Microbial hydroxylation of steroids. Part 12. Hydroxylation of testosterone and related steroids by *Gnomonia fructicola* ATCC 11430

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The fungus *Gnomonia fructicola* ATCC 11430 hydroxylates testosterone at C-2β in 40–60% yield. The enzyme involved has been shown to be an inducible CO sensitive, cyanide insensitive oxygenase, with a requirement for the Δ^4-3-ketosteroid substrate functionality. The use of 2α- and 2β-deuterium labelled substrates has shown that hydrogen loss from C-2 during 2β hydroxylation is isotope dependent and non-stereospecific. This is interpreted in terms of a $\Delta^{2,4}$-dienolic enzyme-bound intermediate form of the substrate.

Key words: *Gnomonia fructicola*, hydroxylation, steroids, testosterone.

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Le champignon *Gnomonia fructicola* ATCC 11430 provoque une hydroxylation en position C-2β de la testostérone, avec un rendement de 40–60%. On a démontré que l'enzyme implique une oxygénase qui peut être induite, qui est sensible au CO et insensible au cyanure et dont le substrat doit contenir une fonctionnalité Δ^4-céto-3 stéroïde. Utilisant des substrats marqués au deutérium dans les positions 2α et 2β, on a pu démontrer que l'hydroxylation dépend de l'isotope et qu'elle n'est pas spécifique. On peut interpréter ce résultat, en suggèrant que le substrat est lié à l'enzyme par le biais d'une forme intermédiaire $\Delta^{2,4}$-diénolique.

Mots clés : *Gnomonia fructicola*, hydroxylation, stéroïdes, testostérone.

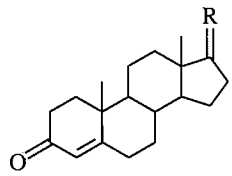
[Traduit par la revue]

The introduction of a hydroxyl functionality into a steroid molecule by fungal biotransformation, first discovered over 40 years ago (1), is still the most efficient route for the preparation of many hydroxysteroids, particularly in the corticosteroid family.

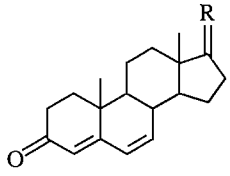
The enzymes responsible for this reaction were slow to yield their secrets, but their essential nature is now understood (2). Their characterization as cytochrome P-450 dependent monooxygenases preceded investigation of enzyme–substrate interactions: the latter have centred on the nature of enzyme–substrate binding through existing electronegative substituents such as substrate carbonyl or hydroxyl groups (2), and on the activation of ketosteroids towards electrophilic hydroxylation at positions adjacent or vinylogous to carbonyl by substrate enolization (3).

A report of the hydroxylation of testosterone (**1**) at C-2β by *Gnomonia fructicola* ATCC 11430 in unspecified yield (4), and the continued question of the intermediacy of a 2β-hydroxylation step in the conversion of testosterone to estradiol by the human aromatase enzyme (5, 6), suggesting a possible role for enzymic 2β hydroxylation of steroids in the control of human breast tumour cell proliferation (7, 8), prompted us to examine both mechanistic and synthetic parameters of the fungal hydroxylation of testosterone at C-2β by *G. fructicola*. In addition, hydroxylation of testosterone at C-2β by mammalian liver enzymes represents one of the major routes of testosterone metabolism in the liver (9, 10), suggesting a further role for the fungal biotransformation as a possible model for mammalian metabolism.

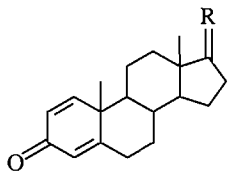
The original report of the steroidal 2β hydroxylating capacibility of *G. fructicola* was concerned largely with the use of Δ^4-3-ketopregnane substrates, reporting 2β hydroxylation of cortexone in 40% isolated yield (4, 11), with a more complex pattern of metabolism for progesterone, including hydroxylation at the C-2β, -6β, -14α, and -16β positions (11). To establish the best substrate for mechanistic purposes, we first investigated the fungal metabolism of the simple Δ^4-3-ketosteroids **1**–**4**, using a simplified procedure for biotransformation that replaced the original conditions (incubation in a complex growth medium (11)) with growth in a simpler medium followed by replacement culture incubation with resting cells in distilled water (for details, see experimental section). Under our conditions, testosterone was routinely converted to the 2β-hydroxy derivative **23** in isolated yields of 40–60%, accompanied by a trace of oxidation at C-17 to give the dione **4**. The results of this and other incubations are summarized in Table 1. Testosterone



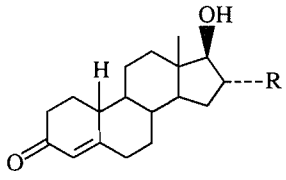
1 R = αH, βOH
2 R = αH, βOAc
3 R = O
4 R = αH, βCOCH$_3$



5 R = αH, βOH
6 R = O



7 R = αH, βOH
8 R = O



9 R = H
10 R = OH

TABLE 1. Transformations of steroids by *G. fructicola*

Substrate	Product (%)
1	**3** (1), **23** (40–60)
2	**3** (1), **23** (30), **31** (2)
3	**28** (4), **30** (5)
4	**32** (5), **33** (8), **34** (12), **35** (21)
5	**6** (16)
7	**8** (7)
9	**10** (6)
11	**36** (3), **37** (20), **38** (3)
12	**39** (7), **40** (19), **41** (17)
13	None
14	**13** (28)
15	None
16	None
17	**1** (2), **3** (1), **23** (17), **28** (2)
18	**19** (5)
18*	**19** (5), **42** (8)
20	**21** (3), **22** (10)

*Following enzyme induction by testosterone.

acetate is similarly transformed but in lower yield, presumably following enzymic hydrolysis of the ester function at C-17, but androst-4-ene-3,17-dione (**3**) underwent oxidation predominantly in the D-ring, yielding the 17β-hydroxy-16-ketone **30**. This compound may be formed by the rearrangement of an intermediate but not isolated 16-hydroxy-17-ketosteroid, a common phenomenon during fungal biotransformation (12). The biotransformation of progesterone gave, as originally reported (11), a mixture of products, from which the 2β and 14α alcohols and 2β,6β and 6β,14α diols were identified.

