THE MICROBIOLOGICAL HYDROXYLATION OF STEROIDS AND RELATED COMPOUNDS

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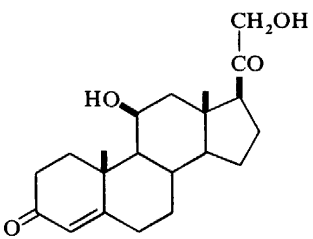
ABSTRACT

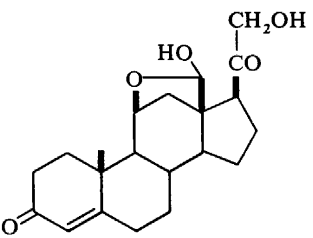
The factors which determine the specific positions at which steroids are hydroxylated by micro-organisms are being investigated, using mono-ketones or keto-alcohols of the androstane series as substrates and infra-red and n.m.r. spectroscopy to determine the structures of the products. Whereas the major product of the hydroxylation of 5α-androstan-3-one by cultures of *Calonectria decora* is 12β,15α-dihydroxy-5α-androstan-3-one, by contrast, with the 17-ketone as substrate, 1β,6α-dihydroxylation occurs. The hydroxyl groups introduced are on centres about 4 Å apart and at distances from the carbonyl 'directing' group which are approximately comparable. With a range of other substrates and with several other organisms results of a similar nature are obtained, revealing the dependence of the substitution pattern on the position of the oxygen function(s) of the substrate.

The regio-specific reactions of organic compounds are usually associated with the presence of functional groups. Substitution is facilitated by bond polarization, i.e. the presence of a good leaving group or of adjacent activation. Substitution at remote sites can only be achieved by transmitting these effects by appropriate means. The organic chemist has become very ingenious at this, constructing scaffolding enabling him to activate remote positions.

In recent years, however, we have seen developments in what is now referred to as 'the functionalization of unactivated carbon'. The first instance was the quite unexpected transannular substitutions[1] observed independently in medium-sized rings by Cope and Prelog. The Barton reaction[2], initially demonstrated with steroidal 20-nitrites, is both of great practical value and of theoretical interest. It made aldosterone (II) available in just three steps from the relatively accessible corticosterone (I) and, in my view,



Corticosterone (I)



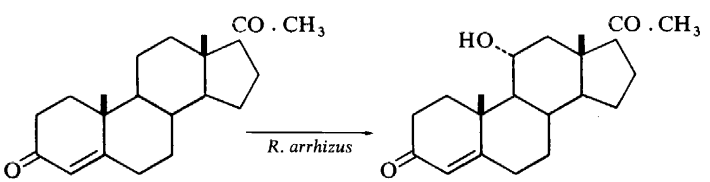
Aldosterone (II)

was partly responsible for the great upsurge in interest in the study of photochemical reactions. It is remarkable that the preparative value of such internal attack by a radical remained unappreciated for so long. Another approach, using an anchored reagent potentiated by irradiation, has been explored successfully by Breslow[3] and also by Baldwin[4].

In aiming at the development of reagents which could act at specified distances from existing functional or orienting groups, the organic chemist can derive much encouragement, inspiration and, in due time, ideas from natural systems. Enzymes possess a remarkable capacity for stereospecific attack on C—H bonds acting at variable distances from binding groups across the enzyme surface, e.g. the dehydrogenation of stearic to oleic acid and related processes, and the ω-hydroxylation of fatty acids. Knowledge of enzyme chemistry is developing rapidly and there is hope that, ere long, we shall get indications of the ways in which nature contrives to be so expert in these processes.

Hench and Kendall's demonstration in 1949 of the effect of cortisone administration to patients afflicted with rheumatoid arthritis presented steroid chemists with the tremendous challenge to find ways of introducing the vital hydroxyl group into the rather inaccessible 11-position in readily available steroidal starting materials. The challenge was accepted enthusiastically and many ingenious methods of 'getting at' the 11-position were devised. Many of these ventures, including total synthesis, were successful and steroid chemistry, and indeed organic chemistry as a whole, was vastly enriched by the tremendous activity stimulated by the need for 11-substituted steroids.

