Formation of 5α steroids by biotransformation involving the 5α-reductase activity of *Penicillium decumbens*

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The biotransformation of a series of Δ^4-3-ketosteroids by the fungus Penicillium decumbens *ATCC 10436 has been investigated. Conversion to the 5α-dihydrosteroid was observed for several substrates of the androstene and pregnene series: the reaction is tolerant of non-polar substituents (Cl and CH_3) at C-4 of the substrate, but does not occur in the presence of a 4-hydroxyl group, or with additional unsaturation at the Δ^1 or Δ^6 positions. A-nor-, B-nor-, 3-deoxy- , and 3,5-cycloandrostanes are not reduced, but 6-methylenetestosterone is converted to a 6-methylene-5α-dihydro derivative. Several biotransformations are reported which involve oxidoreductase activity at C-3 and/or C-17, either concomitant or independent of Δ^4 reduction: the substrate specificity of the oxidoreductase processes has been examined and defined by the use of 3α-hydroxy, 3β-hydroxy, 3-keto, 17β-hydroxy and 17-keto substituted steroids. In this way, the existence in* P. decumbens *of 3β-hydroxy-3-keto and 17β-hydroxy-17-keto oxidoreductases has been demonstrated.* (*Steroids* **59**:642–647, 1994)

Keywords: bioconversion; biotransformation; *Penicillium decumbens*, 5α-reductase; testosterone

Introduction

The 5α-reductase enzymes, which carry out the conversion of Δ^4-3-ketosteroids to 5α-3-ketosteroids (e.g., testosterone, **2**, to 5α-dihydrotestosterone, **6**), occupy a central role in mammalian steroid metabolism.[1] The activity of these enzymes in prostate and other tissue of various mammalian species is well documented,[2–7] and their possible role in the development of human prostatic cancer[8–10] has lead to thorough investigations of the enzymes' cofactor requirements;[3,11–14] kinetics in human[3,15–17] and other[4–6,12,18,19] species; and to the development of inhibitors of various types.[20–25]

In spite of these studies, however, comparatively little is known about the fundamental mechanism of action of 5α-reductase enzymes, a situation due in part to the problems associated with the isolation and purification of these enzymes from mammalian sources on a scale large enough for mechanistic studies.[26,27] Early work by Wilton[28] on a 5α-reductase from rat liver showed that Schiff base intermediates were not a feature of that enzyme's mechanism, and more recently the reduction has been demonstrated to proceed with *trans* stereochemistry (addition of hydrogens at C-4β and C-5α) in the cases of reduction of testosterone by *Penicillium decumbens*[29] and of reduction of the Δ^4 bond of cholesta-4,6-dien-3-one by rat and mouse liver microsomes.[30]

One possible aid to mechanistic studies, the insertion of mammalian enzymes in microbial systems, has resulted in the expression of rat 5α-reductase in *Saccharomyces cerevisiae*,[31] but at the present time this system has not been applied to mechanistic studies. Microbial transformations of steroids provide a convenient alternative to mammalian enzyme catalyzed reactions for both preparative (e.g., 11α- and 11β-hydroxylation[32]) and mechanistic (e.g., stereochemical[29]) studies. In view of this, an early report[33] of the reduction of the Δ^4 bond of progesterone (**4**), giving 5α-dihydroprogesterone (**8**), by *Penicillium decumbens* NRRL 742 (identical with ATCC 10436), prompted us to re-examine this micro-organism's role in the biotransformation of a series of Δ^4-3-keto and related steroids with a view to (a) defining the substrate specificity of this biotransformation from a preparative

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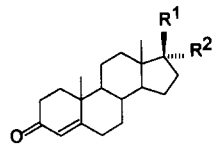
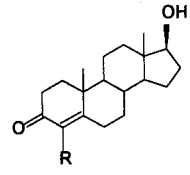
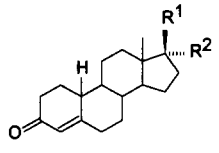


