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Microbiological hydroxylation of 5α-*H*-steroids

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ABSTRACT. The literature data on the use of microrganisms for the hydroxylation of 5α-*H*-steroids of the 5α-*H*-androstane and 5α-*H*-pregnane series and also 5α-*H*-sapogenins are surveyed. A systematic description of the influence of substitution in the steroid molecule on the specificity of the hydroxylation and the activity of the microbial enzymes is given within the framework of the enzyme–substrate interaction model.

The bibliography includes 124 references.

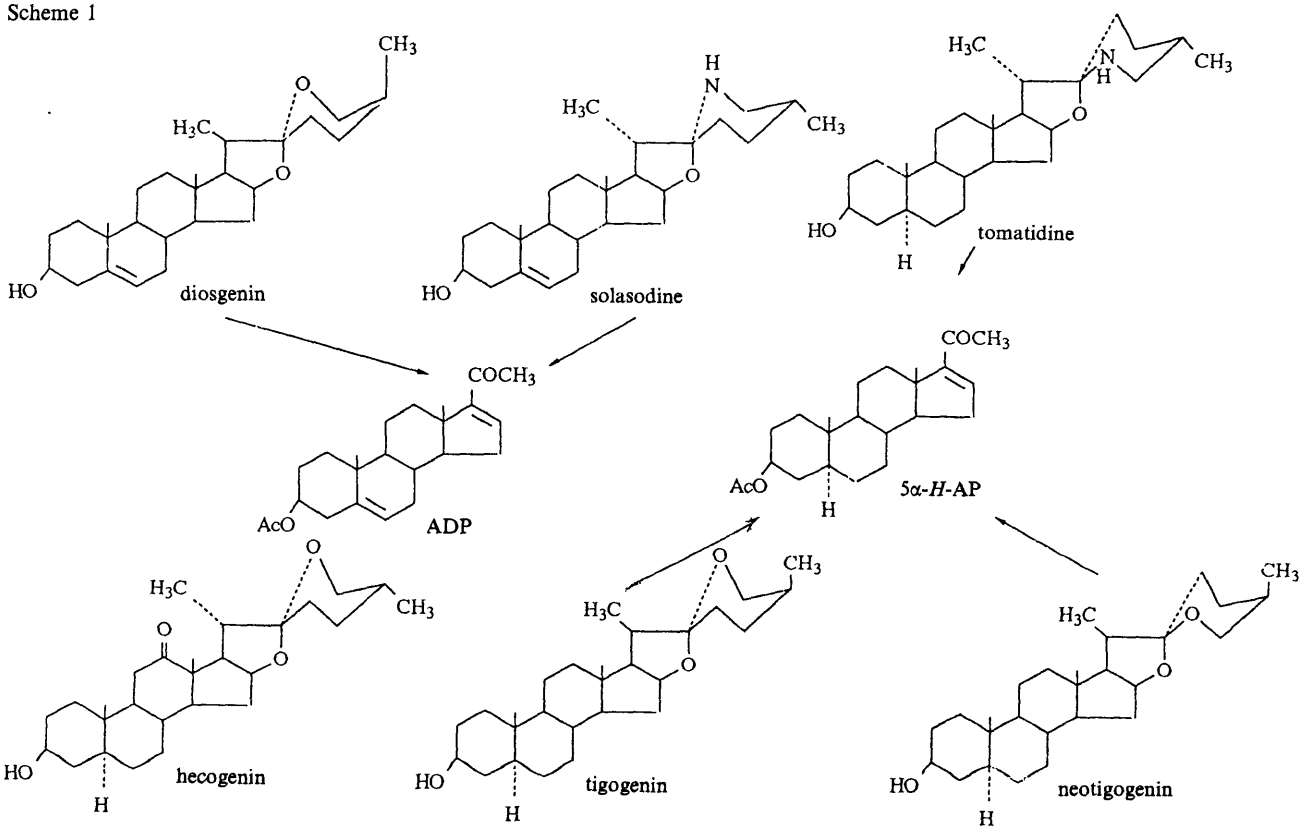
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I. Introduction

The industrial manufacture of corticosteroids is based nowadays mainly on diosgen, solasodin, and their cleavage reaction intermediate 16-dehydropregnenolone 3β-acetate (DPA). The expansion of the manufacture of steroids has become a significantly more difficult in view of the sharp increase in the cost of these raw materials which are purchased in countries with a tropical climate. The only local industrial source of the steroid raw material is solasodine, the requirement for which in the CIS countries is not satisfied. For this reason 5α-*H*-steroids—hecogenin, tigogenin, neotigogenin,[1] and 3β-acetoxy-5α-*H*-Δ^{16}-pregn-20-enone (5α-*H*-AP) obtained from them—are used with increasing frequency as promising alternative sources. So far they cannot compete with their Δ^5-analogues because the *A* and *B* rings are saturated and it is difficult to transform them by chemical means into the Δ^4- and $\Delta^{1,4}$-3-oxo-substituted steroid preparations which are of practical value (Scheme 1).