Remarkably, and almost simultaneously, chemists in at least three laboratories decided to explore the possibility of microbiological introduction of the 11-oxygen atom, a procedure for which the only precedent was the knowledge that 11-hydroxylation of steroids is brought about by isolated adrenal glands. The existence of micro-organisms which would attack steroids was quite certain in view of the absence of steroids and triterpenoids from soil, but the chances of specific 11-hydroxylation were very remote. Peterson and Murray[5] were the first to announce the 11-hydroxylation of a steroid (progesterone (III)) with *Rhizopus arrhizus*.



(III)

Undoubtedly one of the most important factors in achieving rapid success was the use of paper chromatographic methods of screening the products of microbiological transformations. Processes based on these discoveries were rapidly developed on a commercial scale and transformations are nowadays

carried out in vessels of 150000 litres capacity. These pioneering researches have led to the discovery of hydroxylation reactions affecting most of the steroid carbon atoms. They have also led to the uncovering of dehydrogenation processes, oxidation–reduction reactions and the fission of carbon–carbon bonds in both sidechain and nucleus. Elegant investigations have elucidated that in the hydroxylation process atmospheric oxygen, as with several other oxygen-requiring enzyme systems, is used, that unsaturated intermediates are not involved and that stereospecific replacement of hydrogen occurs with retention of configuration[6].

Almost all the substrates employed in the vast exploration of the microbiological reactions of steroids have contained the 3-keto-Δ^4 system (as in III), for good practical reasons, and progesterone has been most frequently used. It seemed not improbable that this structural feature, which might exert a powerful influence on the orientation of a steroid molecule in an enzyme system, determined the positions in which hydroxylation or other processes could occur. As we were aiming at a more fundamental appreciation of the geometry of microbiological hydroxylation, then it was essential to get away from this uniform feature of previous studies.

To embark upon such an investigation more than ten years ago would have been unprofitable; the methods of analysis and of separation of mixtures of steroids and of structure determination were either too inefficient or too tedious. But the advent of analytical and separation methods involving gas–liquid and thin layer chromatography, and of infra-red and n.m.r. spectroscopy for structure determination, radically changed the scene and made a more general exploration of steroid hydroxylation a practical possibility.

We had several objectives in mind, an important one being to make available compounds difficult to get by other means. Then we wished to ascertain whether it is merely the general shape of the molecule that determines the point of microbial attack or whether specific groups are responsible for its orientation before hydroxylation. We also had in mind the extension to purely synthetic steroid-like molecules and were conscious that, whilst monohydroxylation might confer appreciable preparative advantage, dihydroxylation would probably be worth substantially more than twice this. The possibility of being able to introduce functional groups into the terminal rings of synthetic steroid-like materials by micro-organisms seemed an attractive goal. In the long term it is hoped that developments in enzyme chemistry will result in enzymic hydroxylating reagents and, ultimately, the development by organic chemists of non-polypeptide systems modelled on enzymes.

We began by screening 3-, 11- and 17-ketones of the 5α-androstane series with a number of steroid hydroxylating fungi, and chose *Calonectria decora* for our initial study. We have examined its behaviour towards nearly a hundred steroid substrates employing fairly standard conditions[7] (with vigorously growing cultures (80 or 200 ml) in 500 ml shake flasks to which were added ca. 40 mg of the steroid substrate in ethanol or dimethyl sulphoxide (2–6 ml) followed by incubation for up to six days). In general only when the substrate was converted into one or two products were these examined in detail. Separations were frequently made practicable by the

use of preparative thin layer chromatography on metre-long plates using a mechanical applicator.

Determination of the ^{1}H n.m.r. spectra of the polyketones obtained from the hydroxylation products by mild oxidation, together with consideration of solvent shifts, has enormously simplified the task of structure elucidation. Following the original lead of Zürcher[8], we[9] measured the positions of the signals of the 19- and 18-protons (i.e. of the two angular methyl groups) in these ketones and with the aid of a substantial amount of reference data, and the assumption that the chemical and solvent shifts are additive, we have usually been able to determine the positions of the carbonyl groups (cf. *Figure 2*). In addition consideration of the positions and form of the >C*H*OR

