The Mechanism of the Microbial Hydroxylation of Steroids. Part 1. The C-21 Hydroxylation of Progesterone by *Aspergillus niger* ATCC 9142

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The mechanism of the C-21 hydroxylation of progesterone (**1***a*) by *Aspergillus niger* ATCC 9142 to give 11-deoxycorticosterone (**1***b*) has been studied by the use of progesterone derivatives and of C-21 deuterium labelled progesterones. The requirement of the C-21 hydroxylase system for a C-20 carbonyl group is demonstrated and the possiblity of the involvement of this group, in the C-20,21 enol form, in the C-21 hydroxylation reaction has been discussed. However, on the basis of the observed deuterium isotope effect ($K_H/K_D = 1.25$), a mechanism for the microbial C-21 hydroxylation reaction is proposed in which the rate-determining step is the direct insertion of oxygen into a C-21 carbon–hydrogen bond and not one involving enolization of the C-20 carbonyl.

In addition, C-11α and C-15β hydroxylation of both 20α- and 20β-hydroxypregn-4-ene-3-one (**2***a* and **2***b*) by *A. niger* has been observed.

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On a étudié le mécanisme de l'hydroxylation (en position C-21) de la progestérone (**1***a*) par l'*Aspergillus niger* ATCC 9142 conduisant à la déoxy-11 corticostérone (**1***b*); cette étude a été effectuée à l'aide de dérivés de la progestérone et de progestérone marquée au deutérium en position C-21. On a démontré la nécessité d'un groupe carbonyle en C-20 pour rendre efficace le système d'hydroxylase en C-21; on discute aussi de la possibilité de l'implication du groupement carbonyle en position 20 sous forme d'énol C-20,21 lors de la réaction d'hydroxylation en C-21. Toutefois, sur la base de l'observation d'un effet isotopique ($K_H/K_D = 1.25$), on propose un mécanisme pour la réaction d'hydroxylation microbiologique en C-21 dans lequel l'étape déterminante est l'insertion directe de l'oxygène dans le lien carbone–hydrogène en C-21 et on suggère que cette réaction n'implique pas une énolisation du groupement carbonyle en position C-20.

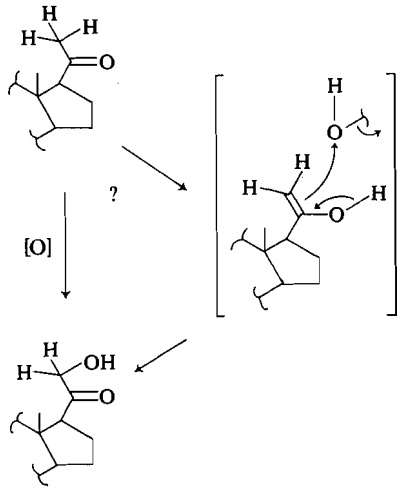
De plus l'*Aspergillus niger* provoque l'hydroxylation en C-11α et en C-15β des hydroxy-20α et -20β pregène-4 one-3 (**2***a* et **2***b*). [Traduit par le journal]

In spite of the wide application of microbial steroid hydroxylation in both industry and research (1) and a thorough investigation of related biochemical processes (2), comparatively little is known about the chemistry of this reaction.

The proposal that microbial hydroxylation at saturated carbon proceeds by direct electrophilic insertion of oxygen into a C—H bond followed from the discovery of a relationship between microbial hydroxylation of steroids at C_n and microbial epoxidation of steroids unsaturated at C_n, demonstrating the electrophilic nature of microbial hydroxylation (3, 4) and the observation that microbial hydroxylation at saturated carbon proceeds with retention of configuration (5–7).

The question of enzymic substrate activation has received little consideration. For hydroxylation at saturated carbon, chemical analogy suggests that activation is unnecessary: oxidation of steroids at saturated carbon has been achieved under electrophilic conditions (8, 9). However, the problem of enzymic substrate activation during hydroxylation adjacent or vinylogous to carbonyl functions is potentially more complex. The substrates most commonly used (1) for microbial hydroxylations have been Δ^4-3-ketosteroids and it has been proposed (1) that, during their hydroxylation at C-2, -6, -17, or -21, activation occurs by enolization and that hydroxylation occurs by electrophilic attack of oxygen at the enol. This is shown for hydroxylation at C-21 of a C-20 ketosteroid in Scheme 1.

Precedent for such a mechanism is provided by



SCHEME 1. Alternative pathways for the C-21 hydroxylation of a C-20 ketosteroid.

the facile oxidation by electrophilic oxygen of steroidal enols to α-hydroperoxy or α-hydroxy ketones (10–14) and by enzymic enolization of ketosteroids (15). Further evidence, albeit circumstantial, is provided by the frequent occurrence of hydroxylations of Δ^4-3,20-diketosteroids at the axial 2β, 6β, 10β (in 19-norsteroids), and 17α positions, whereas microbial hydroxylations at 2α, 6α, 10α, and 17β are unknown (1): analogously, electrophilic attack on the corresponding enols gives exclusively axial substitution of the electrophile (11–13, 16).

To investigate activation by enzymic enolization, the C-21 hydroxylation of progesterone (pregn-4-ene-3,20-dione) (**1***a*) by *Aspergillus niger* ATCC 9142 to give 11-deoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione) (**1***b*) has been examined. The absence of complicating factors such as rigid stereochemistry at C-21 and non-enzymic enolization of the C-20 carbonyl to C-21 (which does not occur under conditions of thermodynamic control (17, 18)) makes this system suitable for the present study.