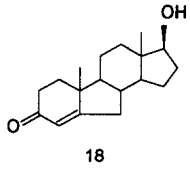
1. $R^1 + R^2 = O$
2. $R^1 = OH, R^2 = H$
3. $R^1 = OAc, R^2 = H$
4. $R^1 = COCH_3, R^2 = H$



9. R = Cl
10. R = OH
11. $R = CH_3$



14. $R^1 = OH, R^2 = H$
15. $R^1 + R^2 = O$



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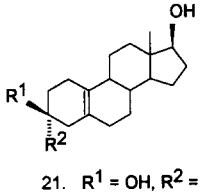
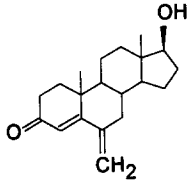


21. $R^1 = OH, R^2 = H$
22. $R^1 + R^2 = O$



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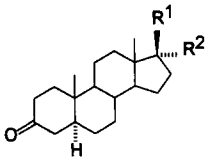
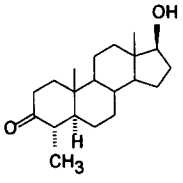


5. $R^1 + R^2 = O$
6. $R^1 = OH, R^2 = H$
7. $R^1 = OAc, R^2 = H$
8. $R^1 = COCH_3, R^2 = H$



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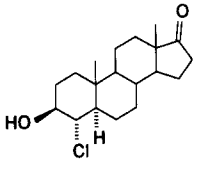

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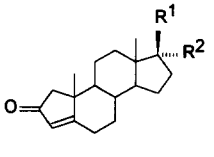
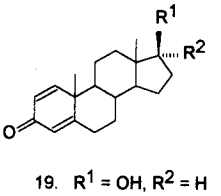
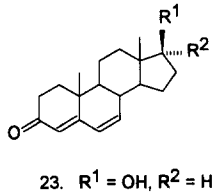


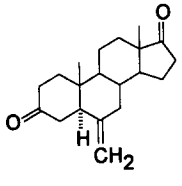
16. $R^1 = OH, R^2 = H$
17. $R^1 + R^2 = O$



19. $R^1 = OH, R^2 = H$
20. $R^1 + R^2 = O$



23. $R^1 = OH, R^2 = H$
24. $R^1 + R^2 = O$



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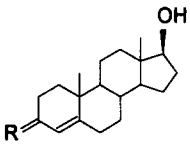
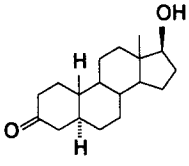


27. $R = CH_2$
28. $R = H_2$
30. R = α^1H, βOH
31. R = α^2H, βOH



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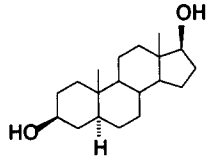

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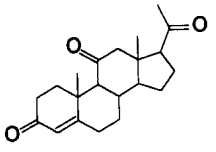

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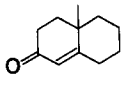

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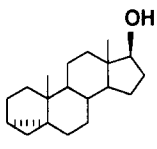

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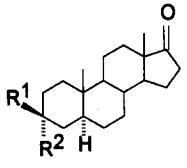
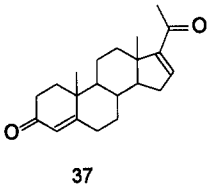


33. $R^1 = OH, R^2 = H$
34. $R^1 = H, R^2 = OH$
35. $R^1 = OH, R^2 = {}^2H$



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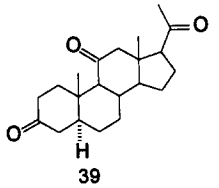

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standpoint; and (b) facilitating a role for the 5α-reductase of *Penicillium decumbens* in future mechanistic studies of the 5α-reductase-catalyzed reaction.