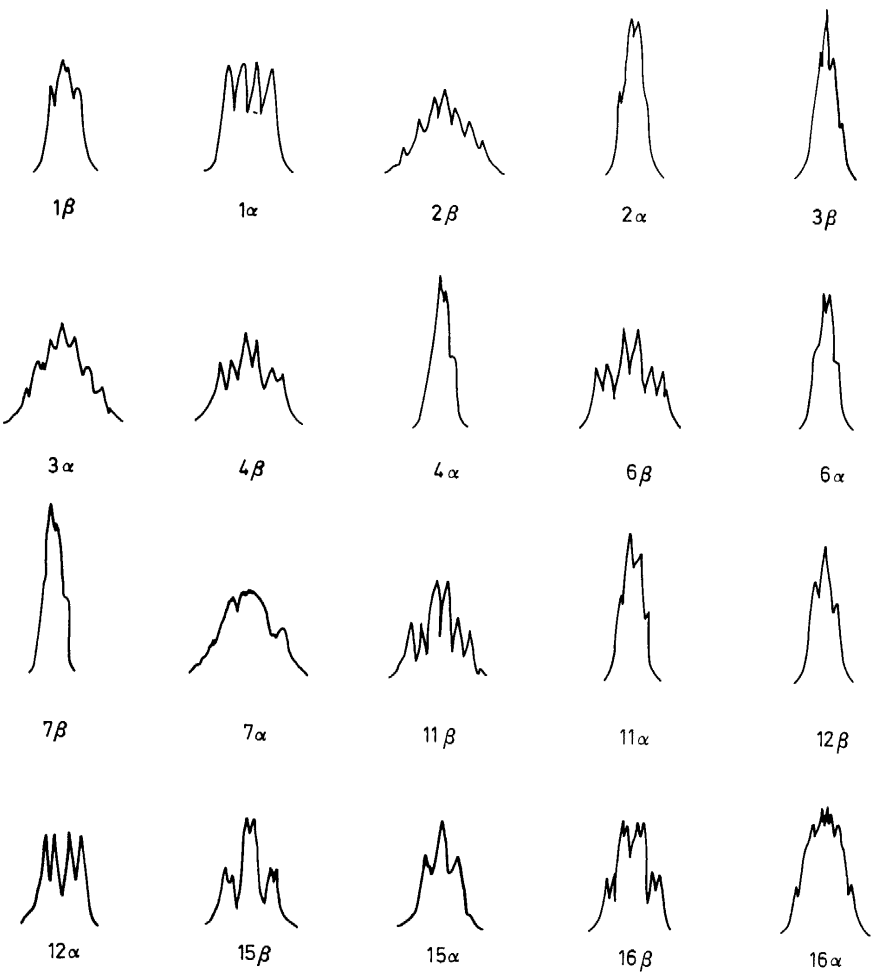


Figure 1. Characteristic shapes of 5α-steroidal >CH.OR n.m.r. signals

resonances (*Figure 1*) in the original products and derivatives gives useful information about the position of the oxygen substituent and especially about its configuration. Infra-red spectra usually provided confirmatory evidence but with some compounds, notably those involving intramolecular interactions [e.g. 12β-OH-17-CO, 1β,11α- and 7β,15α-$(OH)_2$], the data led unequivocally to the establishment of structural features[9]. In spite of this considerable assistance it remained necessary in a number of cases to provide structural proof by effecting links with known compounds.

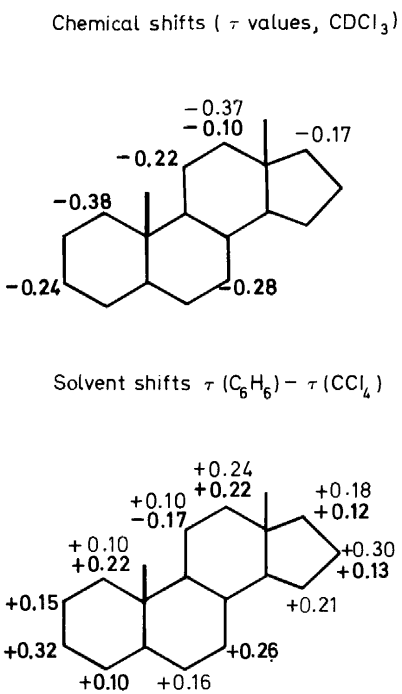
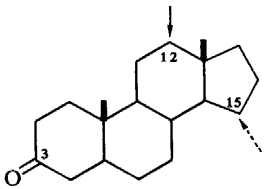
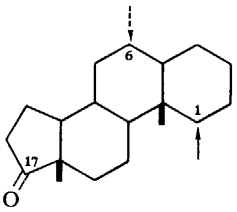