Previous work (19) established that progesterone, 6β-, 11α-, 11β-, and 14α-hydroxyprogesterone, 11-ketoprogesterone, and 19-norprogesterone were hydroxylated at C-21 by *A. niger* but did not report the effect of variation of functionality at C-20: accordingly, 20α- and 20β-hydroxypregn-4-ene-3-one (**2***a* and **2***b*, respectively) were incubated with *A. niger*. In neither case was C-21 hydroxylation observed but C-15β hydroxylated (**2***c* and **2***e*) and C-11α,15β dihydroxylated (**2***d* and **2***f*) products were formed in minor amount. Their structures follow from the p.m.r. data presented in Table 1 and the Experimental section. Thus C-15β hydroxylation is characterized by a downfield shift of the signal of the C-18 methyl group, with respect to the unsubstituted (at C-15) steroid of *ca.* δ 0.3 p.p.m., and hydroxylation at C-11α produces a similar perturbation of the signal of the C-19 methyl group (20). The p.m.r. spectra of **2***c*–**2***f* accord with this and show signals due to methine protons geminal to hydroxyl of the expected multiplicity for, and in the region characteristic of, the proposed structures (20). The location of hydroxyl in **2***c*–**2***f* was confirmed by oxidation to the corresponding known ketones.

Removal of oxygen from C-20 led to a loss of hydroxylase activity in *A. niger*. Incubation of pregn-4-ene-3-one (**3***a*), the 20-methylene derivative **3***b*, and the 20-thiosteroid **3***c* produced no conversion products. The failure of *A. niger* to metabolize **3***c* is of interest in view of the facile thione–enethiol interconversion (21) and the metabolism of sulfur compounds by other strains of *A. niger* (22).

A C-20 carbonyl group is therefore essential for C-21 hydroxylation and activation of C-21 by enolization of this group has been investigated by the use of progesterone labelled with deuterium at C-21. The results are summarized in Table 2.

Progesterone fully substituted with deuterium at the enolizable carbons (**4***a*) gave, upon incubation with *A. niger*, 11-deoxycorticosterone (**5***a*) with loss of only one deuterium atom per molecule. This follows from mass spectral data (Table 2) and from the p.m.r. spectrum of **5***a*, which showed no absorption at δ 4.18 (*cf.* the spectrum of **1***a*, Table 1), and demonstrates that, if enolization is occurring during hydroxylation, it cannot be reversible with exchange of protium with the medium. It also establishes that no loss of label occurs from C-2, -4, -6, or -17 during hydroxylation. In this and subsequent incubations of labelled progesterones, starting material was recovered which showed no loss or scrambling of label.

Progesterone with one deuterium atom at C-21 (**4***b*) was prepared by hydrogenolysis of 21-iodoprogesterone with zinc and monodeuterioacetic acid, and the location of label confirmed by spectral analysis. Thus the p.m.r.

TABLE 1. Proton magnetic resonance data in deuteriochloroform solution* of progesterone, 20α-hydroxypregn-4-ene-3-one, 20β-hydroxypregn-4-ene-3-one, and the products of their metabolism by *A. niger*

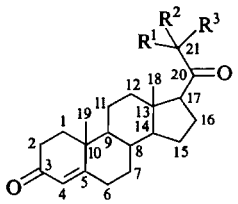
Compound	δ value					
	C-11	C-15	C-18	C-19	C-20	C-21
Progesterone (**1***a*)	—	—	0.67	1.18	—	2.11
11-Deoxycorticosterone (**1***b*)	—	—	0.70	1.20	—	4.18
11-Deoxycorticosterone (**1***b*), C_6D_6 solution	—	—	0.48	0.70	—	3.95
20α-Hydroxypregn-4-ene-3-one (**2***a*)	—	—	0.72	1.20	3.70	1.26
20β-Hydroxypregn-4-ene-3-one (**2***b*)	—	—	0.78	1.18	3.70	1.14
15β,20α-Dihydroxypregn-4-ene-3-one (**2***c*)	—	4.30	1.03	1.19	3.80	1.11
11α,15β,20α-Trihydroxypregn-4-ene-3-one (**2***d*)	4.00	4.30	1.00	1.35	3.80	1.25
15β,20β-Dihydroxypregn-4-ene-3-one (**2***e*)	—	4.28	1.10	1.24	3.90	1.17
11α,15β,20β-Trihydroxypregn-4-ene-3-one (**2***f*)	4.00	4.30	1.10	1.35	3.90	1.18

*Unless otherwise stated.

TABLE 2. Conversion of *d*-progesterones **4***a*–**4***c* to 11-deoxycorticosterone by *A. niger*

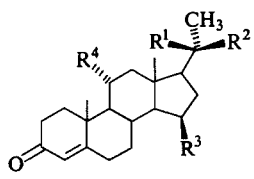
Substrate	Label (%)	Product	Label (%)
2,2,4,6,6,17,21,21-d_9-Progesterone (**4***a*)	d_9, 78*	2,2,4,6,6,17,21,21-d_8-11-DOC (**5***a*)	d_8, 77.2
	d_8, 19		d_7, 18.8
	d_7, 3		d_6, 4
21-*d*-Progesterone (**4***b*)	d_1, 77.5	21-*d*-11-DOC (**1***b* + **5***b* + **5***c*)	d_1, 58
	d_0, 22.5		d_0, 42
2,2,4,6,6,17,21,21-d_8-Progesterone (**4***c*)	d_8, 40	2,2,4,6,6,17,21,21-d_8-11-DOC (**5***a* + **5***d* + **5***e*)	d_8, 16
	d_7, 40		d_7, 40
	d_6, 13.6		d_6, 29
	d_5, 6.4		d_5, 9
			d_4, 6

*Deuterium content values are accurate to ±0.5%.