Experimental

Apparatus, materials, and methods

Melting points were determined on a Kofler heating stage. Infrared spectra were recorded with an Analect 6260FX spectrometer. The NMR spectra were recorded at 200 MHz (^{1}H) or 50 MHz (^{13}C) with a Bruker AC200 spectrometer using $CDCl_3$ as solvent and $CHCl_3$ as internal standard. Mass spectra were obtained with a Kratos 1S instrument operating in EI mode. Thin-layer chromatography was performed on Merck silica gel 60F-254 and flash column chromatography used silica gel, 230-400 mesh, eluting with ether-benzene in a 10% stepwise gradient.

Maintenance and growth of P. decumbens ATCC 10436

This microorganism was maintained on potato dextrose agar slopes, grown at 24°C and stored at 4°C. For preparative biotransformations, 3 L of potato dextrose broth medium (Difco) was distributed over 15 1 L Erlenmeyer flasks, and the flasks stoppered with foam plugs and sterilized at 121°C for 20 min. Once cool, these flasks were inoculated with *P. decumbens*, and placed on a rotary shaker (New Brunswick Scientific, 180 rpm, 1″ orbit) at 24–26°C. After 24 h growth under these conditions, a solution of the substrate (1 g) in 95% ethanol (30 mL) was added (2 mL per flask), and growth allowed to continue under the same conditions for a further 96 h. The fungus was then separated from the growth medium by centrifugation (IEC chemical centrifuge) or by filtration (Buchner funnel). The fungus was extracted by blending with dichloromethane followed by filtration (3x), and extract dried (anhydrous $MgSO_4$) and evaporated. The growth medium was extracted with dichloromethane (continuous extraction, 72 h), and the extract dried and evaporated as above. The extracts were combined and chromatographed to give the products and isolated yields specified in the table. In all cases, the majority of the product (60–80%) was associated with the fungal extract. Omission of the medium extraction gave reduced yields, but facilitated chromatographic purification of the final product. Isolated products and recovered starting materials were identified by comparison (TLC, NMR, and MS) with authentic samples wherever possible, as specified ([a]) in Table 1. Hitherto unreported spectral data are presented below.

Preparation of substrates

The substrates used in this study were commercial samples, with the exception of the following, which were prepared as described and gave satisfactory analytical (TLC, MP) and spectral (NMR, IR, MS) data: 4-chlorotestosterone (**9**),[39] 4-hydroxytestosterone (**10**),[40] 4-methyltestosterone (**11**),[41] A-nortestosterone (**16**),[42] B-nortestosterone (**18**),[43] 6-methylene-testosterone (**25**),[35] 3-methyleneandrost-4-en-17β-ol (**27**),[44] 3,5-cyclo-5α-androstan-17β-ol (**29**),[45] 4a-methyl-3,4,4a,5,6,7,8-octahydro-2(3*H*)-naphthalenone (**40**).[46]

[3-^{2}H]Androst-4-ene-3β,17β-diol (**31**) was prepared as outlined below. Dry *t*-butanol (1.48 g) was added slowly to a stirred mixture of lithium aluminum deuteride (0.28 g) and dry THF (100 mL). When gas evolution had ceased, a solution of testosterone acetate (2 g) in dry THF (50 mL) was added, and the resulting mixture was heated under reflux for 12 h. The mixture was then cooled, water (150 mL) was added, and the bulk of the THF removed on a rotary evaporator. The residue was acidified to pH 3 and extracted with ether. The extract was then washed (5% HCl), dried and evaporated to give a residue which was crystallized from ethyl acetate/hexane to give 1.84 g [3-^{2}H]-androst-4-ene-3β,17β-diol, 17-acetate, identified by comparison with an authentic sample of unlabeled material. This acetate was dissolved in methanol (25 mL), and the solution treated with 10% methanolic potassium hydroxide (86 mL). The resulting mixture was stirred at room temperature for 45 min and then concentrated under reduced pressure without heating to a volume of 15 mL. This solution was poured onto water, and the product extracted with ethyl