Figure 2. N.m.r. signals of 18 and **19** methyl groups in oxo-5α-steroids

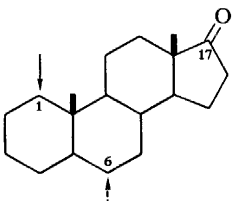
Our more significant results are in *Tables 1* and *2*. Mono-oxygenated substrates are dihydroxylated, the diequatorial (12β, 15α) substitution of the 3- and 4-ketones being in line with the earlier studies[10] on progesterone and 5α-pregnanolone. It has not been possible to obtain useful yields of mono-substitution products in these cases, but this can be done (see later) with certain disubstituted substrates. With the 17- (and 16-) ketone(s) diequatorial substitution is again observed, but now in the 1β,6α-positions (11α and 1β are nearly equivalent as far as the position of the oxygen atom is concerned)[11]. On models we can see a striking preservation pattern by rotating through 180° about a vertical axis between C-8 and C-9. This is illustrated in (IV), (V) and (VI) where the arrows denote the site and stereochemistry of hydroxylation. In general the two hydroxyl groups are introduced on carbon atoms ca. 4 Å apart; their distances from the carbonyl or other 'directing' groups, very closely comparable in the examples mentioned, vary somewhat more



(IV)

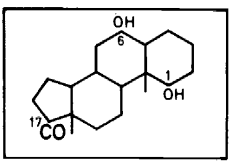


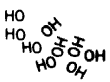
(V)



(VI)

widely. This situation is illustrated in *Figure 3*. Here nine formulae have been superimposed, matching up the hydroxylated carbon atoms and at the same time bringing the carbonyl groups as close together as possible. The correspondence that can be achieved between the hydroxylated carbon atoms is very close and although the variation of the position of the carbonyl group is rather greater, an approximate geometrical relationship is strongly suggested.





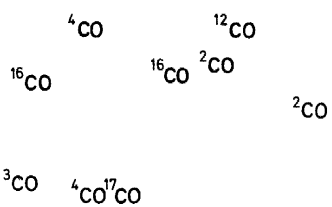


Figure 3

Dioxygenated substrates, perhaps because they are more easily able to penetrate the fungal cell walls, are more readily attacked and single (but sometimes two) hydroxyl groups are introduced. Many of the results in *Table 2* are predictable bearing in mind that terminal ring oxygen functions exert a very positive directing effect and assuming that, as the literature suggests, oxygen functions already in a substrate generally inhibit neighbouring equatorial hydroxylation. It also becomes clear that there is an order of directing power according to the position and nature of the substrate functional groups (e.g. 3-CO, 3β-OH, 3β-OMe > 17-CO but 17-CO > 3α-OH, 3α-OMe).

The overall picture which is emerging for *Calonectria decora* hydroxylations is of the dominant polar group becoming associated with a hydrophilic

region of the hydroxylating enzyme system and thereby determining the orientation in which the steroid is presented at the complementary hydrophobic sites on the enzyme complex where hydroxylation occurs. This would explain why the groups in rings A and D direct in opposite senses, and why they are more effective than those in the central rings. In these cases the molecule, without clear guidance, gets oriented in more than one way in the enzyme system and consequently converted into a multiplicity of products. The more efficient conversions of many of the dioxygenated substrates are readily understood if a hydroxylating site on the enzyme can also act as a binding site. More precise views are not justified on the basis of the kind of evidence we have available and are unlikely to be formulated until studies are possible with isolated enzyme systems[12].