Scheme 1



Data have now been accumulated concerning the possibility of microbiological methods for the effective transformation (hydroxylation and dehydrogenation) of 5α-*H*-steroids. However, they have never been treated systematically and are therefore unavailable for deliberate practical application. Furthermore, most of them refer to the 5α-*H*-androstane series and an appreciably smaller number to the 5α-*H*-pregnane series, the practical value of which is incomparably greater. Nevertheless, close examination shows that there is a close analogy between these series, which makes it possible to compare the results and permits their *a priori* transfer from the androstane to the pregnane series. The principal aim of the review is to present a systematic picture reflecting the influence of substituents in various positions in the steroid nucleus on the microbiological hydroxylation of 5α-*H*-steroids; the examination of the transformations of the *A* ring in 5α-*H*-steroids with the aid of microbiological dehydrogenation and subsequent 9α-hydroxylation effected by bacteria remains outside its framework. We believe that it is also undesirable to include in the review and to consider data on the transformation of 5β-*H*-steroids, which are not only less available but are also factually non-comparable. Thus it is known that the geometrical isomerism at the C(5) atom, which controls the junction between the *A* and *B* rings, is one of the most important factors influencing the interaction of steroid substrates with steroid hydroxylases.[2] We shall illustrate this using as an example androsterone and its 3β-hydroxy-isomer, which is hydroxylated by the mould *Penicillium* sp. in the 1α- and 12β-positions respectively. Their *A/B-cis*-analogues are hydroxylated only in the 7β-position regardless of the configuration of the 3-hydroxy-group.[3] The mould *Aspergillus tamarii* hydroxylase androsterone and its 3β-hydroxy-isomer in the 11β- and 11α-positions, whereas their *A/B-cis*-analogues are hydroxylated in the 7β-position.[4] Yet another example of compounds of the pregnane series is 16α,17α-epoxy-5α-*H*-pregnan-3-one, which is transformed with the aid of *Rhizopus nigricans* into the 11α-hydroxy-derivative (60), while its 5β-*H*-isomer is also transformed in the same way but with much difficulty.[5] The critical change in the stereochemistry of the substrate (from *A/B-trans-* to *A/B-cis-*) undoubtedly affects the steric structure of the enzyme – substrate complex; as can be seen from the foregoing, this factor becomes decisive and requires independent consideration.

Studies on the microbiological transformations of steroids refer mainly to compounds with Δ^4-3-oxo or Δ^5-3β-hydroxy-groups and have been surveyed in a number of communications.[2,6–11] The efforts of many investigators have been directed in this connection to the study of the mechanisms of the microbiological transformations and to the specificity of the action of microorganisms as a function of their positions in the classification system and on the stereochemical features of the steroid substrates. In contrast to dehydrogenation, the study of the mechanism of the hydroxylation reaction is complicated by the considerable lability of the steroid hydroxylases.[12] The question why the same culture changes the mode of hydroxylation when the steroid substrate is altered remains unelucidated. The question arises whether it is a consequence of the functioning of different enzymes in the cell. The causes of the microbiological hydroxylation of steroids have not been finally established. According to the existing hypotheses, the microbiological transformation of steroids is induced in certain cases by the need for their detoxication, while in others it occurs as a stage in the assimilation of steroids as a source of carbon. The latter has been confirmed in relation to bacterial hydroxylation. Bacteria begin to cleave the steroid molecule with hydroxylation of the 9α-, 22-, and 26-positions. However, there are no data concerning the causes of the hydroxylation in the 16α-position characteristic of streptomycetes.[6–11] With the exception of the examples of the hydroxylation by schizomycetes described, in the vast majority of cases steroids have been hydroxylated with the aid of representatives of all classes of fungi in the 1α-, 1β-, 2α-, 2β-, 3β-, 4β-, 5β, 6α-, 6β-, 7α-, 7β-, 8β-, 9α-, 10β-, 11α-, 11β-, 12α-, 12β-, 14α-, 15α-, 15β-, 16α-, 16β-, 17α-, 18-, and 21-positions.[6–11] This review, which constitutes a first attempt at a systematic treatment and analysis of the scattered data concerning the transformation of 5α-*H*-steroids, including the results published in 1992, deals with examples of the hydroxylation by fungal cultures only. All the species of fungi used for the transformation of 5α-*H*-steroids are presented in Table 1.

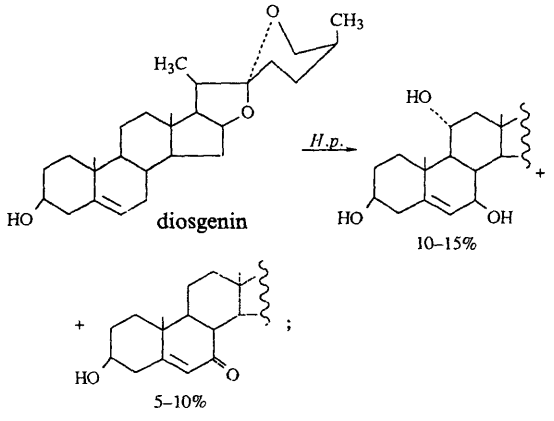
Table 1. The taxonomy of the fungi hydroxylating 5α-*H*-steroids.