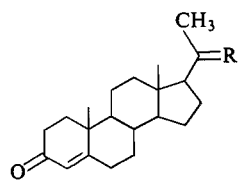
1

a $R^1 = R^2 = R^3 = H$
b $R^1 = R^2 = H; R^3 = OH$



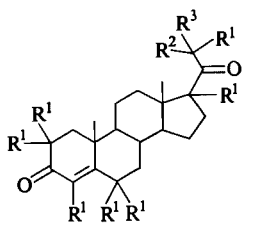
2

a $R^1 = R^3 = R^4 = H; R^2 = OH$
b $R^1 = OH; R^2 = R^3 = R^4 = H$
c $R^1 = R^4 = H; R^2 = R^3 = OH$
d $R^1 = H; R^2 = R^3 = R^4 = OH$
e $R^1 = R^3 = OH; R^2 = R^4 = H$
f $R^1 = R^3 = R^4 = OH; R^2 = H$



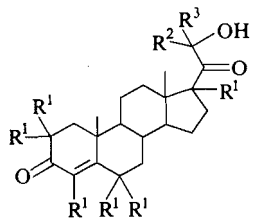
3

a $R = H_2$
b $R = CH_2$
c R = S



4

a $R^1 = R^2 = R^3 = D$
b $R^1 = R^2 = H; R^3 = D$
c $R^1 = R^2 = D; R^3 = H$



5

a $R^1 = R^2 = R^3 = D$
b $R^1 = R^2 = H; R^3 = D$
c $R^1 = R^3 = H; R^2 = D$
d $R^1 = R^2 = D; R^3 = H$
e $R^1 = R^3 = D; R^2 = H$

signal of the C-21 methyl at δ 2.11 corresponded to approximately two protons, while mass spectral analysis of **4***b* confirmed the presence of deuterium at C-21 (see Experimental).

Incubation of **4***b* with *A. niger* gave 11-deoxycorticosterone which consisted of unlabelled material **1***b* and the diastereomeric monodeuterioalcohols **5***b* and **5***c*. Mass spectral analysis showed all the label to be present at C-21: intense ions were observed at *m/e* 299 (M − CHDOH) and 271 (M − C_2HDO_2H) corresponding to the loss of C-21 and C-20 + C-21 and their substituents, respectively, whereas the ^{13}C corrected intensities of the corresponding ions at *m/e* 300 and 272, expected if label were located in the steroid nucleus, were zero. Analysis of the p.m.r. spectrum (C_6D_6 solution) of the mixture of **1***b*, **5***b*, and **5***c* showed **5***b* and **5***c* to be present in equimolar amounts. Two absorptions of equal area at δ 3.86 and 3.98, attributable to the C-21 hydrogens of **5***b* and **5***c*, were complicated by the presence of an AB quartet (J = 19 Hz) centered at δ 3.95 and assigned to the C-21 hydrogens of **1***b* but nevertheless their assignment to **5***b* and **5***c* is unambiguous. No attempt was made to assign these signals individually to **5***b* or **5***c*.

The formation of **5***b* and **5***c* in equimolar amounts shows that the two hydrogens originally present at C-21 may be lost with equal facility: by implication, the replacement of hydrogen by hydroxyl is nonstereospecific. The deuterium content of the product (**1***b* + **5***b* + **5***c*) thus enables the deuterium isotope effect of the C-21 hydroxylation to be calculated as $K_H/K_D = 1.3 \pm 0.1$.[1]

This was confirmed following synthesis of 2,2,4,6,6,17,21,21-d_8-progesterone (**4***c*) (Scheme 2) and incubation with *A. niger*. The synthesis involved loss of some deuterium during acid catalyzed hydrolysis of the intermediate 2,2,4,6,6,17,21,21-d_8-pregn-4-ene-3,20-dione 21-(2′-tetrahydropyranyl) ether but spectral analysis of **4***c* clearly indicated that deuterium content at C-21 was 2.0 atoms per molecule and that loss had taken place from C-2, -4, or -6. Thus the mass spectrum of **4***c* showed ions at *m/e* 126–129 from the fragment C-1-6 + C-19, a fragment appearing from unlabelled material **1***a* at *m/e* 124 (23) and observed in the mass spectrum of 2,2,4,6,6,17,21,21,21-d_9-progesterone (**4***a*) at *m/e* 129. The p.m.r. spectrum of **4***c* showed absorption in the region δ 2.0–2.6, essentially absent from the spectrum of **4***a* and attributable to protons adjacent or vinylogous to carbonyl; the signal of the C-21 methyl of **4***c* appeared as a singlet corresponding to approximately one proton at δ 2.11.

The 11-deoxycorticosterone formed from **4***c* consisted of the diastereomers **5***d* and **5***e*, analogous to **5***b* and **5***c*, in equimolar amount, together with **5***a* which contained a maximum

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[1]If % d_1-progesterone is x and % d_0-progesterone is $(100 - x)$ then, in the absence of an isotope effect,