Table 1 Biotransformations by *Penicillium decumbens*

Substrate	Products (% yield, bioconversion activity)
1 (androstenedione)	**5** (42, 5α-reductase)[a]
2 (testosterone)	**5** (37, 5α-reductase + 17-oxidoreductase)[a]
	6 (28, 5α-reductase)[a]
3 (testosterone acetate)	**7** (68, 5α-reductase)[a]
5 (androstandione)	none
6 (dihydrotestosterone)	**5** (70, 17-oxidoreductase)[a]
9 (4-chlorotestosterone)	**13** (15, 5α-reductase + 17-oxidoreductase)
10 (4-hydroxytestosterone)	none
11 (4-methyltestosterone)	**12** (23, 5α-reductase)
14 (19-nortestosterone)	**15** (45, 17-oxidoreductase)[a]
	32 (10, 5α-reductase)[a]
16 (A-nortestosterone)	**17** (80, 17-oxidoreductase)[a]
18 (B-nortestosterone)	none
20 (Δ^1-testosterone)	**19** (3, 17-oxidoreductase)[a]
22 ($\Delta^{5(10)}$-estrenedione)	**21** (22, 3-oxidoreductase)
23 (Δ^6-testosterone)	**24** (76, 17-oxidoreductase)[a]
25 (6-methylenetestosterone)	**26** (10, 5α-reductase + 17-oxidoreductase)
27 (3-methylenetestosterone)	none
28 (3-deoxytestosterone)	none
29 (3,5-cycloandrostanol)	none
30 (androstenediol)	**5** (36, 5α-reductase + 17-oxidoreductase + 3-oxidoreductase)[a]
	33 (30, 5α-reductase + 17-oxidoreductase)[a]
31 (3-d-androstenediol)	**5** (29, 5α-reductase + 17-oxidoreductase + 3-oxidoreductase)[a]
	33 + 35 (18, 5α-reductase + 17-oxidoreductase)[a]
33 (androstanolone)	**5** (10, 3-oxidoreductase)[a]
34 (epiandrostanolone)	none
36 (androstanediol)	**5** (10, 17-oxidoreductase + 3-oxidoreductase)[a]
	33 (60, 17-oxidoreductase)[a]
37 (16-dehydroprogesterone)	**4** (8, 16(17)-reductase)[a]
38 (11-ketoprogesterone)	**39** (40, 5α-reductase)[a]
40 (octalenone)	none

[a] Material identified by TLC, MP, and spectral comparison with an authentic sample.

acetate. The combined extract was washed, dried, and evaporated to give a residue which was crystallized from aqueous ethanol to give [3-^{2}H]-androst-4-ene-3β,17β-diol (**31**), identified by comparison with an authentic sample of unlabeled material. MS (m/z, %) 291(M^+, 20), 273(100), 258 (15), 240 (10), 220 (12), deuterium content >98%; ^{13}C NMR (DMSO-D_6, identical with unlabeled material except for δ 66.6 (weak t, C-3).

Analysis of products

Hitherto unreported spectral data are presented below.

4α-Methyl-5α-androstan-17β-ol-3-one (12). m.p. 204–206°C (lit.[47] m.p. 206–207°C); ^{1}H NMR δ 0.74 (3H, s, H-18), 0.83 (3H, s, H-19), 0.95 (3H, d, H-4), 3.61 (1H, t, H-17) ppm; ^{13}C NMR δ 11.1 (C-18), 11.5 (C-19), 12.7 (C-4 methyl), 21.0 (C-11), 23.4 (C-15), 25.5 (C-6), 30.6 (C-16), 31.4 (C-7), 35.0 (C-8), 35.9 (C-10), 36.7 (C-12), 38.0 (C-1), 39.2 (C-2), 42.9 (C-13), 45.0 (C-5), 50.9 (C-14), 53.6 (C-4), 54.2 (C-9), 81.6 (C-17), 212.0 (C-3) ppm; MS m/z (%) 304(100), 289(8), 271(12), 260(18), 245(62).