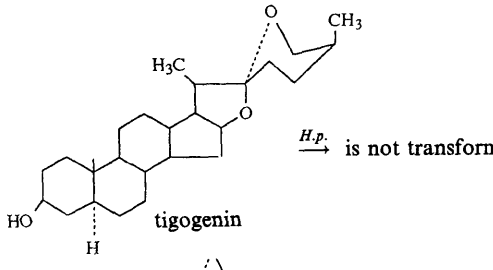
Class	Genus	Species	Abbreviated designation	Refs.
Ascomycetes	*Calonectria*	*decora*	*C.d.*	[13, 16, 19, 20, 30, 34–36, 100–103]
	Diaporthe	*celastrina*	*D.c.*	[31]
	Didymella	*vodakii*	*D.v.*	[64]
	Glomerella	*cingulata*	*Gl.c.*	[58]
		fusaroides	*Gl.f.*	[58]
		lagenarium	*Gl.l.*	[58]
	Gymnoascus	*reesii*	*G.r.*	[97]
	Ophiobolus	*herpotrichus*	*O.h.*	[27]
Basidiomycetes	*Corticium*	*praticola*	*C.pr.*	[38]
	Daedalea	*rufescens*	*D.r.*	[28, 35]
	Hypholoma	*sublateritium*	*H.s.*	[99]
	Leptoporus	*fissilis*	*L.f.*	[37]
	Polystictus	*sanguineus*	*P.s.*	[38]
Deuteromycetes	*Aspergillus*	*nidulans*	*A.n.*	[38]
		ochraceus	*A.o.*	[17, 18, 21, 25, 26, 29, 34, 36, 38, 68, 80, 93, 98, 111]
		tamarii	*A.t.*	[4]
	Beauveria	sp.	*B.sp*	[62]
	Chaetomella	*oblonga*	*Ch.o.*	[113]
		raphigera	*Ch.r.*	[113]
	Curvularia	*clavata*	*C.cl.*	[82, 87, 88, 117]
		lunata	*C.l.*	[59, 61, 65, 68, 105–107]
	Epicoccum	*humicola*	*E.h.*	[114]
		neglectum	*E.n.*	[114]
		oryzae	*E.o.*	[114]
		yuccae	*E.u.*	[114]
	Fusarium	*graminearum*	*F.g.*	[71]
		sulfureum	*F.s.*	[57]
	Gliocladium	*roseum*	*G.r.*	[116]
	Phoma	sp.	*Ph.sp.*	[60]
	Penicillium	sp.	*P.sp.*	[3, 70, 77]
		urticae	*P.u.*	[18]
	Pestalotia	*funerea*	*P.f.*	[64, 110]
	Spondylocladium	*australe*	*Sp.a.*	[66]
		xylogenum	*Sp.x.*	[66]
	Sporotrichum	*epigaeum*	*S.e.*	[63]
		sulfurescens	*S.s.*	[55, 63]
	Wojnowicia	*graminis*	*W.g.*	[27]
Phycomycetes	*Absidia*	*regnieri*	*A.r.*	[33]
	Circinella	sp.	*C.sp.*	[67]
	Cokeromyces	*recurvatus*	*C.r.*	[115]
	Cunninghamella	*blakesleeana*	*C.bl.*	[107]
		bainieri	*C.b.*	[124]
		elegans	*C.el.*	[47, 89–96]
	Helicostylum	*piriforme*	*H.p.*	[41–46, 84, 86, 87]
	Mucor	*griseo-cyanus*	*M.g.-c.*	[56]
	Thieghemella	*orchidis*	*T.o.*	[107]
	Rhizopus	*arrhizus*	*R.a.*	[24, 35, 38, 54, 69]
	Rhizopus	*circinans*	*R.c.*	[24, 35]
		nigricans	*R.n.*	[5, 14, 22, 23, 26, 29, 34–36, 54, 79, 81, 111, 112]
	Syncephalastrum	*racemosum*	*S.r.*	[33, 85]

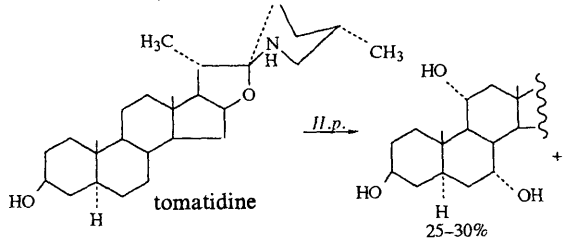
II. Hydroxylation of 5α-*H*-sapogenins

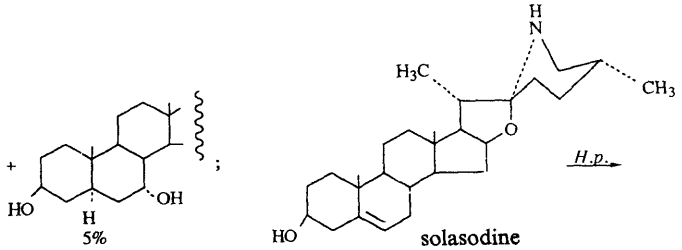
After the discovery of the ability of lower fungi to hydroxylate progesterone,[39] a number of attempts were made to obtain microbiologically hydroxylated steroid sapogenins, because they were regarded as promising initial substrates in the synthesis of steroid hormones (Scheme 1). The first result of such transformations were negative despite the fact that fairly wide ranging microorganisms and conditions in their cultivation were employed for this purpose.[40] However, later Japanese workers obtained with the aid of phycomycetes *H.p.* the hydroxy-derivatives of both Δ^5- and 5α-*H*-sapogenins (diosgenin, tomatidine solasodine, and 5,6-dihydrosolasodine.[41–46] The fact that diosgenin and tomatidine, a 5α-*H*-steroid alkaloid, undergo the same type of transformation and the complete absence of a microbiological transformation of tigogenin compared with diosgenin (Scheme 2) merits attention.[44]