$(100 - x)d_0$-progesterone → $(100 - x)d_0$-11-DOC

and

xd_1-progesterone → $1/3xd_0$-11-DOC + $2/3xd_1$-11-DOC

In the presence of an isotope effect y, where $y = K_H/K_D$, then

$(100 - x)d_0$-progesterone → $(100 - x)d_0$-11-DOC

and

xd_1-progesterone → $(1/3)(x/y)d_0$-11-DOC + $(x - (1/3)(x/y))d_1$-11-DOC

Thus,

[1] final % d_1-11-DOC = $(x - (1/3)(x/y)) = z$

and

[2] final % d_0-11-DOC = $(100 - x) + (1/3)(x/y)$ = $(100 - z)$

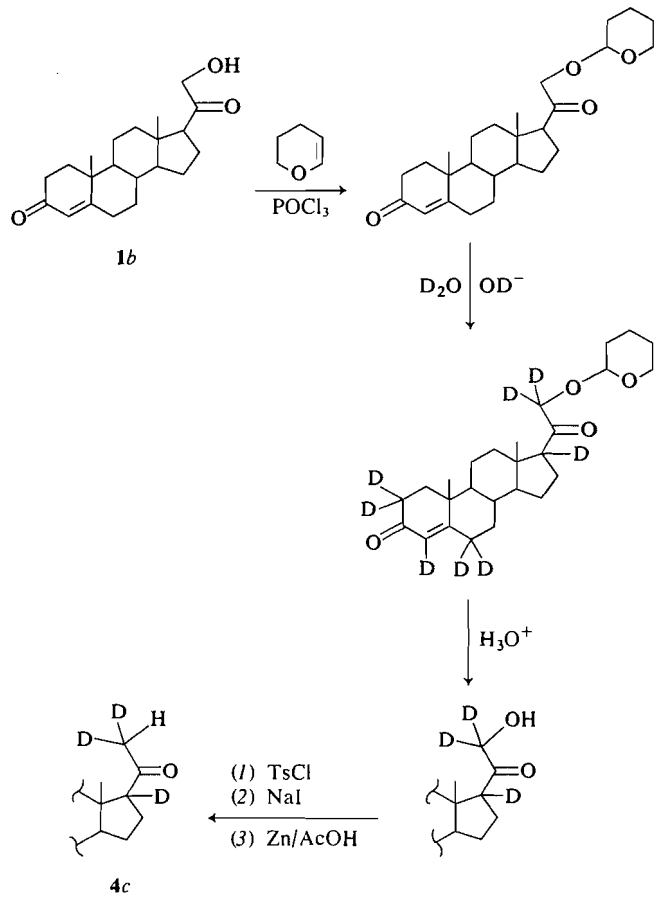
Found

$x = 75.5 \pm 0.5$

$z = 58.0 \pm 0.5$

Therefore, from [1] or [2]

$y = 1.3 \pm 0.1$



SCHEME 2. Synthesis of 2,2,4,6,6,17,21,21-d_8-progesterone (**4***c*).

of eight deuterium atoms per molecule. Proton magnetic resonance analysis was facilitated by the absence of material unlabelled at C-21 and confirmed the results of the incubation of **4***b*. Calculation of the deuterium isotope effect for the conversion of **4***c* to 11-deoxycorticosterone gave $K_H/K_D = 1.2 \pm 0.1$, in close agreement with the value obtained from the incubation of **4***b*.

The magnitude of this effect eliminates a mechanism of C-21 hydroxylation in which enolization of the C-20 carbonyl, with a rate determining loss of hydrogen from C-21, is the slow step. Primary deuterium isotope effects of between 5 and 10 for base (24–26) and acid (27) catalyzed enolizations of ketones, where the loss of α hydrogen is rate determining, have been reported. However, the effect is consistent with a mechanism of C-21 hydroxylation involving rate determining electrophilic insertion into a C—H or C—D bond. The observed value is similar to those of analogous chemical reactions such as the autoxidation of cholesterol at C-7α ($K_H/K_D = 1.8$) (28) and the insertion of a carbene into a C—H or C—D bond ($K_H/K_D = 1.8$) (29). The latter process has been the subject of calculations (30) which indicate a transition state with little C—H bond lengthening, consistent with an isotope effect of small magnitude (31). By analogy, the isotope effect of the C-21 hydroxylation may be the result of rate limiting electrophilic insertion of oxygen into a C—H or C—D bond, with little lengthening of that bond in the transition state.

This interpretation is supported by the isotope effects of enzymic hydroxylations of steroids

at nonenolizable positions. Hydroxylations at C-7β of estr-4-ene-3,17-dione (32), C-7α of cholesterol (28), C-6β of taurochenodeoxycholic acid (28), and C-11α of progesterone (6) all involve isotope effects close to unity.

This result does not, however, eliminate the C-20,21 enol from involvement in C-21 hydroxylation. Enolization may occur in which loss of hydrogen from C-21 is not rate limiting: Hine and co-workers (33, 34) have observed catalysis of the dedeuteration of α-deuteriocarbonyl compounds in which loss of deuterium is only partly rate controlling. Similar catalysis, in which the rate determining step may involve the carbonyl center (34), may occur in enzymic enolization of the C-20 carbonyl. In this event, the deuterium content of the product would depend on the relative rates of loss of protium or deuterium from C-21 and would therefore be subject to a primary isotope effect whose magnitude in this situation is unknown.[2] Exchange of deuterium at C-21 with protium from the medium does not occur during hydroxylation: the observed isotope effect is not therefore the result of an equilibration of protium and deuterium during reversible enolization.

The complexity of interpretation of the kinetic isotope effects of enzymic hydroxylations has been discussed fully (28, 32) and the isotope effect of the C-21 hydroxylation of progesterone is not open to unambiguous interpretation. It appears, however, that the effect is the result of a mechanism in which any of the C-21 C—H bonds may undergo direct insertion of oxygen, leading to the formation of 11-deoxycorticosterone directly. The microbial conversion of steroidal C-methyl groups into hydroxymethyl is well known (1) and is performed on androst-4-ene-3,17-dione by *A. niger* ATCC 9142 (35).

The C-21 hydroxylation of progesterone by *A. niger* ATCC 9142 is therefore a reaction directly analogous in mechanism to hydroxylation at saturated carbon not adjacent or vinylogous to carbonyl and not one in which the substrate is activated to electrophilic attack by enolization. The latter remains an attractive hypothesis however for axial hydroxylation at the enolizable positions of Δ^4-3-ketosteroids and will receive further consideration.

[2]J. Hine, private communication.

Experimental

Apparatus, Materials, and Methods

Melting points were determined on a Kofler heating stage and are uncorrected. Infrared spectra were recorded with a Perkin-Elmer 137, p.m.r. spectra with a Varian HA 100, using deuteriochloroform as solvent (unless otherwise stated) and TMS as internal standard, and mass spectra were obtained with an AEI MS902. Deuterium content was estimated by repeated scanning of the relevant ions and application of the appropriate corrections for ^{13}C. Accurate mass measurements were made by reference to the perfluorokerosene spectrum. Optical rotation data were obtained in chloroform solution with a Perkin-Elmer 141 polarimeter.