Table 1. Mono-oxygenated substrates with *Calonectria decora*

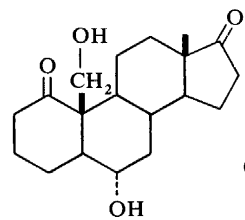
(Substrates are all 5α-androstane derivatives except where indicated. Under Conditions reference is made to use of ethanol (E) or dimethyl sulphoxide (D) as solvent for the substrate and incubation time in days. Percentage yields are based on material actually converted, i.e. allowing for recovered substrate.)

Substrate	Conditions	Substrate recovered %	Main products	%
3-CO	E5	23	**12β,15α-**$(OH)_2$	52
	D4	0	**6α,12β,15α-**$(OH)_3$	28
3-CO-Δ^4	E2	31	**12β,15α-**$(OH)_2$	55
3β-OH	E6	33	**12β,15α-**$(OH)_2$	18
Estr-4-en-3-one	D6	3	**12β,15α-**$(OH)_2$	35
4-CO	D4	0	**11α,15α-**$(OH)_2$	36
			12β,15α-$(OH)_2$	36
2-CO	D4	12	**6α,12β-** $(OH)_2$	26
			6α,11α- $(OH)_2$	11
15-CO	D6	4	14β-**6α,12β-** $(OH)_2$	21
14β-15-CO	D6	23	14β-**7β,12β-** $(OH)_2$	34
			7β,12β,14β-$(OH)_3$	10
17-CO	E4	40	**1β,6α-** $(OH)_2$	47
17β-OH	E6	41	**1β,6α-** $(OH)_2$	17
16-CO	D4	31	**6α,11α-** $(OH)_2$	33
			1β,6α- $(OH)_2$	9
12-CO	D6	8	**6α,** **15α-**$(OH)_2$	15
			1β,6α, **15α-**$(OH)_3$	12

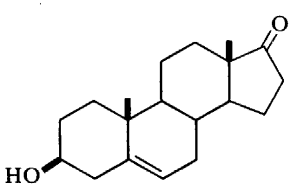
In a further investigation of the conversion of 5α-androstan-17-one into the 1β,6α-diol (VI) it was discovered[13] that the use of dimethyl sulphoxide for the substrate addition made a notable difference, as has been observed in other instances. Although the diol (VI) is formed first it is rapidly transformed, probably through the 1-keto-6α-alcohol, into the diketo-diol (VII) in which 19-hydroxylation has occurred, very rare in steroid hydroxylations. Seeking ways of taking advantage of this discovery we have now found that the 6α-OH-*i*-steroid (IX), obtained from dehydroisoandrosterone (VIII) via the 17-ketal, can be converted into the keto-triol (X), potentially useful since it can lead to 11-substituted 19-nor compounds.

Table 2. Di-oxygenated substrates with *Calonectria decora*

Substrate	Conditions	Substrate recovered %	Main products	%
3,11-$(CO)_2$	D2	27	**15**α- OH	18
			3β,**15**α-$(OH)_2$	23
3,12-$(CO)_2$	E4	10	**15**α- OH	11
			3β,**15**α-$(OH)_2$	26
3,17-$(CO)_2$-Δ^4	E2	4	**15**α- OH	36
3,7-$(CO)_2$	D2	26	3β,**12**β- $(OH)_2$	19
6,17-$(CO)_2$	E2	27	**1***β*- OH	64
7,17-$(CO)_2$	D6	25	**1***β*- OH	55
11,17-$(CO)_2$	E2	4	**6**α- OH	21
3β-OH-17-CO	D4	6	**12**β,**15**α-$(OH)_2$-3-CO	32
			3β,**12**β,**15**α-$(OH)_3$	21
3α-OH-17-CO	D4	0	**1**β,3α,**6**α- $(OH)_3$	37
			3α,**12**β,**15**α-$(OH)_3$	25
3β-OMe-17-CO	E4	12	**12**β,**15**α-$(OH)_2$	30
3α-OMe-17-CO	D4	3	**1**β, **6**α- $(OH)_2$	60



(VII)



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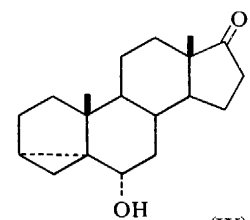
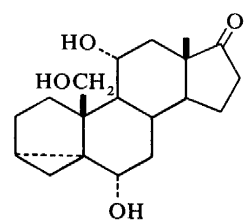