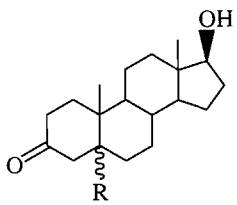
In view of the experiments outlined above, further work concentrated on steroids of the androstane skeleton, which carried the 17β-alcohol functionality. Substrate specificity was investigaed by using a series of substrates (**5**, **7**, **9**, and **11**–**17**); in only one instance (**11**) was 2β hydroxylation observed, and then in minor amount together with other reactions. Alternative modes of biotransformation that were noted included alcohol oxidation (for **5**, **7**, **12**, and **17**), carbonyl reduction (for **11**, **12**, and **14**), 16α hydroxylation (for **9** and **11**), and 5β hydroxylation of **12**.

Product structure determination relied heavily on the interpretation of ^{13}C nmr data (see Experimental), the following shifts being particularly characteristic: for 16α hydroxylation (products **10**, **37**, and **38**) the regiochemistry of hydroxylation is given by downfield shifts of signals assigned to C-17 and C-15 compared with their position in the corresponding 16-unsubstituted steroids (13), and stereochemistry is confirmed by a γ upfield shift at C-14 of 2.8–3.3 ppm; for 5β hydroxylation (products **40** and **41**), regiochemistry is reflected in downfield shifts at C-4 and C-6, together with a γ upfield shift at C-1, and stereochemistry by the latter and by the characteristic C-9 signal of 5β steroids (13); for 2β hydroxylation (products **23**, **28**, **34**, and **38**), regiochemistry is given by upfield shifts at C-1, and characteristic shifts at C-4, -5, -9, and -10, and stereochemistry by a downfield shift of the C-19 signal by 3–5 ppm (13).

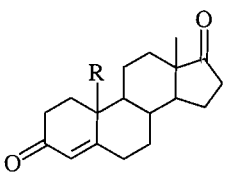
The α stereochemistry of reduction of the C-3 carbonyl group in 5α steroids (product **38**) is reflected in γ upfield shifts for C-1 and C-5, while the β stereochemistry of reduction (products **36** and **37**) is apparent from direct comparison with literature values reported for C-1–C-5, C-10, and C-19 for 3β-hydroxy-5α-androstanes (13). The stereochemistry of reduction at C-3 in the 5β-androstane series, giving the 3α-hydroxy product **41**, is confirmed by the absence of a γ-*gauche* upfield shift on the C-1 resonance in the spectrum of **41** compared with that of **40**.

It is apparent from the above that the 2β-hydroxylase enzyme requires a Δ^4-3-keto steroid substrate with a C-19 methyl group for normal activity. It is noteworthy that neither 19-nortestosterone (**9**) nor the 19-functionalized steroids **13** and **14** are substrates for this enzyme. Complete removal of the C-3 oxygen function (compounds **15** and **16**) produces steroids that are not hydroxylated, while reduction at C-3 (compound **17**) is reversed by fungal oxidoreductases prior to hydroxylation (see Table 1).

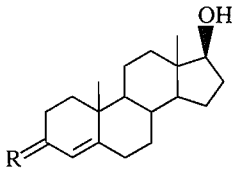
The occurrence of 16α hydroxylation (substrates **9**, **11**, and possibly **3**) may involve the 2β-hydroxylase enzyme binding the substrate in an inverted manner, a common phenomenon for fungal steroid hydroxylases (2), and other steroid metabolizing enzymes (14) as suggested in Fig. 1, but in the absence of



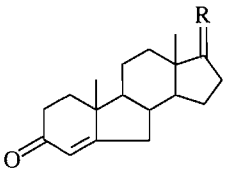
11 R = αH
12 R = βH



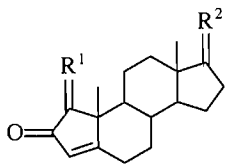
13 R = CH_2OH
14 R = CHO



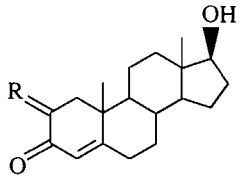
15 R = H_2
16 R = CH_2
17 R = αH, βOH



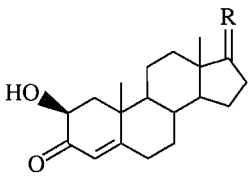
18 R = αH, βOH
19 R = O



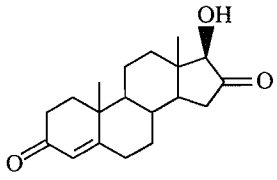
20 R^1 = H_2, R^2 = αH, βOH
21 R^1 = H_2, R^2 = O
22 R^1 = R^2 = αH, βOH