Column chromatography was performed on silica gel (Whatman's SG 31) and thin-layer chromatography on Merck silica $PF_{254+366}$.

Aspergillus niger ATCC 9142 was obtained from the American Type Culture Collection, Washington, D.C. and was maintained on 2% malt agar slopes.

Preparation of Substrates

Pregn-5-ene-3,20-dione Bis(ethylene acetal)

This was prepared by the method described (36) from progesterone (7.5 g). Recrystallization from acetone gave the title compound (6.8 g, 71%), m.p. 179–181° (lit. (36) m.p. 177–180°).

Pregn-5-ene-3,20-dione 3-(Ethylene Acetal)

This was prepared from pregn-5-ene-3,20-dione bis(ethylene acetal) (6 g) by the method described (37). Recrystallization from acetone gave the desired compound (3.8 g, 84%), m.p. 175–176° (lit. (37) m.p. 178–180°).

20α-Hydroxypregn-5-ene-3-one Ethylene Acetal

Sodium (9.3 g) was added portionwise to a refluxing solution of pregn-5-ene-3,20-dione 3-(ethylene acetal) (3.5 g) in 2-propanol (300 ml). When addition was complete refluxing was continued for 15 min. The solution was then poured onto ice-water (300 ml) and the precipitated solid collected by filtration, washed with water, and dried *in vacuo*. Recrystallization from ether gave the product (2.5 g, 71%), m.p. 177–179°, $[\alpha]_D^{21}$ −41.9° (*c*, 1.0) (lit. (38) m.p. 178–180°, $[\alpha]_D$ −43°).

20α-Hydroxypregn-4-ene-3-one (2a)

Sulfuric acid (8% v/v; 15 ml) was added to a solution of 20α-hydroxypregn-5-ene-3-one ethylene acetal (2.5 g) in methanol (50 ml). The solution was refluxed for 15 min, neutralized with 5% sodium bicarbonate, poured onto ice-water, and the precipitate collected by filtration and washed with water. Two crystallizations from ethyl acetate gave **2a** (1.9 *g*, 85%), m.p. 160–163°; $[\alpha]_D^{20}$ +95.4° (*c*, 1.0) (lit. (39) m.p. 161–162°, $[\alpha]_D^{22}$ +98.6°); p.m.r.: included signals at δ 0.72 (3H, s, C-18 methyl), 1.20 (3H, s, C-19 methyl), 1.26 (3H, d, J = 6 Hz, C-21 methyl), 3.70 (1H, quintet, J = 6,6,6,6 Hz, C-20 H), 5.75 (1H, d, J = 1 Hz, C-4 H).

20β-Hydroxypregn-4-ene-3-one (2b)

This was prepared from progesterone (5 g) by the method described (40). Recrystallization from aqueous methanol gave the product (3.1 g, 64% overall), m.p. 172–174°, $[\alpha]_D^{25}$ +85.2° (*c*, 1.0) (lit. (40) m.p. 174–175°, $[\alpha]_D^{20}$ +86°); p.m.r.: included signals at δ 0.78 (3H, s, C-18 methyl), 1.14 (3H, d, J = 6 Hz, C-21 methyl),

1.18 (3H, s, C-19 methyl), 3.70 (1H, m, C-20 H), 5.75 (1H, d, J = 1 Hz, C-4 H).

Pregn-4-ene-3-one (3a)

This was prepared from 3β-hydroxypregn-5-ene-20-one (3 g) by the route described (41). Recrystallization from methanol gave the product (1.4 g, 47% overall), m.p. 102–105°, $[\alpha]_D^{20}$ +124° (*c*, 0.9) (lit. (41) m.p. 102–104°, $[\alpha]_D$ +121°).

20-Methylpregna-4,20(21)-diene-3-one (3b)

This was prepared from 3β-hydroxypregn-5-ene-20-one (5 g) by the route described (42). The crude product (1.96 g) was purified by chromatography and crystallization from aqueous methanol to give the title compound (1.7 g, 34% overall), m.p. 153–156°, $[\alpha]_D^{18}$ +110° (*c*, 0.96) (lit. (42) m.p. 152–154°, $[\alpha]_D^{27}$ +112° (*c*, 1.35)).

20-Thiopregn-4-ene-3-one (3c)

This was prepared from 3β-hydroxypregn-5-ene-20-one (6 g) by the route described (43). Chromatography of the crude product on alumina gave the title compound (0.8 g, 11% overall), m.p. 112–115°.

2,2,4,6,6,17,21,21,21-d_9-Progesterone (4a)

Sodium (1 g) was added to a solution of progesterone (1 g) in dry dioxan (50 ml) and deuterium oxide (12 ml) added to this solution. The solution was refluxed for 7 days, cooled, neutralized to pH 8 with acetic acid, poured onto ice-water (150 ml), and rapidly extracted with ether. The ether extract was washed with water, dried, and evaporated to give a residue which, on crystallization from ether – light petroleum, gave the desired compound (0.64 g, 63%), m.p. 120–122°, $[\alpha]_D^{25}$ +165° (*c* 1.02, EtOH); p.m.r.: included signals at δ 0.67 (3H, s, C-18 methyl), 1.18 (3H, s, C-19 methyl), 2.0–2.6 (*ca.* 0.2H, m, protons adjacent or vinylogous to carbonyl); m.s.: *m/e* 323(100), 322(30), 321(5), 279(50), 278(10), 129(70), 128(21); analysis of ions at *m/e* 320–323 gave the following molecular isotopic composition: d_9, 78%; d_8, 19%; d_7, 3%; d_6, 0%.