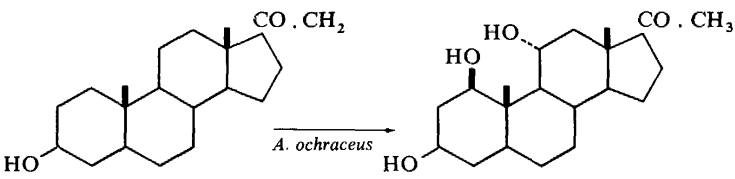
(IX)

C. decora



(X)

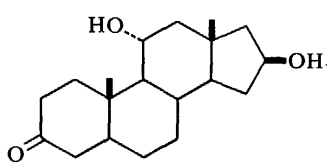
Aspergillus ochraceus is well-known as an 11α-hydroxylator and with 27 substrates, including many of those listed in *Tables 1* and *2*, we have invariably observed 11α-hydroxylation: with the 3-monoketones, 6β-substitution also takes place, switched in the 17-ketone to the 7β-position. Recently it has been shown[12], using cell-free cultures, that of the two independently-acting steroid hydroxylases which this fungus produces, the one (11α) is induced by progesterone, while the other (6β) requires 11α-hydroxypregn-4-en-3-one. We have encountered[11] a striking variation from the uniform behaviour of this organism in its conversion of 5α-pregnan-3β-ol-20-one (XI) into the 1β,11α-diol (XII). This is also notable as a rare example of the introduction



(XI) (XII)

of two almost contiguous equatorial hydroxyl groups and for the extraordinary stability (unaffected by boiling with 2N-hydrochloric acid in dioxan) of the derived acetonide.

Rhizopus nigricans mainly introduces an 11α-hydroxyl group into 3-keto-Δ^4-steroids, but hydroxylation does sometimes occur in the 6β- or 7β-positions. It was therefore surprising to discover[14] that, in the absence of 17-substituents, 16β-hydroxylation of steroids very rapidly follows the initial 11α-attack. The more significant results are set out in *Table 3*. Once again we find a clear dependence of the substitution pattern on the position of oxygen functions in the substrate. Some of the more striking observations reveal the similarity between the products (XIII and XIV) from the 3- and



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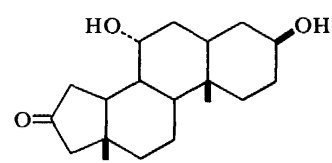
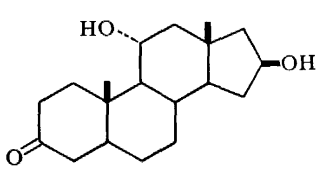


(XIV)



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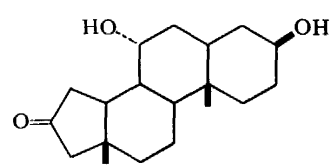


(XVI)

16-ketones, and again between those (XV and XVI) from the 3,16-keto-alcohols produced in quite high yields. In these cases we see the conventional 11α-attack by *Rhizopus nigricans* switched, in the absence of the directing 3-keto group, into the corresponding, similarly located, 7-position under the direction of the 16 (or 17)-keto-group. Moreover the 16β-substitution in ring D (in XIII) is now paralleled by 3-substitution in ring A (in XIV).