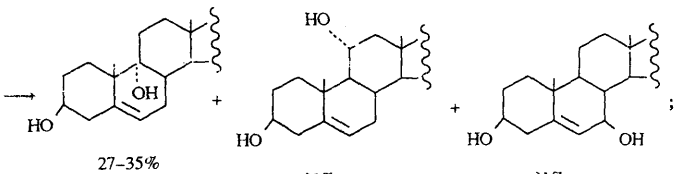
Scheme 2

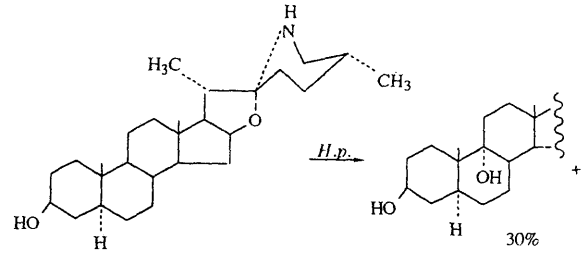




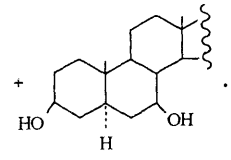








5,6-dihydrosolasodine



The principal site of hydroxylation is determined by the structure of the substrate. Diosgenin and tomatidine are transformed predominantly with formation of 11α-hydroxy-derivatives, while solasodine and 5,6-dihydrosolasodine are converted into 9α-hydroxy-derivatives.[41,46] It must be emphasised that in the transformation of tomatidine by the *H.p.* culture the nitrogen atom in the *F* ring does not hinder the 11α-hydroxylation.

A repeated attempt to obtain the hydroxy-derivatives from 5α-spirostanols was undertaken 20 years after the studies mentioned above. 1β,7β-Dihydroxy-derivatives of hecogenin were obtained with the aid of *C. el.*[47] In contrast to *H.p.* and *C. el.*, the cultures of the *Gymnoascus, Scopulariopsis, Fusarium, Dendrostibella,* and *Hypomyces* fungi do not hydroxylate 5α-*H*- and Δ^5-sapogenins, but, like bacteria, transform the *A* ring and cleave the side chain.[48–53]

III. Hydroxylation of 5α-*H*-androstanes

The hydroxylation by fungi of 5α-*H*-androstanes has been most widely investigated. Analysis of the results of the transformations of different 5α-*H*-androstanes (monoketones, diketones, ketoalcohols), chosen as the simplest model compounds for the interpretation of the behaviour of the microorganisms, permitted the main conclusions concerning the mechanism of the microbiological hydroxylation of the steroids. The hydroxylating activity in relation to 5α-*H*-androstanes has been investigated for fungi belonging to different classes of ascomycetes, basidiomycetes, deuteromycetes, and phycomycetes, which are known to be capable of hydroxylating Δ^4-3-oxosteroids in the 11α-positions (*A.o., R.n.*), the 11β-position (*C.cl.*), the 12β- and 15α-position (*C.d., P.u., P.*sp.), and the 21-position (*W.g., O.h.*).[2,6,7] Cultures of *A.t.*,[4] *D.r.*,[28] *A.r.*,[33] *L.f.*,[37] *D.c.*,[31] and *R.c.*,[24] which had not been investigated previously, were also tested. The results of these tests are presented in Tables 2 and 3.

Table 2. Hydroxylation of 5α-*H*-androstanes by fungi.

Substituents in substrate	Micro-organism	Initial compound, %	Principal hydroxylation sites	Yield, %	Secondary hydroxylation sites	Yield[a], %	Refs.
1	2	3	4	5	6	7	8
2,7-$(CO)_2$	*L.f.*	15	16β	22	16β, 18	6	[37]
2,7-$(CO)_2$	*W.g.*	15	2β, 17β	19	17β	9	[27]
					2β,18	10	
3-CO	*A.o.*	56	6β, 11α	51	11α	11	[21]
3-CO	*A.r.*	55	9α, 15β	8	9α	4	[33]
3-CO	*W.g.*	72	9α, 17β	13	–	–	[27]
3-CO	*D.r.*	90	not transformed		–	–	[28]
17-CO	*A.o.*	65	7β, 11α	27	–	–	[21]
3,6-$(CO)_2$	*D.r.*	9	3β, 16β	39	3β, 15β	12	[28]
					3β, 6α, 16β	25	
3,6-$(CO)_2$	*L.f.*	21	16β	12	3β, 16β	8	[37]
3,7-$(CO)_2$	*W.g.*	32	3β, 17β	41	3α, 17β	27	[27]
3,7-$(CO)_2$	*L.f.*	4	16β, 18	7	3β, 18	5	[37]
3,7-$(CO)_2$	*O.h.*	12	3β, 17β	62	–	–	[27]
3,7-$(CO)_2$	*A.r.*	0	3β, 11α	6	Δ^1-17-CO	3	[33]
					11α	1	
3,7-$(CO)_2$	*S.r.*	3	3β, 12α	13	7α, 9α	5	[33]
		3	3β, 12β	6	1β	3	
					11α	2	
					12α	2	
					7α, 9α, 11α	2	
3,7-$(CO)_2$	*D.c.*	7	3β, 16α	48	3β, 16β	11	[31]
3,7-$(CO)_2$	*R.a.*	3	16β	15	16α	3	[24]
			3β, 16β	31	11α, 16β	3	
					17α	2	
3,7-$(CO)_2$	*R.c.*	7	3β, 11α	22	11α, 16β	5	[24]
			16β	10	3β, 16β	6	
3,7-$(CO)_2$	*D.r.*	3	3β, 7α, 16β	33	3β, 7α, 16-CO	3	[28]
					3β, 7β, 16β	13	
3,11-$(CO)_2$	*A.r.*	0	17β, ($\Delta^{1,4}$)	6	16β ($\Delta^{1,4}$)	4	[33]
					17-CO($\Delta^{1,4}$)	2	
					16-CO	2	
3,11-$(CO)_2$	*S.r.*	–	16β($\Delta^{1,4}$)	18	17β($\Delta^{1,4}$)	9	[33]
					17-CO($\Delta^{1,4}$)	9	
3,11-$(CO)_2$	*D.c.*	0	3β, 16α	55	16α	18	[31]
					3β, 11, 16-$(CO)_2$	10	
3,11-$(CO)_2$	*R.c.*	17	6α	20	3β, 7β	3	[24]
			16β	15	9α, 16β	2	
			7β	10			
3,11-$(CO)_2$	*D.r.*	5	3β, 16β	28	3β, 15β	15	[28]
3,11-$(CO)_2$	*L.f.*	11	16β	13	16β, 18	5	[37]
3,11-$(CO)_2$	*R.a.*	16	3β, 16β	22	16β	7	[24]
			9α, 16β	23	9α, 16α	7	
3,11-$(CO)_2$	*O.h.*	46	16β	11	16-CO	38	[27]
			17β	11	16-CO, 3β	21	
3,11-$(CO)_2$	*W.g.*	24	17β	28	3α, 17β	8	[27]
					3β, 17β	13	
					3β, 16β	6	
3,11-$(CO)_2$	*P.u.*	—	15α	31	—	—	[18]
			3β, 15α	15	–	–	
3,16-$(CO)_2$	*L.f.*	15	18	16	—	—	[37]
3,17-$(CO)_2$	*W.g.*	7	1α, 17β	7	1α, 3α, 9α, 7β	1	[27]
			1α, 3β	6	7α	1	