21-d-Progesterone (4b)

Zinc dust (1.2 g) was added to a solution of 21-iodopregn-4-ene-3,20-dione (1.2 g) (44) in monodeuterioacetic acid (10 ml) and the mixture stirred for 10 min at room temperature. The solution was then filtered, water (100 ml) added to the filtrate, and this solution extracted with ether. The ethereal extract was washed with 2 M sodium bicarbonate, dried, and evaporated. The residue was chromatographed to give, after recrystallization from ether – light petroleum, the product (0.4 g, 46%), m.p. 120–122°; p.m.r.: included signals at δ 0.67 (3H, s, C-18 methyl), 1.18 (3H, s, C-19 methyl), 2.11 (*ca.* 2H, s, C-21 CH_2D), 5.76 (1H, broad s, C-4 H); m.s.: *m/e* 315(100), 314(30), 273(60), 272(20), 271(7), 125(30), 124(180); analysis of ions at *m/e* 314, 315 gave the following molecular isotopic composition: d_1, 77.5%; d_0, 22.5%.

Pregn-4-ene-3,20-dione 21-(2′-Tetrahydropyranyl) Ether

Phosphorous oxychloride (1 g) was added to a solution of 11-deoxycorticosterone (2.5 g) and 2,3-dihydropyran (0.64 g) in dichloromethane (50 ml) and the resulting solution stirred at 35° for 15 min. Ether (300 ml) was then added and the solution washed with 2 M sodium bicarbonate and water, dried, and evaporated to give a residue which, on crystallization from pentane, afforded the product (1.87 g, 62%), m.p. 110–113°, $[\alpha]_D^{18}$ +186° (*c*, 1.07) (lit. (45) m.p. 111–112°, $[\alpha]_D^{25}$ +183°).

2,2,4,6,6,17,21,21-d_8-Pregn-4-ene-3,20-dione 21-(2′-Tetrahydropyranyl) Ether

Sodium (1 g) was added to a solution of pregn-4-ene-3,20-dione 21-(2′-tetrahydropyranyl) ether (1.5 g) in dry dioxan (100 ml). Deuterium oxide (20 ml) was then added and the resulting solution treated as described in the preparation of 2,2,4,6,6,17,21,21,21-d_9-progesterone (**4a**). The title compound (1.36 g), after recrystallization from hexane, melted at 110–113°, $[\alpha]_D^{18}$ +181° (*c*, 0.94); m.s.: analysis of ions at *m/e* 414-422 gave the following molecular isotopic composition: d_8, 74%; d_7, 18%; d_6, 7%; d_5, 1%.

2,2,4,6,6,17,21,21-d_8-11-Deoxycorticosterone

Hydrochloric acid (37%, 5 drops) was added to a solution of 2,2,4,6,6,17,21,21-d_8-pregn-4-ene-3,20-dione 21-(2′-tetrahydropyranyl) ether (1.36 g) in hexane (100 ml) and methanol (100 ml). The mixture was stirred at room temperature for 2 h and evaporated to give a residue which was dissolved in ether, washed with 2 M sodium bicarbonate, dried, and evaporated. Recrystallization from aqueous methanol gave the product (1.0 g, 86%), m.p. 139–142°, $[\alpha]_D^{20}$ +181° (*c*, 1.0); m.s.: analysis of ions at *m/e* 330–338 gave the following molecular isotopic composition: d_8, 41%; d_7, 39%; d_6, 14%; d_5, 6%.

2,2,4,6,6,17,21,21-d_8-21-Iodopregn-4-ene-3,20-dione

This was prepared from 2,2,4,6,6,17,21,21-d_8-11-deoxycorticosterone (1 g) by the method described (44). The crude product (1.2 g) was used directly.

2,2,4,6,6,17,21,21-d_8-Progesterone (4c)

This was prepared from 2,2,4,6,6,17,21,21-d_8-21-iodopregn-4-ene-3,20-dione (1.2 g) by the procedure described for the preparation of 21-*d*-progesterone, using acetic acid in place of monodeuterioacetic acid. The product, after purification by chromatography and crystallization from aqueous methanol (0.48 g, 57%), melted at 120–121°; p.m.r.: included signals at δ 0.66 (3H, s, C-18 methyl), 1.18 (3H, s, C-19 methyl), 2.11 (*ca.* 1H, s, C-21 CHD_2), 2.0–2.6 (*ca.* 0.6H, m, protons adjacent or vinylogous to carbonyl); m.s.: *m/e* 322(80), 321(70), 320(25), 280(0.1), 279(2), 278(11), 277(37), 276(25), 275(11), 129(17), 128(60), 127(43), 126(37); analysis of ions at *m/e* 314–322 gave the following molecular isotopic composition: d_8, 40%; d_7, 40%; d_6, 13.6%; d_5, 6.4%.

Incubations with A. niger ATCC 9142

Incubations were performed in a 5-l fermentation vessel, mechanically stirred and aerated with air sterilized by passage through a Whatman Gamma 10 filter. Growth medium (2.5 l, containing Czapek Dox nutrient 33.4 g/l) was autoclaved in the fermentation vessel at 15 p.s.i. for 20 min, and then allowed to cool. A spore suspension obtained by shaking an agar slope of *A. niger* with sterile glass beads in water was then transferred to the vessel with a sterile syringe; this process was repeated so that two slopes were used to inoculate 2.5 l of medium. After 2 days growth at 28°, a solution of the substrate (1 g) in ethanol (50 ml) was added to the flask and growth allowed to continue for a further 4 days. The mycelium and medium were then separated by centrifugation, and both extracted with dichloro-

methane. The extract was dried over sodium sulfate and evaporated. Incubations with smaller quantities of substrate were performed with proportionately smaller fermentation volumes.