Table 3, Rhizopus nigricans incubations

Substrate	Conditions	Substrate recovered %	Products	%
(a) Mono-oxygenated substrates				
3-CO	D6	46	**11α,16β-**$(OH)_2$	33
estran-3-one	D6	59	3β, **11α,16β-**$(OH)_3$	38
			11α,16β-$(OH)_2$	23
16-CO	E6	34	**3β,7α-** $(OH)_2$	46
17-CO	D6	59	**3α, 11α-** $(OH)_2$	10
			3β,7β- $(OH)_2$	9
			6α,11α- $(OH)_2$	9
estran-17-one	D6	60	**3β,7β-** $(OH)_2$	29
(b) Di-oxygenated substrates				
3,6-$(CO)_2$	E2	6	3β, **16β-**$(OH)_2$	26
			3β, **16α-**$(OH)_2$	23
			16α- OH	23
3β-OH-6-CO	D5	0	**16α-** OH	43
7α-OH-3-CO	D6	0	**16β-** OH	33
			3β, **16β-**$(OH)_2$	24
11α-OH-3-CO	D5	0	3β, **16β-**$(OH)_2$	33
			16β- OH	20
16β-OH-3-CO	D2	0	**11α-** OH	71
17β-OH-3-CO	E2	8	**11α-** OH	33
			6α OH	90
3β-OH-16-CO	D2	0	7α OH	90
3β-OH-17-CO	E2	0	7β- OH	48
7,17-$(CO)_2$	E4	0	**3α-** OH	37
17β-OH-7-CO	E4	0	**3α-** OH	52
11,16-$(CO)_2$	E2	22	**3α-** OH	34
			3β- OH	22
17β-OH-11-CO	E4	26	**3α-** OH	47
			4α- OH	25

As with *Calonectria decora*, mono-oxygenated substrates are twice hydroxylated whereas only one hydroxyl group is introduced into the keto-alcohols and diketones. The emerging picture (cf. *Figure 4*) of the enzyme system of *Rhizopus nigricans* is of three centres in a very symmetrical arrangement [3, 11 and 16 ≡ 3, 7 (or 6) and 16]. That corresponding to the 3 position could be simply a binding site, especially for the 3 and 16 carbonyl groups, while those corresponding to the 11 (or 6 or 7) and 16 positions need to be both binding and hydroxylating sites. Most of the deviations from this pattern can be explained either, if we envisage the substrate being associated with the enzyme in a 'capsized' mode, i.e. with the angular methyl groups oriented

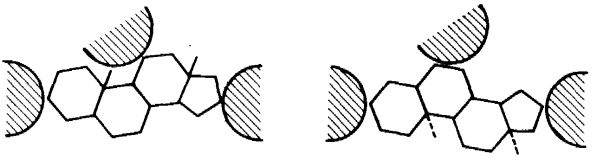
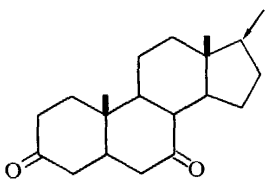


Figure 4. Representation of the binding and hydroxylating sites of *Rhizopus nigricans*

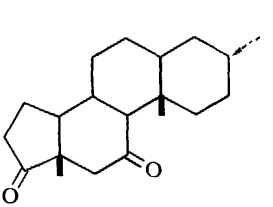
in the reverse direction by a half-revolution around a 3, 16-axis, or better, if the third site is also regarded as a dual-purpose site. It is noteworthy that the stereochemistry of the 3- and 16-hydroxylation, and also that at 7, varies with the substitution of the substrate, in sharp contrast to the great stereospecificity observed with *C. decora.*

These new modes of attack with *R. nigricans*, i.e. in the 7-position, in the terminal rings, and with the stereochemical variations, make its use, already well-established, even more attractive.

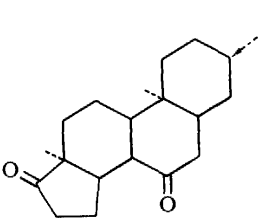
Brief mention can be made of comparable results with two other organisms. Mono-oxygenated ring A or ring D steroids are not attacked by *Ophiobolus herpotrichus* but this fungus does hydroxylate diketones, with one group in either ring B or ring C, in the unsubstituted terminal ring[13]. Thus, the 17-substitution (XVII) of the 3,7-dione is closely matched by the 3-substitution (XVIII) and (XIX) of the 11,17-dione and of the 7,17-dione respectively. In



(XVII)



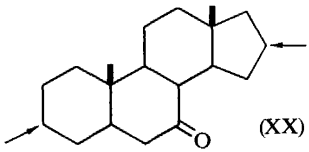
(XVIII)



(XIX)