23 R = αH, βOH
24 R = αH, βD
25 R = αD, βH
26 R = αD, βOH
27 R = αH, β^{18}OH



28 R = O
29 R = αH, βOAc



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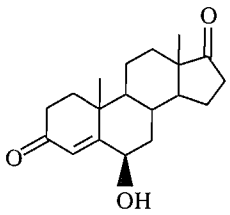

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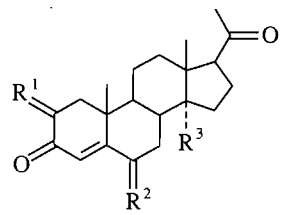
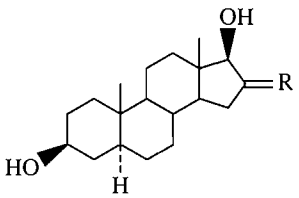
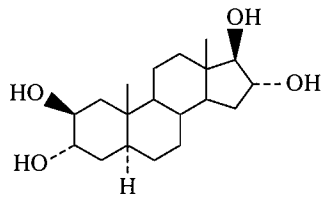


32 R^1 = αH, βOH, R^2 = H_2, R^3 = H
33 R^1 = R^2 = H_2, R^3 = OH
34 R^1 = R^2 = αH, βOH, R^3 = H
35 R^1 = H_2, R^2 = αH, βOH, R^3 = OH



36 R = H_2
37 R = αH, βOH



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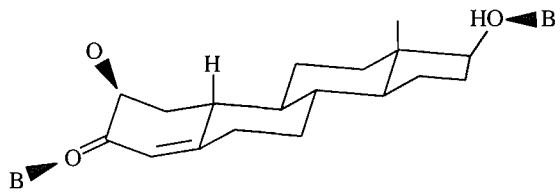
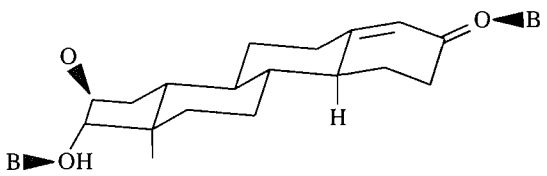


Normal binding

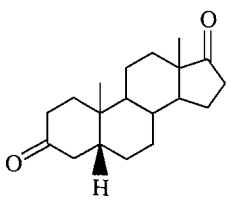


Inverted binding

B = binding site
O = oxidation site

FIG. 1. Possible method for 16α hydroxylation by a 2β-hydroxylase.

isolated enzymes this proposal must remain speculative. As previously noted (4), the 2β hydroxylase is able to tolerate an *A*-nor-$\Delta^{3(5)}$-2-one skeleton such as that of *A*-nortestosterone (**20**), giving a low yield of hydroxylation product **22**, analogous in structure to that observed for the normal Δ^4-3-keto skeleton. The *B*-norsteroid **18** was not hydroxylated under normal incubation conditions, the only biotransformation product (**19**) being the result of oxidation at C-17, but when testosterone was used as an enzyme inducer during the fungal growth period, as described in the experimental section, and **18** subsequently used as substrate, a low yield of the *B*-nor-$\Delta^{1,4}$-diene **42** was



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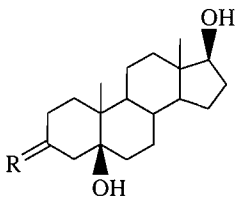
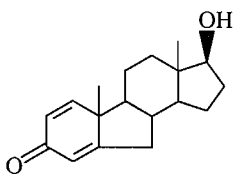


40 R = O
41 R = αH, βOH



42

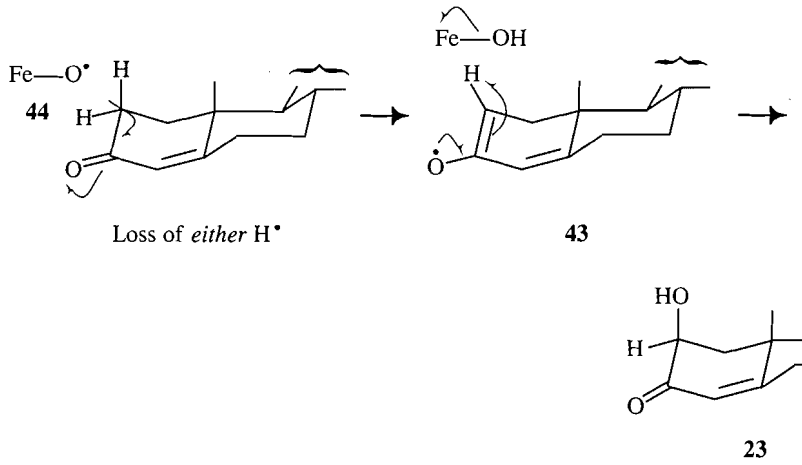
obtained. This may have arisen by acid-catalysed dehydration of a 2β-hydroxy intermediate in the growth medium (pH 4.6 at the end of the incubation), but in the absence of an isolated intermediate this proposal is speculative. The use of testosterone as an enzyme inducer was also investigated for incubations with substrates **7**, **9**, **11**, and **20**, but in each case the products were the same as those obtained from non-induced fungus.

We have addressed the question of enzymic activation of the substrate towards electrophilic oxidation via formation of the $\Delta^{2,4}$-dienol **43** (Scheme 1) by the use of testosterone substrates labelled with deuterium at C-2β and C-2α (**24** and **25**, respectively), and the results of these experiments are summarized in Table 2. A meaningful interpretation depends upon a fuller knowledge of the nature of the 2β-hydroxylase enzyme, and to this end the experiments summarized in Table 3 were performed.