Progesterone (1a)

One gram gave a crude extract (0.434 g) which was subjected to preparative t.l.c. (ether) and gave: progesterone (0.16 g from ether – light petroleum), R_f 0.87, identified by m.p. and spectral comparison with an authentic sample. And *11-deoxycorticosterone* (0.21 g from aqueous methanol), R_f 0.6, identified by m.p. and spectral comparison with an authentic sample; p.m.r.: included signals at δ 0.70 (3H, s, C-18 methyl), 1.20 (3H, s, C-19 methyl), 4.18 (2H, broad s, C-21 H), 5.73 (1H, broad s, C-4 H); p.m.r. (C_6D_6) included signals at δ 0.48 (3H, s, C-18 methyl), 0.70 (3H, s, C-19 methyl), 3.95 (2H, ABq, J = 19 Hz, C-21 H), 5.81 (1H, broad s, C-4 H).

20α-Hydroxypregn-4-ene-3-one (2a)

One gram gave a crude extract (0.537 g) which was subjected to preparative t.l.c. (ethyl acetate) and gave: 20α-hydroxypregn-4-ene-3-one (**2a**) (0.3 g from aqueous methanol), R_f 0.6, identified by m.p. and spectral comparison with an authentic sample. And *15β,20α-dihydroxypregn-4-ene-3-one (2c)* (0.028 g from ethyl acetate – light petroleum), R_f 0.43 (ethyl acetate), 0.16 (ether), 0.40 (chloroform–methanol–water 188:12:1), m.p. 210–212°, M^+ 332.2358 ($C_{21}H_{32}O_3$ requires M^+ 332.2351), $[\alpha]_D^{18}$ +60° (*c*, 0.4); i.r.: ν_{max} (KBr) 3470, 1670, 1620 cm^{-1}; p.m.r.: included signals at δ 1.03 (3H, s, C-18 methyl), 1.11 (3H, d, J = 6 Hz, C-21 methyl), 1.19 (3H, s, C-19 methyl), 3.80 (1H, quintet, J = 6,6,6,6 Hz, C-20 H), 4.30 (1H, t, J = 7,7 Hz, C-15 H), 5.75 (1H, d, J = 1 Hz, C-4 H).

Oxidation of **2c** was performed as follows: a solution of **2c** (0.015 g) in acetone (5 ml) was stirred at 25° during the addition of standard chromic acid reagent (46) until the reagent was present in excess. Water (15 ml) was then added and the mixture extracted with ether. The extract was washed with 2 M sodium bicarbonate, dried, and evaporated to give a residue (0.014 g) which on crystallization from aqueous methanol gave pregn-4-ene-3,15,20-trione (0.011 g), m.p. 154–156°, $[\alpha]_D^{24}$ +198° (lit. (47) m.p. 155–157°, $[\alpha]_D^{23}$ +200°). And *11α,15β,20α-trihydroxypregn-4-ene-3-one (2d)* (0.016 g from ethyl acetate – light petroleum), R_f 0.23 (ethyl acetate), 0.1 (ether), 0.25 (chloroform–methanol–water 188:12:1); M^+ 348.2298 ($C_{21}H_{32}O_4$ requires M^+ 348.2300); m.p. 230–232°, $[\alpha]_D^{18}$ +55° (*c*, 1.3); i.r.: ν_{max} (KBr) 3470, 1670, 1620 cm^{-1}; p.m.r.: included signals at δ 1.00 (3H, s, C-18 methyl), 1.25 (3H, d, J = 6 Hz, C-21 methyl), 1.35 (3H, s, C-19 methyl), 3.80 (1H, quintet, J = 6,6,6,6 Hz, C-20 H), 4.00 (1H, sextet, J = 10,10,6 Hz, C-11 H), 4.30 (1H, t, J = 7,7 Hz, C-15 H), 5.75 (1H, d, J = 1 Hz, C-4 H).

Oxidation by the method described above gave pregn-4-ene-3,11,15,20-tetraone (0.008 g), m.p. 214–216°, $[\alpha]_D^{20}$ +309° (*c*, 0.5) (lit. (48) m.p. 218–221°, $[\alpha]_D^{23}$ +325°).

20β-Hydroxypregn-4-ene-3-one (2b)

One gram gave a crude extract (0.4 g) which was subjected to preparative t.l.c. (ethyl acetate) and gave 20β-hydroxypregn-4-ene-3-one (0.246 g from aqueous methanol), identified by m.p. and spectral comparison with an authentic sample. And *15β,20β-dihydroxypregn-4-ene-3-one (2e)* (0.04 g from diisopropyl ether–hexane), R_f 0.35 (ethyl acetate), 0.32 (ether), 0.49 (chloroform–methanol–water 188:12:1); M^+ 332.2356 ($C_{21}H_{32}O_3$ requires M^+ 332.2351), m.p. 188–190°, $[\alpha]_D^{24}$ +44° (*c*, 0.8); i.r.: ν_{max} (KBr) 3470, 1670, 1620 cm^{-1}; p.m.r.: included signals at δ 1.10 (3H, s, C-18 methyl), 1.17 (3H, d, J = 6 Hz, C-21 methyl), 1.24 (3H, s, C-19 methyl), 3.90 (1H, m, C-20 H), 4.28 (1H, t, J = 7,7 Hz, C-15 H), 5.76 (1H, d, J = 1 Hz, C-4 H).

Oxidation by the method described above gave pregn-4-ene-3,15,20-trione, m.p. 153–156°, $[\alpha]_D^{23}$ +196° (*c*, 1.0). And *11α,15β,20β-trihydroxypregn-4-ene-3-one (2f)* (0.027 g from ether), R_f 0.22 (ethyl acetate), 0.15 (ether), 0.3 (chloroform–methanol–water 188:12:1); M^+ 348.2303 ($C_{21}H_{32}O_4$ requires M^+ 348.2300), m.p. 248–252° (dec.), $[\alpha]_D^{23}$ −24° (*c*, 1.0); i.r.: ν_{max} (KBr) 3470, 1670, 1620 cm^{-1}; p.m.r.: included signals at δ 1.10 (3H, s, C-18 methyl), 1.18 (3H, d, J = 6 Hz, C-21 methyl), 1.35 (3H, s, C-19 methyl), 3.90 (1H, m, C-20 H), 4.00 (1H, sextet, J = 10,10,5 Hz, C-11 H), 4.30 (1H, t, J = 7,7 Hz, C-15 H), 5.75 (1H, d, J = 1 Hz, C-4 H).