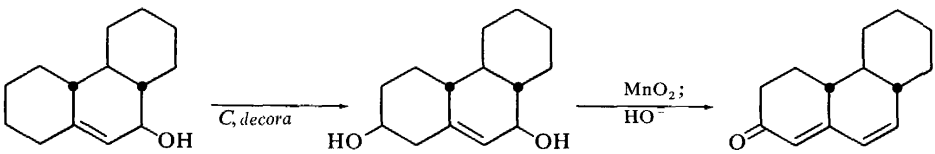
the latter case it seems likely that the substrate presents its other face (i.e. the 'capsized' mode) to the enzyme surface, expressed in the formula (XIX) (cf. *Figure 4*) by rotation both about a vertical axis through the centre of the molecule and by 180° about a horizontal 3,17- (or A,D-) axis. Such matching up of the structures, best seen of course on models, not only rationalizes the position of hydroxylation but also the 3α-substitution in (XVIII) and 3β-attack in (XIX), i.e. below or in the plane of the drawing.

Daedalea rufescens did nothing to the usual ring A and ring D mono-oxygenated steroids but gave monohydroxylation products in good yields with diketones of the ring AB, AC or BD types. Terminal ring substitution is the normal pattern here and eventually we discovered that, quite different from any of our other experiences, this fungus will satisfactorily metabolize ring B and C monoketones; thus with androstan-7-one it gives almost exclusively 3,16-dihydroxylation as in (XX) (see *Table 4*). This is a very clear indication of preparative potentialities, i.e. functionalization in both terminal rings of a synthetic steroid-like tri- or tetra-cyclic intermediate.



(XX)

Some exploration of the conversion of steroid-like synthetic compounds has been undertaken. The tricyclic alcohol (XXI) (a better substrate than the ketone) with *Calonectria decora* gives the diol (XXII), its structure being established by its transformation into the conjugated dienone (XXIII). As

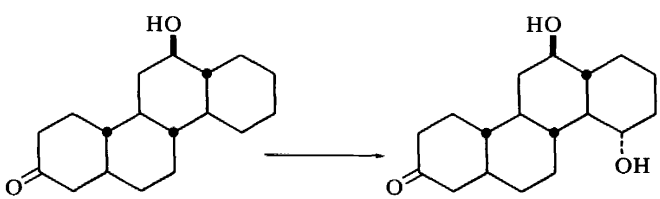


(XXI) (XXII) (XXIII)

Table 4. Daedalea rufescens transformations
(using ethanol as solvent and 6 days incubation)

Substrate	Products	%
6-CO	**3**β,6α,**16**β-$(OH)_3$	20
7-CO	**3**β,7α,**16**β-$(OH)_3$	45
3,6-$(CO)_2$	3β,6α,**16**β-$(OH)_3$	19
	3β, **16**β-$(OH)_2$-6-CO	36
3,7-$(CO)_2$	3β,7α,**16**β-$(OH)_3$	50
	7α,**16**β-$(OH)_2$-3-CO	22
3,11-$(CO)_2$	3β, **16**β-$(OH)_2$	28
	3β, **15**β-$(OH)_2$	14
7,17-$(CO)_2$	**3**β,7α,17β-$(OH)_3$	50

we expected, the same organism monohydroxylated the synthetic keto-alcohol (XXIV) in high yield in the terminal ring, but neither the product nor the recovered starting material showed appreciable optical activity. This was only to be expected in view of the comparative lack of substrate specificity



(XXIV) (XXV)

observed in these hydroxylation processes. The structure (XXV) assigned to the keto-diol is in accord with the physical data; complete confirmation is being sought by an x-ray diffraction study.

In this lecture the emphasis has been on the comparative structures of these semi-natural products to the exclusion of much interesting organic chemistry. I could have dwelt on the intriguing problems posed by our need of unusual substrates, e.g. the 2- and 16-ketones[15] or the 15-oxygenated compounds, for which a microbiological route[7] was rather attractive to us. I could have related how we encountered the surprisingly facile *trans* → *cis* conversion of the 1,6-dioxo-steroids[16] and the embarrassing rust-catalysed conversion of 6β,11α-dihydroxyandrost-4-en-3-one into the 11α-hydroxy-3,6-diketone as a result of doing extractions in steel drums. Instead I have directed attention to the broad substitution patterns which can now be discerned. There is certainly more to do, new possibilities are continually opening up, but already there are clear indications of a much greater scope for using micro-organisms to functionalize a wide range of organic compounds. We are a bit farther along the road to x-Å hydroxylation.

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