12 (3H, s, 19H), 3.31 (1H, d, J = 3.76, 6β-H), 3.85 (IH, tt, J = 5.8 and J = 10.8, 3α-H). 4.05 (1H, brs 7αH).

16α,17α-epoxy-5α-androstan-3β-ol (**9**) (0.5g) gave 16α,17α-epoxy-5α-androstan-3-one (**13**) (12%) as an oil. M^+ 288. Found 288.2081. $C_{19}H_{28}O_2$ calculated 288.2089. FTIR: ν_{max}/cm^{-1} 1705. δ_H ($CDCl_3$, 400 MHz) 0.69 (3H, s, 18-H), 0.78 (3H, s, 19H), 3.06 (1H, d, J = 4 Hz, 17β-H), 3.31 (1H, d, J = 4 Hz, 16β-H).

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