The enzyme uses molecular oxygen as the source of hydroxyl, giving 2β-hydroxytestosterone-2-^{18}O (**27**, ^{18}O content 50%) when the reaction was performed under an $^{18}O_2$ enriched atmosphere (^{18}O content 62%); it is insensitive to the presence of cyanide but is inhibited by carbon monoxide at saturation concentration in water, and is substrate inducible, the reaction being completely suppressed by the presence of the protein synthesis inhibitor, cycloheximide, unless preinduction with testosterone was carried out. All of these properties are consistent with the 2β-hydroxylase enzyme being a cytochrome P-450 dependent mono-oxygenase (15*a*), although in the absence of an isolated, purified enzyme this cannot be definitively proven.

The active site species of cytochrome P-450 dependent mono-oxygenases has been formulated as the ironoxy radical complex **44** of Schemes 1 and 2, and the current consensus of opinion favours the radical abstraction/recombination mechanism for hydroxylation outlined in Scheme 2 (15*b*). The data presented in Table 2 can now be interpreted on the basis of this mechanistic outline.

The substrates whose hydroxylation is summarized in Table 2 were prepared as described elsewhere (16). The β-labelled isomer (62% d_1, 85% of label in the β position (16)) was hydroxylated to give a product that contained 39% d_1 species, the location of label at C-2α being apparent from ^{1}H nmr integration, and from ^{2}H nmr and ^{13}C nmr data. The α-labelled isomer (80% d_1, 87% of label in the α position (16)) was hydroxylated to give 2β-hydroxytestosterone with 75% d_1 in the 2α position. It is therefore apparent that, within error, label originally present at C-2α is maintained during the hydroxyla-



SCHEME 1. Possible route for 2β hydroxylation of testosterone.

TABLE 2. Transformations of deuterium labelled testosterones by *G. fructicola*

Substrate (% composition)*	2β-hydroxytestosterone (% composition)*	% ^{2}H retention
1 (38) + **24** (53) + **25** (9)	**23** (61), **26** (39)	63
1 (20) + **24** (10) + **25** (70)	**23** (25), **26** (75)	94

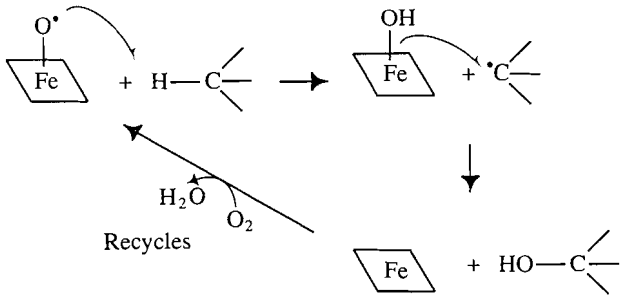
*±2.5%

TABLE 3. Transformations of testosterone by *G. fructicola*

Conditions of incubation	Product (%)
Normal	**23** (40–60)
Under $^{18}O_2$ atmosphere	**27** (58)
In 5 × 10^{-4} M KCN	**23** (50)
Under 15% CO atmosphere	0
In 3 × 10^{-4} M cycloheximide	0
In 3 × 10^{-4} M cycloheximide, cells preinduced with testosterone	**23** (10)

tion (94% retention). While this result alone would indicate hydroxylation with retention of configuration (the mode of cytochrome P-450 dependent hydroxylation most commonly observed (15*b*)), the data obtained from the β-labelled substrate, which retains 63% of the original label, show that this cannot be the case.

The analysis of the data of Table 2 depends on the relationship between stereoelectronic effects (SEE) (favouring axial (β) loss or addition of groups from C-2 (17)), and the intramolecular kinetic isotope effect (KIE) favouring loss of H over D, by a factor that may be as large as 11 in cytochrome P-450 hydroxylations (18). As outlined in Scheme 1, the loss of H from C-2 to the enzyme would be exclusively from the β face if the KIE and SEE are cooperative (for 2α-deuterium labelled substrate, **25**). However, these effects become counteractive for the C-2β labelled substrate, **24**, in which case the ratio of loss from the α and β sides is the result of an SEE/KIE competition. In both the cases reported in Table 2, recovered substrate shows no loss or epimerization of label, confirming that the phenomena reported are indeed a consequence of the hydroxylation process. The final stereochemistry of hydroxylation (conversion



SCHEME 2. Mechanism of C hydroxylation by cytochrome P-450 dependent mono-oxygenase enzymes.

of **43** to **23**) would be expected, proceeding under SEE control, to produce exclusive β product (17).

The magnitude of the SEE for hydrogen loss from C-2 during enolization of Δ^4-3-ketosteroids has been estimated at 4:1 (β:α) (17). The data of Table 2 suggest that, of the 39% d_1 species obtained from the β-labelled isomer, 30% was formed by epimerization of label from the β to the α position during the reaction (the remaining 9% being obtained from the 9% of **25** originally present). In this event, the ratio of β:α loss from C-2 of **24** can be estimated as (53–30):30 or 1:1.3, requiring an intramolecular KIE of ca. 1.3 × 4 or 5.2, well within the observed range of such effects (18, 19). The above analysis is approximate, ignoring as it does the role of intermolecular KIE's, but as these are usually close to unity in cytochrome P-450 dependent hydroxylation (19, 20), their contribution is generally small.