Table 2 (cont'd).

1	2	3	4	5	6	7	8
3,17-$(CO)_2$	*S.s.*	–	11α	22	3β	7	[55]
3,17-$(CO)_2$	*A.r.*	0	9α	14	3β, 9α	5	[33]
			7α, 9α	13	7α	10	
3,17-$(CO)_2$	*A.r*	–	9α	11	7α	2.4	[33]
					1β, 9α	0.7	
3,17-$(CO)_2$	*C.cl.*	–	11β	–	7α	–	[82]
3,17-$(CO)_2$	*D.c.*	6	3β, 9α	6	9α	3	[31]
3,17-$(CO)_2$	*S.r.*	0	3β, 7α	7	3β	7	[33]
			3β, 7α, 17β	3	3β, 9α	2	
3,17-$(CO)_2$	*D.r.*	0	3β, 7β, 17β	24	3β, 11α, 17β	13	[28]
					3β, 6α, 17β	11	
3,17-$(CO)_2$	*A.o.*	–	11α	66	–		[21, 93]
3,17-$(CO)_2$	*A.t.*	–	6β	25[b]	–	–	[4]
			11β	30[b]	–	–	
3,17-$(CO)_2$	*P.sp.*	–	1α	17	–	–	[70]
6-CO	*D.r.*	21	3β, 6α, 16β	16	3β, 6α, 16-CO	5	[28]
					3β, 16β	4	
					3β, 15β	2	
6,17-$(CO)_2$	*L.f.*	17	2β	13	–	–	[37]
7-CO	*D.r.*		3β, 7α, 16β	41	–	–	[28]
			3β, 7β, 16β	16	–	–	
7,11-$(CO)_2$	*D.r.*	0	3β, 7α, 16β	42	–	–	[28]
7,16-$(CO)_2$	*D.r.*	0	3β, 7α	39	–	–	[28]
7,17-$(CO)_2$	*D.r.*	0	3β, 7α, 17β	32	3-CO, 7β, 17β	5	[28]
			3β, 7β, 17β	16			
7,17-$(CO)_2$	*A.r.*	33	5α	6	–	–	[33]
7,17-$(CO)_2$	*S.r.*	4	1α, 17β	23	12β, 17β	7	[33]
					11α, 17β	11	
					5α, 17β	4	
7,17-$(CO)_2$	*W.g.*	13	2α	34	3β	11	[27]
			2α, 17β	30	3β, 17β	5	
7,17-$(CO)_2$	*L.f.*	12	2β	24	–	–	[37]
7,17-$(CO)_2$	*O.h.*	38	3β, 17β	43	3β	32	[27]
7,17-$(CO)_2$	*R.c.*	38	3β	15	11α, 17β	8	[24]
			11α	18	4α	6	
					3β, 17β	5	
7,17-$(CO)_2$	*R.a.*	11	3α	31	3β	6	[24]
					3β, 17β	6	
					3α, 17β	2	
					3α, 11α	6	
					3β, 11α	2	
					11α	3	
7,17-$(CO)_2$	*D.c.*	0	3α	52	1α	4	[31]
					17β	7	
11-CO	*D.r.*	10	3β, 14α, 16β	12	3β, 16-CO	7	[28]
					3β, 16α	5	
11-CO	*O.h., W.g.*	50—58	not isolated	–	–	–	[27]
11,17-$(CO)_2$	*A.r.*	34	–	–	17β	28	[33]
11,17-$(CO)_2$	*S.r.*	37	7α, 17β	27	5α, 17β	9	[33]
					7α	5	
11,17-$(CO)_2$	*D.c.*	32	7α, 17β	11	7α	10	[31]
11,17-$(CO)_2$	*L.f.*	39	not isolated	–	–	–	[37]
11,17-$(CO)_2$	*O.h.*	63	3α	45	17β	44	[27]

Table 2 (cont'd).