4α-Chloro-5α-androstan-3β-ol-17-one (13). m.p. 186–188°C (lit.[48] m.p. 189–191°C); ^{1}H NMR δ 0.78 (3H, s, H-18), 1.22 (3H, s, H-19), 3.20/3.28 (1H, d of t, H- 3, α), 3.64 (1H, t, H-17) ppm; ^{13}C NMR δ 13.0, 13.8 (C-18 and C-19), 20.4 (C-11), 21.8 (C-15), 24.4 (C-6), 28.6 (C-2), 30.4 (C-7), 31.6 (C-12), 34.8 (C-8), 35.8 (2C, C-1 and C-16), 38.0 (C-10), 47.7 (C-13), 51.4, 51.8 (C-5 and C-14), 54.5 (C-9), 71.9 (C-4), 77.6 (C-3) and 220.7 (C-17) ppm; MS m/z (%) 324/326 (35/12), 288 (50), 255 (12), 244 (18), 217 (30) relative to 55 (100); M^+ 324.1881/326.1857 ($C_{19}H_{29}{}^{35}ClO_2$ requires 324.1856, $C_{19}H_{29}{}^{37}ClO_2$ requires 326.1833).

Estr-5(10)-ene-3β,17β-diol (21). m.p. 128–131°C (lit.[49] m.p. 130–132°C); ^{1}H NMR δ 0.75 (3H, s, H-18), 3.68 (1H, t, H-17), 4.0–4.1 (1H, m, H-3α); ^{13}C NMR δ 11.3 (C-18), 22.9, 23.0 (C-11 and C-15), 25.2 (C-1), 26.6 (C-7), 30.0 (C-16), 30.7 (C-2), 31.2 (C-6), 37.1 (C-12), 39.1 (2C, C-4 and C-8), 43.5 (C-13), 46.4 (C-9), 50.0 (C-14), 66.0 (C-3), 82.0 (C-17), 124.8 (C-5) and 129.7 (C-10) ppm; MS m/z (%) 276 (93), 258 (89), 243 (14), 225 (12), 214 (28) relative to 91 (100).

6-Methylene-5α-androstane-3,17-dione (26). m.p. 141–143°C; ^{1}H NMR δ 0.85 (3H, s, H-18), 0.90 (3H, s, H-19), 4.45 and 4.83 (each 1H, s, 6-methylene H's) ppm; ^{13}C NMR δ 11.7 (C-18), 13.8 (C-19), 20.9 (C-11), 21.7 (C-15), 31.4 (C-12), 35.8 (C-16), 36.8 (C-8), 37.7 (C-7), 38.0 (C-2), 38.3 (C-10), 40.5 (2C, C-1 and C-4), 47.8 (C-13), 50.8 (C-9), 51.2 (C-14), 54.5 (C-5), 107.3 (C-6 methylene), 147.2 (C-6), 211.0 (C-3) and 219.5 (C-17) ppm; IR ν_{max} 1702, 1739 cm^{-1}; MS m/z (%) 300 (100), 285 (20), 267 (17), 256 (99), 243 (64); M^+ 300.2072 ($C_{20}H_{28}O_2$ requires 300.2089).

[3-^{2}H]-5α-androstan-3β-ol-17-one (35). Identical with authentic unlabeled material except for ^{1}H NMR δ 3.4–3.65 (0.3H, m, C-3αH); MS m/z (%) 291 (100), 290 (47), indicating d_1 70%, d_0 30%.

Results

The results of the biotransformations of the substrates used in this study are presented in the accompanying table. The use of potato dextrose broth medium (see Experimental), in contrast to the complex medium used by Miller and Hessler,[33] facilitated isolation of products by providing relatively clean extracts of both fermentation broth and fungal mycelia, resulting in higher isolated yields (e.g., 42% for **1** in comparison with the reported yield of 18%). Furthermore, since the bulk (typically >75%) of the product was found to be associated with the harvested fungal mycelia in all cases, extraction of the growth medium need not be carried out unless maximum recovery of product is essential.