The phenomenon of non-stereospecific hydrogen loss followed by stereospecific hydroxylation is one that we have previously reported for enzymic hydroxylation (19, 20), and the present analysis is analogous to that given by us for the steroid 6β-hydroxylase enzyme of *Rhizopus arrhizus* (3, 12), where the stereochemistry of hydrogen loss is also isotope-dependent. This phenomenon may be a general one for hydroxylations at sites subject to stereoelectronic effects due to their proximity to carbonyl groups or other centres of π-electron density.

The data reported herein for the 2β-hydroxylase enzyme of *G. fructicola* therefore suggest that this enzyme is a cytochrome P-450 dependent mono-oxygenase, operating via a stepwise oxidation mechanism, the stereochemical details of which are subject to substrate stereoelectronic and kinetic isotope effects.

Experimental

Apparatus, material, and methods

Melting points were determined on a Kofler heating stage and are uncorrected. Infrared spectra were recorded with an Analect 6260FX spectrometer. Proton nmr spectra were recorded at 80 MHz with a Bruker WP80CW or at 200 MHz with an AC200 spectrometer, using CDCl, as solvent (except where stated) and TMS as internal standard. Carbon spectra were recorded at 50.3 MHz (JMOD mode) using the latter instrument with chloroform as solvent and internal standard. Deuterium spectra were recorded at 30.72 MHz (AC200) or at 76.8 MHz (Bruker AM500, McMaster University), using chloroform as solvent and natural abundance $CDCl_3$ as internal standard. Mass spectra were obtained with an AEI MS30/Kratos DS55 system. Isotopic abundances were determined from the molecular ion or M − H_2O spectral regions following standard correction procedures for natural ^{13}C abundances, and are accurate to ±2.5%. Thin-layer chromatography was performed on Merck silica gel 60F-254, and flash column chromatography on silica gel (230–400 mesh, Merck 9385).

Maintenance of microorganisms

Gnomonia fructicola ATCC 11430 was grown at 24–26°C on slopes of potato dextrose agar containing 0.5% strawberry petiole extract (21), then stored at 4°C and transferred at intervals not exceeding 8 weeks.

Preparation of substrates

With the exception of those listed below, the substrates used were commercial samples. Hitherto unreported spectral data are tabulated below for substrates not commercially available. 3-Deoxytestosterone (**15**): preparation, ref. 22; ^{13}C nmr, δ: 11.0 (C-18), 19.3 (C-2), 19.5 (C-19), 21.0 (C-11), 23.4 (C-15), 25.8 (C-6), 30.5 (C-16), 32.0, 32.5 (C-3, C-7) 36.0 (C-8), 36.8 (C-12), 37.1 (C-10), 37.9 (C-1), 42.7 (C-13), 50.9 (C-14), 54.6 (C-9), 81.7 (C-17), 117.2 (C-4), and 144.0 (C-5) ppm. 3-Methyleneandrost-4-en-17β-ol (**16**): preparation, ref. 23; ^{13}C nmr, δ: 11.1 (C-18), 18.6 (C-19), 21.1 (C-11), 23.5 (C-15), 27.3 (C-2), 30.6 (C-16), 32.4 (2C, C-6, C-7), 36.1 (C-8), 36.8 (C-12), 37.4 (C-1), 37.7 (C-10), 43.0 (C-13), 50.9 (C-14), 54.4 (C-9), 82.0 (C-17), 108.0 (=CH_2), 122.8 (C-4), 144.0 (C-3), and 149.0 (C-5) ppm. Androst-4-ene-3β,17β-diol (**17**): preparation, ref. 24; ^{13}C nmr, δ: 11.0 (C-18), 19.0 (C-19), 20.6 (C-11), 23.4 (C-15), 29.5 (C-2), 30.5 (C-16), 32.1, 32.6 (C-6, C-7), 35.4 (C-1), 36.0 (C-8), 36.6 (C-12), 37.4 (C-10), 42.9 (C-13), 50.8 (C-14), 54.6 (C-9), 67.9 (C-3), 81.8 (C-17), 123.5 (C-4), and 147.4 (C-5) ppm. *B*-Norandrost-4-en-17β-ol-3-one (**18**): preparation, ref. 25. *A*-Norandrost-4-en-17β-ol-3-one (**20**): preparation, ref. 26. Androst-4-en-17β-ol-3-one-2β-d_1 (**24**): preparation, ref. 16. Androst-4-en-17β-ol-3-one-2α-d_1 (**25**): preparation, ref. 16.

Fungal biotransformations

General procedure

G. fructicola was grown in a medium of potato dextrose broth (200 mL) in a 1-L Erlenmeyer flask, maintained on a rotary shaker (180 rpm) at 24–26°C. After a growth period of 5 days, the fungus was collected by centrifugation and resuspended in distilled water (200 mL) in a 1-L Erlenmeyer flask. In a typical procedure, 15 such flasks were used to transform 1 g of substrate, added at the rate of 70 mg in 2 mL 95% ethanol per flask. Incubations with substrate were then performed under conditions identical with those described above (24–26°C, 180 rpm), for a 5-day period, following which the fungus was removed by filtration and products isolated by continuous extraction of the aqueous medium (methylene chloride), and purified by column chromatography. Extraction of the fungal biomass led to the recovery of starting material in most cases. This latter process was not routinely performed and was not quantified.

Controlled atmosphere and inhibitor experiments

The experiments summarized in Table 3 were performed in closed 1-L vessels ($^{18}O_2$ and 15% CO atmospheres), or by replacement of distilled water in the above procedure by the appropriate inhibitor solution. Preinduction of enzymes by testosterone was carried out by the addition of testosterone (4 mg in 1 mL 95% ethanol per flask) to the growing fungus 4 days after inoculation, followed by a further 24-h growth period. Subsequent procedures were carried out as described above. In all cases, yields reported in Table 3 refer to isolated, purified products.