Oxidation by the method described above gave pregn-4-ene-3,11,15,20-tetraone, m.p. 216–219°, $[\alpha]_D^{20}$ +317° (*c*, 0.4).

Pregn-4-ene-3-one (3a)

One gram gave a crude extract (0.621 g). Analysis by t.l.c. indicated the presence of starting material (R_f 0.66, ether) only. Chromatography gave **3a** (0.508 g from methanol), identified by m.p. and spectral comparison with an authentic sample.

20-Methylpregna-4,20(21)-diene-3-one (3b)

A 0.2 g sample gave a crude extract (0.137 g), t.l.c. analysis of which indicated the presence of starting material (R_f 0.6, ether) only. Preparative t.l.c. gave **3b** (0.12 g from aqueous methanol), identified by m.p. and spectral comparison with an authentic sample.

20-Thiopregn-4-ene-3-one (3c)

A 0.24 g sample gave a crude extract (0.157 g). The only steroid detectable by t.l.c. analysis was **3c** (R_f 0.23, ether), which was recovered by chromatography on alumina.

2,2,4,6,6,17,21,21,21-d_9-Progesterone (4a)

One gram gave a crude extract (0.46 g) which was subjected to preparative t.l.c. (ether) and gave 2,2,4,6,6,-17,21,21,21-d_9-progesterone (0.11 g from ether – light petroleum), R_f 0.76, identified by m.p. and spectral comparison with an authentic sample; m.s.: analysis of ions at *m/e* 320–323 gave the following molecular isotopic composition: d_9, 77%; d_8, 20%; d_7, 3%; the mass spectrum was identical with that of authentic **4a**. And *2,2,4,6,6,17,21,21-d_8-11-deoxycorticosterone (5a)* (0.124 g from aqueous ethanol), R_f 0.4, identified by m.p. and spectral comparison with unlabelled material **1b**; p.m.r.: included signals at δ 0.70 (3H, s, C-18 methyl), 1.20 (3H, s, C-19 methyl) but no absorption in the region 4.18 (C-21 CH_2OH); m.s.: *m/e* 338(20), 337(5), 336(1), 335(0.05), 305(100) (M − CD_2OH), 277(50) (M − $C_2D_2O_2H$); analysis of ions at *m/e* 335–338

gave the following molecular isotopic composition: d_8, 77.2%; d_7, 18.8%; d_6, 4%.

21-d-Progesterone (4b)

A 0.35 g sample gave a crude extract which was subjected to preparative t.l.c. (ether) and gave 21-*d*-progesterone (0.062 g from ether – light petroleum), R_f 0.8, identified by m.p. and spectral comparison with an authentic sample; p.m.r.: included signals at δ 0.67 (3H, s, C-18 methyl), 1.18 (3H, s, C-19 methyl), 2.11 (*ca.* 2H, s, C-21 CH_2D), 5.76 (1H, broad s, C-4 H); m.s.: analysis of ions at *m/e* 314, 315 gave the molecular isotopic composition as d_1, 78%; d_0, 22%. The mass spectrum was identical with that of **4***b*. And *21-d-11-deoxycorticosterone (**1***b* + **5***b* + **5***c*)* (0.074 g from aqueous methanol), R_f 0.4, identified by m.p. and spectral comparison with an authentic sample of unlabelled material; p.m.r. (C_6D_6): included signals at δ 0.48 (3H, s, C-18 methyl), 0.70 (3H, s, C-19 methyl), 3.86 (*ca.* 0.3H, broad s, C-21 C*H*DOH), 3.95 (*ca.* 0.5H, ABq, J = 19 Hz, C-21 CH_2OH), 3.98 (*ca.* 0.3H, broad s, C-21 CD*H*OH), 5.82 (1H, broad s, C-4 H); m.s.: *m/e* 331(15), 330(9), 300(26), 299(100) (M − CHDOH), 272(12), 271(50), 270(1.1) (M − $C_2DO_2H_2$); analysis of ions at *m/e* 330,331 gave the following molecular isotopic composition: d_1, 58%; d_0, 42%.

2,2,4,6,6,17,21,21-d_8-Progesterone (4c)

One gram gave a crude extract (0.594 g) which afforded, after preparative t.l.c. (ether), 2,2,4,6,6,17,21,21-d_8-progesterone (0.214 g from aqueous methanol), R_f 0.8, identified by m.p. and spectral comparison with an authentic sample. And *2,2,4,6,6,17,21,21-d_8-11-deoxycorticosterone (**5***a* + **5***d* + **5***e*)* (0.092 g from aqueous methanol), R_f 0.4, identified by m.p. and spectral comparison with an authentic sample of unlabelled material; p.m.r. (C_6D_6): included signals at δ 0.48 (3H, s, C-18 methyl), 0.70 (3H, s, C-19 methyl), 3.86 (*ca.* 0.25H, broad s, C-21 CD*H*OH), 3.95 (*ca.* 0.25H, broad s, C-21 C*H*DOH); m.s.: *m/e* 338(10), 337(15), 336(10), 335(3), 334(2), 333(0.1), 305(100) (M − CD_2OH), 277(45), 276(40) (M − $C_2D_2O_2H$); analysis of ions at *m/e* 333–338 gave the following molecular isotopic composition: d_8, 16%; d_7, 40%; d_6, 29%; d_5, 9%; d_4, 6%.

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