Product structures were determined by spectral and chromatographic comparisons with authentic samples wherever possible (see Table 1). In other cases (products **12**, **13**, **21**, and **26**), structures were assigned on the basis of infrared (IR), mass spectroscopy (MS), and nuclear magnetic resonance (NMR) spectral analyses. Determination of substituent stereochemistry was facilitated by analysis of JMOD ^{13}C NMR data: In all cases, resonances were assigned by comparison with spectra of analogous steroids,[34,35] and are entirely consistent with the proposed structures.

Discussion

As noted by earlier workers,[33] reduction of androst-4-ene-3,17-dione (**1**) proceeded cleanly to give 5α-androstane-3,17-dione (**5**) as the only isolable product. In the case of testosterone (**2**), 5α-reduction was accompanied by some oxidation at C-17 to produce a mixture of dihydrotestosterone (**6**) and the diketone **5**. This C-17 oxidoreductase activity was circumvented by the use of testosterone acetate (**3**) as substrate, the 17-acetoxy group being unchanged following incubation with *P. decumbens*, when dihydrotestosterone acetate (**7**) was isolated in 68% yield.

The effect of substitution at C-4 was explored by use of substrates **9** to **11**. 4-Hydroxytestosterone (**10**) was recovered unchanged following incubation with *P. decumbens*, but 4-chlorotestosterone (**9**) was reduced in low yield to produce **13**, the result of 5α-reductase activity coupled with oxidoreductase activities in both C-17 and C-3. In contrast, 4-methyltestosterone (**11**) was cleanly reduced to 4α-methyl-5α-dihydrotestosterone, clearly reflecting a difference in substrate specificities of the C-3 and C-17 oxidoreductase enzymes. This difference was again apparent from the results of incubations of the nor-steroids **14**, **16**, **18**, and **22**. 19-Nortestosterone (**14**) gave the corresponding dione (**15**) as the major product, but some reduction of the Δ^4 bond, giving 19-nor-5α-dihydrotestosterone (**32**), was apparent. In contrast, although neither A-nortestosterone (**16**) nor B-nortestosterone (**18**) were substrates for 5α-reduction, the former was efficiently oxidized at C-17, but the latter was recovered unchanged from biotransformation. Estr-5(10)-ene-3,17-dione (**22**) gave the 3β alcohol **21** as the only detectable biotransformation product.

The effect of additional unsaturation was explored by biotransformations of Δ^1-androstenedione (**20**), Δ^6-testosterone (**23**) and 6-methylenetestosterone (**24**). Only the latter was reduced at C-4(5), the two former substrates being subject only to C-17 oxidoreductase activity. Conversion of 6-methylenetestosterone to the corresponding 5α-dihydro compound **26** by *P. decumbens* is particularly interesting as various 6-methylene steroids are known to be irreversible

inhibitors of mammalian 5α-reductases, with **26** being only weakly active in this respect.[20,36] The 5α-reductase activity of *P. decumbens* is clearly dependent on the presence of oxygen at C-3, evidenced by the failure of this organism to produce 5α steroids from substrates **27**, **28**, or **29**, the latter in particular being surprisingly unreactive in view of the presence of the labile 3,5-cyclopropyl unit in place of the double bond. The 5α-reductase activity also requires a steroidal substrate for expression, since dienone **40** was recovered unchanged from incubations with *P. decumbens*. In variance with this apparently high substrate selectivity on the part of the 5α-reductase system, the biotransformation of 16-dehydroprogesterone (**37**) by *P. decumbens* gave only progesterone (**4**), albeit in low isolated yield. This is in contrast 5α-reduction of both progesterone[33] and 11-ketoprogesterone (**38**) by *P. decumbens*, the latter giving the corresponding 5α-dihydro compound in 40% yield.