Substrates

Isolated yields of products are listed in Tables 1 and 2. The following paragraphs contain (in order) substrate; recovered substrate from aqueous medium (%); products (%), in order of elution from column chromatography, together with hitherto unpublished spectral and (or) analytical data.

Androst-4-en-17β-ol-3-one (testosterone, **1**), 17; androst-4-ene-3,17-dione (**3**), 1, identified by comparison with authentic material; androst-4-ene-2β,17β-diol-3-one (**23**), 40–60; ^{13}C nmr, δ: 11.3 (C-18), 22.5 (C-11), 22.8 (C-19), 23.3 (C-15), 30.4 (C-16), 33.0 (C-7), 34.5 (C-6), 35.8 (C-8), 36.5 (C-12), 39.5 (C-1), 41.4 (C-10), 43.4 (C-13), 50.3, 50.5 (C-9, C-14), 68.6 (C-2), 81.4 (C-17), 118.8 (C-4), 175.2 (C-5), 199.9 (C-3) ppm, identified by comparison with authentic material (27).

Androst-4-en-17β-ol-3-one, 2β-d_1 (**24**), 9; ^2H nmr, δ: 2.40 (C-2β^2H) ppm; androst-4-ene-2β,17β-diol-3-one, 2α-d_1 (**23** + **26**), total 25, ^1H nmr included signals at δ: 3.63 (1H, t, C-17αH), 4.15 (0.61 H, dd, C-2αH), and 5.78 (1H, s, C-4H) ppm; ^2H nmr δ: 4.16 (C-2α^2H) ppm.

Androst-4-en-17β-ol-3-one, 2α-d_1 (**25**), 9; ^2H nmr, δ: 2.32 (C-2α^2H) ppm; androst-4-ene-2β,17β-diol-3-one, 2α-d_1, (**23** + **26**), total 20; ^1H nmr included signals at δ: 3.63 (1H, t, C-17αH), 4.15 (0.25 H, dd, C-2αH), and 5.79 (1H, s, C-4H) ppm; ^2H nmr δ: 4.16 (C-2α^2H) ppm.

Androst-4-en-17β-ol-3-one, 17 acetate (testosterone acetate, **2**), 0; androst-4-ene-3,17-dione, 1; androst-4-ene-2β,17β-diol-3-one (**23**), 30; androst-4-en-6β-ol-3,17-dione (**31**), 2, identified by comparison with authentic material (20).

Androst-4-ene-3,17-dione (**3**), 5; androst-4-en-2β-ol-3,17-dione (**28**), 4, identified by comparison with an authentic sample (27); androst-4-ene-17β-ol-3,16-dione (**30**), 5; ^1H nmr included signals at δ: 0.78 (3H, s, C-18H), 1.2 (3H, s, C-19H), 3.75 (1H, s, C-17αH) and 5.7 (1H, s, C-4H) ppm, identified by spectral, tlc, and mp comparisons with data from authentic material (28).

Pregn-4-ene-3,20-dione (progesterone, **4**), 10; pregn-4-en-2β-ol-3,20-dione (**32**), 5, identified by comparison of spectral and physical data with those reported (11); pregn-4-en-14α-ol-3,20-dione (**33**), 8, identified by comparison with reported spectral data (29, 30); pregn-4-ene-2β,6β-diol-3,20-dione (**34**), 12; ^1H nmr including δ: 0.68 (3H, s, C-18H), 1.48 (3H, s, C-19H), 2.1 (3H, s, C-21H), 4.2–4.4 (2H, m, C-2 and -6 H's), and 5.82 ppm, obtained in admixture with pregn-4-ene-6β,14α-diol-2,20-dione (**35**), 21. The latter compound was purified by repeated chromatography and had physical and spectral data identical with those reported (29, 30).

Androsta-4,6-dien-17β-ol-3-one (**5**), 25; androsta-4,6-diene-3,17-dione (**6**), 16, identical with an authentic commercial sample.

Androsta-1,4-dien-17β-ol-3-one (**7**), 15; androsta-1,4-diene-3,17-dione (**8**), 7, identical with an authentic samples prepared by Jones' oxidation of **7**.

19-Norandrost-4-en-17β-ol-3-one (19-nortestosterone, **9**), 12; 19-norandrost-4-ene-16α,17β-diol-3-one (**10**), 6, mp 172–174°C (lit. (31) mp 185–186°C); ^1H nmr included peaks at δ: 0.78 (3H, s, C-18H), 3.49 (1H, t, C-16βH), 4.10 (1H, br s, C-17αH), and 5.81 (1H, s, C-4H) ppm; ^{13}C nmr δ: 12.2 (C-18), 25.7 (C-11), 26.5 (C-1), 30.6 (C-7), 33.7 (C-15), 35.4 (C-6), 36.4 (2C, C-2 and C-12), 40.0 (C-8), 42.6 (C-10), 43.6 (C-13), 47.6 (C-14), 49.5 (C-9), 78.4 (C-16), 89.7 (C-17), 124.7 (C-4), 166.5 (C-5), and 200.0 (C-3) ppm; ms *m/z* (%): 290 ($M^{+\cdot}$, 90), 272 (100), 257 (25), 254 (35).