1	2	3	4	5	6	7	8
11,17-$(CO)_2$	*R.a.*	11	4α, 17β	27	7β, 17β	8	[24]
			3α, 17β	20			
11,17-$(CO)_2$	*R.c.*	19	4α, 17β	54	7β, 17β	18	[24]
16-CO	*D.r.*	58	not trans-formed	–	–	–	[28]
17-CO	*D.r.*	50	not trans-formed	–	–	–	[28]
17-CO	*H.p.*	–	not trans-formed	–	–	–	[86]
17-CO	*O.h.*, *W.g.*	74—87	not trans-formed	–	–	–	[27]
17-CO	*C.el.*	50	1β, 7α	14	1β, 7-CO	6	[92]
3α-OH, 6-CO	*L.f.*	0	16β, 18	39	16β	31	[37]
3β-OH, 6-CO	*L.f.*	–	17β	40	17-CO	3	[37]
			16β	27	16-CO	3	
3β-OH, 6-CO	*A.r.*	5	9α	23	14α	9	[33]
					11α	5	
3β-OH, 7-CO	*L.f.*	0	16β	24	16β, 18	6	[37]
3α-OH, 7-CO	*W.g.*	6	17β	55	–	–	[27]
3β-OH, 7-CO	*W.g.*	4	17β	63	3-CO, 17β	3	[27]
					17-CO	5	
					7α, 17β	2	
3β-OH, 7-CO	*S.r.*	0	12α	51	9α	19	[33]
					12β	8	
3β-OH, 11-CO	*D.r.*	0	16β	17	15β	8	[28]
					14α, 16β	5	
					16-CO	3.5	
3β-OH, 11-CO	*L.f.*	5	16β, 18	24	–	–	[37]
3β-OH, 11-CO	*W.g.*	48	6β	48	17β	22	[27]
3β-OH, 11-CO	*D.r.*	0	16β	17	15β	8	[28]
					14α, 16β	5	
					16-CO	3.5	
3β-OH, 16-CO	*L.f.*	22	18	21	–	–	[37]
3α-OH, 17-CO	*R.a.*	0	11α	30	7β	8	[24]
3α-OH, 17-CO	*P.sp.*	–	12β	12[b]	–	–	[3]
3β-OH, 17-CO	*P.sp.*	–	7α	11.8[b]	–	–	[3]
3α-OH, 17-CO	*A.t.*	–	11β	30[b]	11α	5	[4]
3α-OH, 17-CO	*A.o.*	–	11α	82	–	–	[18]
3α-OH, 17-CO	*C.el.*	0	7β	43	7α	10	[92]
3β-OH, 17-CO	*R.a.*	–	7β	57	7-CO	1	[24]
			7α	20	6α	8	
3β-OH, 17-CO	*W.g.*	5	1α	22	14α	13	[27]
					9α	7	
					5α	7	
3β-OH, 17-CO	*F.g.*	–	15α	–	–	–	[71]
3β-OH, 17-CO	*A.t.*	–	11β	45[b]	–	–	[4]
3β-OH, 17-CO	*D.c.*	0	9α	40	3-CO, 9α	15	[31]
					7α	12	
3β-OH, 17-CO	*M.g.-c.*	–	7α	–	7α, 14α	–	[56]
3β-OH, 17-CO	*R.c.*	7	7β	43	6α	25	[24]
3β-OH, 17-CO	*A.r.*	4	9α	19	9α, 14α	2	[33]
					7β	12	
					7α, 14α	4	
3β-OH, 17-CO	*S.r.*	0	7β	27	7-CO	10	[33]
			7β, 17β	24			
3α-OAc, 17-CO	*C.el.*	15	6β, 11β	22	1β	8,0	[92]

Table 2 (cont'd).