The presence of an active C-17 oxidoreductase enzyme in *P. decumbens* is apparent from several of the biotransformations described above. The observation that *P. decumbens* is able to convert androst-4-ene-3β,17β-diol (**30**) into both 5α-androstane-3,17-dione (**5**) and 5α-androstan-3β-ol-17-one (**33**) prompted a more thorough examination of the role of a C-3 oxidoreductase in biotransformations by this organism. The question of the role of such an enzyme in the 5α-reduction of **30** was addressed by the use of the C-3α-deuterium labeled substrate **31**. It is tempting to assume that reduction of the Δ^4 bond of **30** and **31** occurs following oxidation at C-3 to produce the normal substrate testosterone: in this event, product **33** should arise from subsequent reduction at C-3 of 5α-androstane-3,17-dione (**5**). However, incubation of the latter with *P. decumbens* did not lead to the formation of any C-3 reduced products and, furthermore, the C-3 redox balance in this series of substrates appears to lie in the opposite direction: the alcohol **33**, used a substrate, gave the C-3 ketone **5**. The failure of *P. decumbens* to oxidize the corresponding C-3α alcohol **34** suggests the C-3β-OH/C-3 carbonyl oxidoreductase stereospecificity.

The use of the C-3α labeled substrate **31** indicates that some reduction of the Δ^4 bond may occur directly at the C-3 alcohol level without the requirement of a C-3-keto intermediate. The C-3 reduced product so obtained, **35**, was shown by MS and NMR analysis to have retained 75% of the deuterium originally present at C-3α in the substrate. This result suggests either a reversible oxidoreductase [which returns the bulk (75%) of the original hydrogen isotope] being closely linked with Δ^4 reduction, or the existence of two concurrent reaction pathways, one of which proceeds via reversible C-3 oxidation (25%) and one which is independent of such an event (75%). ^{1}H NMR analysis of the product obtained from conversion of 4-[^{2}H]androst-4-ene-3β,17β-diol to 4-[^{2}H]androstan-3β-ol-17-one by *P. decumbens* confirms the usual *trans* addition of hydrogens to C-4β and C-5α,[29] suggesting an unexceptional progenesis for this product, and a role for a normal 5α-reductase in its formation.

The direct reduction of the C=C bond of an allylic alcohol during biotransformation is unusual, but not unique, having been reported previously for reduction of Δ^4-3β-ols in the cholestane series.[37] More common are reductions which occur following oxidation to the ketone, typified by the reduction of cinnamyl alcohol to 3-phenylpropanol by yeast,[38] but in these latter cases the reactions are characterized by loss of deuterium from the carbinol carbon, a situation at variance with the result reported herein.

It is apparent from the results of the biotransformations discussed above that: (a) although the 5α-reductase system of *P. decumbens* has a wide substrate specificity within the steroid series, maximum isolated yields are obtained from substrates such as testosterone acetate for which no other metabolic routes are open; (b) 17β-hydroxy steroids are frequently converted to the corresponding 17-ketosteroid by *P. decumbens*, a reaction which may accompany the 5α-reduction reaction in susceptible substrates; and (c) substrates which possess a 3β-hydroxy group are susceptible to oxidation by *P. decumbens*, but this process is apparently not a prerequisite for 5α-reduction.

The preparative value of biotransformation for the conversion of Δ^4-3-ketones to 5α-3-ketones is apparent from the efficient production of steroids such as 5α-dihydrotestosterone acetate (**7**) and 5α-pregnane-3,11,20-trione (**39**), and selective reduction of 6-methylenetestosterone to give the *exo*-methylene compound **26**, while the regioselectivity of alcohol oxidation (e.g., conversion of **36** to the 17-ketone **33**) suggests a role for this organism in the selective oxidation of polyfunctional steroidal alcohols. Now that the substrate specificity of the *P. decumbens* 5α-reductase has been defined, and its basic mode of action explored, the mechanistic ramifications of this work for the mammalian enzyme will be further developed once a purified, cell free enzyme system has been derived from this fungal source.

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