5α-Androstan-17β-ol-3-one (**11**), 12; 5α-androstane-3β,17β-diol (**36**), 3, analytical and spectral data identical with those reported (13); 5α-androstane-3β,17β,16α-triol (**37**), 20, mp 238–240°C (lit. (32) mp 256–260°C corr.); ^1H nmr included signals at δ: 0.66 (3H, s, C-18H), 0.73 (3H, s, C-19H), 3.26 (d, J = 5.6 Hz, C-17αH), 3.30–3.45 (1H,

m, C-3αH), and 3.88 (1H, d of t, J = 2.1, 5.6 Hz, C-16βH) ppm; ^{13}C nmr δ: 11.5, 11.7 (C-18, C-19), 19.6 (C-11), 27.8 (C-6), 30.8, 30.9 (C-2, C-7), 33.1 (C-15), 34.3 (C-8), 34.8 (C-10), 36.2 (2C, C-1 and C-12), 37.5 (C-4), 42.7 (C-13), 44.1 (C-5), 48.0 (C-14), 53.8 (C-9), 69.6 (C-3), 77.3 (C-16), and 88.9 (C-17) ppm; converted to the triacetate, mp 172–175°C (lit. (32) mp 174–176°C); ^{1}H nmr included signals at δ: 0.75, 0.77 (each 3H, s, C-18H and C-19H), 2.0, 2.01, 2.04 (each 3H, s, $COCH_3$), 4.5–4.75 (1H, m, C-3αH), 4.89 (1H, d, J = 5.6 Hz, C-16βH) ppm; ^{13}C nmr δ: 12.2 (C-19), 13.0 (C-18), 20.4 (C-11), 21.0, 21.1, 21.4 (acetyl CH_3) 27.5 (C-2), 28.4 (C-6), 31.4 (C-7), 32.3 (C-15), 34.0 (C-4), 34.9 (C-8), 35.6 (C-10), 36.7, 36.8 (C-1, C-12), 43.6 (C-13), 44.7 (C-5), 49.1 (C-14), 54.1 (C-9), 73.6 (C-3), 78.1 (C-16), 86.3 (C-17), 170.6, 170.6, 170.7 (acetyl CO) ppm; 5α-androstane-2β,3α,16α,17β-tetrol (**38**), 3, mp 247–250°C; ^{1}H nmr included signals at δ: 0.63 (3H, s, C-18H), 0.88 (3H, s, C-19H), 3.28 (1H, d, J = 5.4 Hz, C-17αH), 3.35–3.7 (2H, complex m, C-2α and C-3β H's), and 3.90 (1H, d of t, J = 2, 5.4 Hz, C-16βH) ppm; ^{13}C nmr (DMSO-d_6) δ: 11.9 (C-18), 13.7 (C-19), 19.4 (C-11), 27.6 (C-6), 31.1 (C-4), 30.9 (C-7), 33.1 (C-15), 33.9 (C-8), 35.2 (C-10), 36.4 (C-12), 38.3 (C-1), 43.0 (C-13), 48.2 (C-14), 54.8 (C-9), 69.5, 70.6 (C-2, C-3), 77.2 (C-16), and 89.2 (C-17) ppm; the signal from C-5 was obscured by solvent peaks; ms, m/z (%): 324 ($M^{+\cdot}$), (25), 306 (80), 291 (40), 288 (45), 273 (40), 262 (43), 249 (75), 231 (100). **38** converted to the tetraacetate, mp 82–84°C from methanol; ^{1}H nmr included signals at δ: 0.76 (3H, s, C-18H), 0.87 (3H, s, C-19), 2.00, 2.03, 2.035, 2.04 (total 12H, each s, $COCH_3$), 4.87 (2H, br s, C-17α and C-2α or C-3β H), 4.75–5.0 (1H, m, C-2α or C-3β H), and 5.10 (1H, d of t, J = 2, 5.5 Hz, C-16β H) ppm; ^{13}C nmr δ: 13.0 (C-18), 13.7 (C-19), 20.0 (C-11), 21.1, 21.2, 21.3 (2C) (acetyl CH_3), 27.7 (C-6), 29.3 (C-4), 31.2 (C-7), 32.3 (C-15), 34.4 (C-8), 35.1 (C-10), 36.7 (C-12), 37.7 (C-1), 39.6 (C-5), 43.6 (C-13), 49.0 (C-14), 54.7 (C-9), 69.8, 70.2 (C-2, C-3), 78.1 (C-16), 86.2 (C-17), 169.6, 169.8, 170.7, 170.9 (acetyl CO) ppm.