1	2	3	4	5	6	7	8
3β-OAc, 17-CO	*C.el.*	0	7β	31	7α	3	[92]
					7-CO	3	
6β-OH, 3-CO	*L.f.*	0	10β, 18	34	–	–	[37]
6α-OH, 17-CO	*R.c.*	37	11α	56	3β	5	[24]
6α-OH, 17-CO	*R.a.*	4	11α	72	11α, 17β	5	[24]
7α-OH, 3-CO	*D.r.*	28	16β	53	3β, 16β	28	[28]
7β-OH, 3-CO	*D.r.*	33	3β	18	–	–	[28]
7β-OH, 11-CO	*A.r.*, *S.r.*	22—63	–	–	17-CO	15—25	[33]
9α-OH, 3-CO	*D.c.*	3	3β, 16α	47	16α	15	[31]
9α-OH, 3-CO	*A.r.*	82	not isolated				[33]
9α-OH, 3-CO	*S.r.*	10	3β, 12α	12	–	–	[33]
11α-OH, 3-CO	*A.r.*	49	not isolated				[33]
11α-OH, 3-CO	*S.r.*	25	3β, 7β	27	7β	17	[33]
					3β	4	
11α-OH, 3-CO	D.c.	28	16α	9	–	–	[31]
12α-OH, 3-CO	D.c.	0	3β, 16α	48	16β	19	[31]
12α-OH, 3-CO	S.r., *A.r.*	53—65	–	–	3β	6—10	[33]
17β-OH, 3-CO	*A.t.*	–	11β	20[b]	–	–	[4]
17β-OH, 3-CO	*D.c.*	54	3β, 9α, 17-CO	18	3β, 9α	9	[31]
17β-OH, 3-CO	*A.r.*	5	9α	14	7α, 9α, 17-CO	7	[33]
					9α, 14α	2	
17β-OH, 3-CO	*L.f.*	67	not isolated				[37]
17β-OH, 3-CO	*A.o.*	7	11α	79	6α, 11α	2	[26]
17β-OH, 3-CO	*R.a.*	21	11α	23	3β, 7β	5	[24]
			6α	12	3β, 6α	4	
					3β, 11α	4	
					5α	4	
17β-OH, 3-CO	*D.c.*	54	3β, 9α, 17-CO	18	9α, 17-CO	4	[31]
					3β, 9α	9	
17β-OH, 3-CO	*R.c.*	19	6α	33	–	–	[24]
			11α	26	–	–	
17β-OH, 3-CO	*R.sp.*	–	1α	–	–	–	[70]
17β-OH, 3-CO	*C.el.*	–	7α	18	9α	0.5	[93]
					6α	3	
					12α	0.8	
17β-OH, 3-CO	*D.r.*, *O.h.*	81	not isolated				[27]
17β-OH, 3-CO	*W.g.*	7	1α	5	3β	2	[27]
			1α, 3α, 9α	2			
17β-OH, 3-CO	*S.r.*	0	3β, 7α	27	7β	9	[33]
					9α	7	
17β-OH, 6-CO	*S.r.*	19	1α, 17-CO	23	–	–	[33]
17β-OH, 7-CO	*W.g.*	14	2α	58	3β, 7α	5	[27]
			2α, 7α	15			
			3β	11			
17β-OH, 7-CO	*D.c.*	0	3β	26	3α, 17-CO	16	[31]
					3α	7	
17β-OH, 7-CO	*A.r.*	18	3β	24	3α, 17-CO	6	[33]
17β-OH, 7-CO	*S.r.*	0	3β, 12β	23	3β	17	[33]
					12β	1	
					1β	7	
17β-OH, 11-CO	*W.g.*	39	3β	35	3α	11	[27]
17β-OH, 11-CO	*A.r.*, *S.r.*	22—63	–	–	17-CO	15—25	[33]
17β-OH, 11-CO	*D.c.*	8	7α	18	7,17-$(CO)_2$	5	[31]

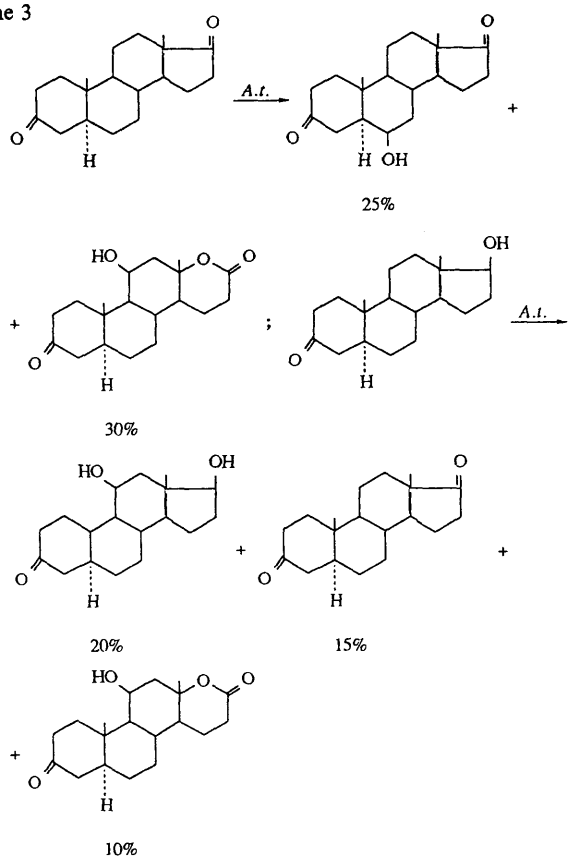
Table 2 (cont'd).

1	2	3	4	5	6	7	8
3β, 7α-$(OH)_2$-17-CO	*D.c.*	70	3-CO	56	–	–	[31]
3β, 7β-$(OH)_2$-17-CO	*A.o.*	16	7β, 11α	78	–	–	[26]

[a] Here and in the subsequent tables, the yields are quoted relative to the converted substrate.
[b] The yields of the product isolated relative to the substrate introduced into the reaction.