5β-Androstan-17β-ol-3-one (**12**), 7; 5β-androstane-5,17β-diol-3-one (**40**), 19, mp 154–165°C (dec.); ^{1}H nmr included signals at δ: 0.75 (3H, s, C-18H), 1.0 (3H, s, C-19H), 3.0 (1H, half of ABq, J = 15 Hz, C-4H), and 3.65 (1H, t, J = 8 Hz, C-17α H) ppm; ^{13}C nmr δ: 11.1 (C-18), 16.2 (C-19), 21.4 (C-11), 23.4 (C-15), 28.7 (C-17), 30.6 (C-16), 31.2 (C-1), 35.0 (C-8), 36.6 (C-12), 37.0, 37.3 (C-2, C-6), 40.4 (C-10), 43.1 (C-13), 44.1 (C-9), 49.4 (C-4), 51.3 (C-14), 78.6 (C-5), 81.7 (C-17), and 210.8 (C-3) ppm; ms, m/z (%): 288 ($M - H_2O$, 10), 274 (10), 246 (10), 236 (18) relative to 71 (100); converted to the 17-monoacetate, mp 174–176°C (lit. (33) mp 176–178°C); ^{1}H nmr included signals at δ: 0.78 (3H, s, C-18H), 0.98 (3H, s, C-19H), 2.02 (3H, s, $COCH_3$), 3.0 (1H, half of ABq, J = 15 Hz, C-4H), and 4.57 (1H, t, J = 8 Hz, C-17αH) ppm; ^{13}C nmr δ: 12.1 (C-18), 16.2 (C-19), 21.1 (acetyl CH_3), 21.3 (C-11), 23.6 (C-15), 27.6 (C-16), 28.7 (C-7), 31.2 (C-1), 34.7 (C-8), 36.3, 36.9 (C-6, C-12), 37.3 (C-2), 40.8 (C-10), 42.7 (C-13), 44.0 (C-9), 49.5 (C-4), 51.0 (C-14), 78.5 (C-5), 82.6 (C-17), 170.8 (acetyl CO), and 210.6 (C-3) ppm; 5β-androstane-3α,5,17β-triol (**41**), 17; mp 218–221°C (lit. (34) mp 220–223°C); ^{1}H nmr included signals at δ: 0.73 (3H, s, C-18H), 0.90 (3H, s, C-19H), 3.4–3.7 (1H, m, C-3β H), and 3.64 (1H, t, J = 8 Hz, C-17α H) ppm; ^{13}C nmr δ: 11.1 (C-18), 16.4 (C-19), 20.8 (C-11), 23.4 (C-15), 28.2 (C-7), 29.8, 30.1 (2C) (C-1, C-2, C-16), 35.1 (C-8), 36.7, 36.9 (C-6, C-12), 39.8 (C-10), 42.0 (C-9), 43.0 (C-13), 43.5 (C-4), 51.2 (C-14), 68.1 (C-3), 75.7 (C-5), and 81.8 (C-17) ppm; ms, m/z (%): 308 (2), 290 (26), 275 (10), 273 (10), 272 (30), 257 (15), 236 (100).

Androst-4-en-19-ol-3,17-dione (**13**) was recovered quantatively and unchanged from incubation with *G. fructicola*.

Androst-4-en-19-al-3,17-dione (**14**) was converted to the alcohol **13** in 85% isolated yield by *G. fructicola*.

Androst-4-en-17β-ol (**15**) and 3-methyleneandrost-4-en-17β-*ol* (**16**) were recovered unchanged in yields of 28 and 36% respectively from the aqueous medium following incubation with *G. fructicola*. Extraction of the fungal biomass yielded the balance of the substrates.

Androst-4-ene-3β,17β-diol (**17**), 0; androst-4-en-17β-ol-3-one (**1**), 2; androst-4-ene-3,17-dione (**3**), 1; androst-4-ene-2β,17β-diol-3-one (**23**), 17; and androst-4-en-2β-ol-3,17-dione (**28**), 2; all of which were identified by comparison with authentic samples (see above).

B-*Norandrost-4-en-17β-ol-3-one* (**18**), 24; *B*-norandrost-4-ene-3,17-dione (**19**), 5; spectral data identical with those reported (25).

B-*Norandrost-4-en-17β-ol-3-one* (**18**), with induced enzymes, 45; *B*-norandrost-4-ene-3,17-dione (**19**), 5, identified as reported above; *B*-norandrosta-1,4-dien-17β-ol-3-one (**42**), 8, mp 156–159°C (lit. (35) mp 157–159°C); ^{1}H nmr included signals at δ: 0.80 (3H, s, C-18H), 1.10 (3H, s, C-19H), 3.68 (1H, t, J = 8 Hz, C-17αH), 6.0 (1H, s, C-4H), 6.15 and 7.02 (2H, ABq, C-2 and C-1 H's) ppm; ^{13}C nmr δ: 11.5 (C-18), 20.5 (C-19), 20.8 (C-11), 23.9 (C-15), 30.6 (C-16), 32.7 (C-6), 36.4 (C-12), 38.5 (C-8), 44.9 (C-13), 48.2 (C-10), 50.6 (C-14), 53.4 (C-9), 81.3 (C-17), 123.7 (C-4), 128.4 (C-2), 152.6 (C-1), 174.1 (C-5), and 186.7 (C-3) ppm.

A-*Norandrost-3(5)-en-17β-ol-2-one* (*A*-nortestosterone, (**20**), 60; *A*-norandrost-3(5)-ene-2,17-dione (**21**), 3, mp 163–166°C (lit. (36) mp 166–166.5°C), identified by comparison with an authentic sample produced by Jones' oxidation of *A*-nortestosterone; *A*-norandrost-3(5)-ene-1β,17β-diol-2-one (**22**), 10, mp 236–238°C (lit. (37) mp 240–243°C); ^{1}H nmr included peaks at δ: 0.80 (3H, s, C-18H), 1.10 (3H, s, C-19H), 3.62 (1H, t, J = 8 Hz, C-17α H), 3.75 (1H, s, C-1H), and 5.80 (1H, s, C-4H) ppm; ^{13}C nmr δ: 11.1 (C-18), 15.5 (C-19), 23.0 (C-11), 23.3 (C-15), 28.1 (C-6), 30.0 (C-16), 31.5 (C-7), 35.7 (C-8), 36.4 (C-12), 43.4 (C-13), 50.0 (C-10), 50.4 (C-14), 53.7 (C-9), 81.6 (C-17), 83.0 (C-1), 122.1 (C-3), 186.3 (C-5), and 207.4 (C-2) ppm; ms, m/z (%): 290 (78), 275 (52), 259 (20), relative to 57 (100).

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