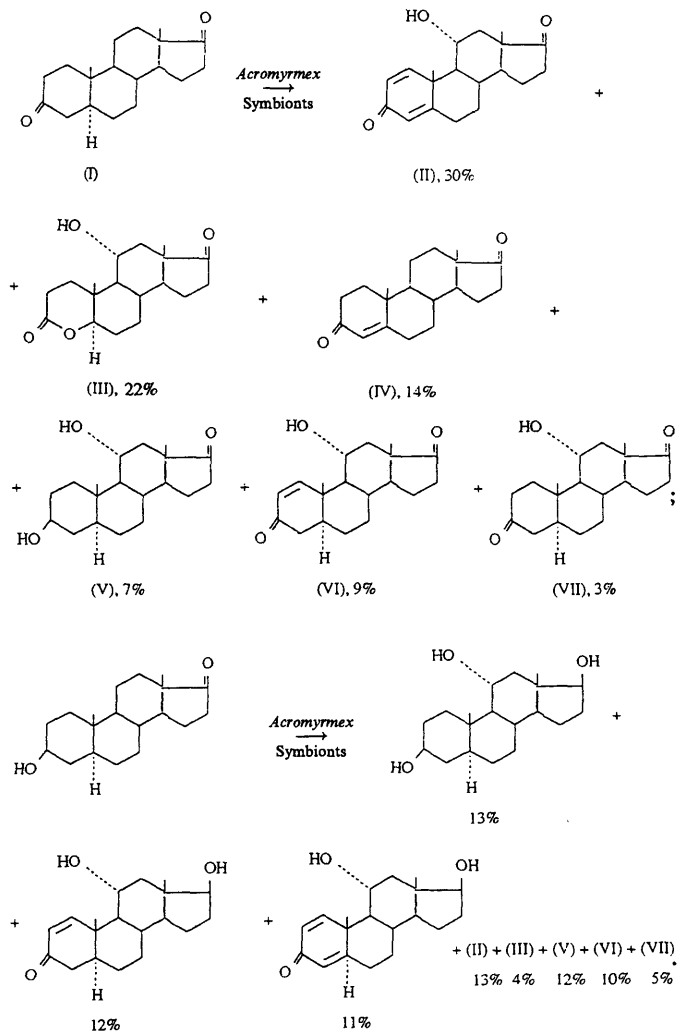
In assessing the data in Table 2, attention should be drawn to what we believe to be two important factors. Strange as it may seem, among all the species investigated, only the strain *A.t.* forms 11β-monohydroxylated compounds in the series of 5α-*H*-androstanes in a sufficiently high yield. However, the lactonisation of the *D* ring is characteristic of the culture and unfortunately this process competes with the main 11β-hydroxylation reaction. The presence of 3-oxo-group in the substrate activates the cleavage of the *D* ring with formation of the lactone, whilst in the incubation of *A.t.* with 3-hydroxy-5α-*H*-androstane *D*-homoisomerisation does not occur* (Scheme 3).

Scheme 3



The second phenomenon which merits attention refers to unidentified fungi—symbionts of ants, which have not been investigated either before the study mentioned above[32] or after it. It follows from Scheme 4 that the symbiont fungi catalyse three processes in the series of 5α-*H*-androstanes: (a) the hydroxylation of the sites specified by the directing influence of the existing substituents in the molecule; (b) the Baeyer–Villiger oxidation; (c) dehydrogenation of the *A* ring. The last two processes occur only in the presence of an oxygen-containing substituent in the 11-position. Examples comprising 5α-*H*-androstanes with oxygen-containing functional groups in the 3- and 17-positions confirm the constancy of the overall mode of the process as the nature of the oxygen-containing substituent at C(3) is varied (Scheme 4).

Scheme 4



It is regrettable that similar studies have not been made with compounds of the 5α-*H*-pregnane series, because the simultaneous activation of the *A* and *C* rings in the pregnane series appears to be an urgent task. The rare combination of the hydroxylating and dehydrogenating activities in the *Acromyrmex* fungi can be

explained, in our view, by the fact that the fungi constitute a microbial consortium whose members perform different transformations of the steroid molecule. The ability of fungi of the genus *Septomyxa* to transform 5α-*H*-steroids into the $\Delta^{1,4}$-3-oxo-compound is known in principle, but this does not entail hydroxylation.[72–74]

Table 3. Hydroxylation by *Calonetria decora* and *Rhizopus nigricans* cultures of 5α-androstanes monosubstituted and disubstituted by oxygen-containing groups.

Substituents in substrate	*Calonetria decora* (Refs. 13, 19, 20, 30)					*Rhizopus nigricans* (Refs. 14, 22, 23)				
	recovered substrate, %	principal hydroxylation sites	Yield, %[a]	Secondary hydroxylation sites	Yield, %[a]	recovered substrate, %	principal hydroxylation sites	Yield, %[a]	secondary hydroxylation sites	Yield, %[a]
1-CO	75	complex mixture	–	–	–					
2-CO	13	6α,12β	26	6α, 11α	11	51	6α, 16α	10	6α,16α(2-CO → 2α-OH)	2.5
3-CO(EtOH)	23	12β, 15α	40—52	12β, 15α(3-CO → 3β-OH)	13	46	11α, 16β	18	6β, 11α	4
3-CO(ДМСО)	0	6α, 12β, 15α	28			–	11α, 16β	33	6β, 11α	4
									11α	1
3β-OH(EtOH)	33	12β, 15α	18	–	–					
4-CO	0	12β,15α	41	–	–	40	11α, 16β	9	11α, 16β(4-CO → 4β-OH)	7
		11α, 15α	40	–	–					
6-CO	90	does not react				90	does not react			
7-CO	72	"		12β, 15α	4	90	3α, 16β	34	4β,16β	16
11-CO	40	1β, 6α	11	6α	3	62	4α, 16β	7	3α,16β	5
									3β, 16β	3
15-CO	4	14β, 6α, 12β	21	2α, 12β	8	59	not isolated			
16-CO	31	6α, 11α	26—33	1β, 6α	7	34	3β, 7α	46		
17-CO	32—75	1β, 6α	15—47	1-CO, 6α, 19	38	59	3α, 11α	10	3α, 6α	8
12-CO	8	6α, 15α	15	1β, 6α, 15α	12	–	3β, 7β	9	3β,11β	2.5
							6α, 11α	9		
							3β, 7β	29		
17β-OH	41	1β, 6β	18	6α, 11α	7					
15α-OH-2-CO	0	6α	13	–	–					
17β-OH-2-CO	4	6α	31	–	–	17	6α	15	–	
6α-OH-3-CO	–	–	–	–	–	0	16α	30	17α	7
6β-OH-3-CO							16β	27	11α,16β	4
							11α	14		
						0	16β	39	16β(3-CO → 3β-OH)	2
							11α, 16β	30		
							11α	14		
7α-OH-3-CO						0	16β	33		
							16β(3-CO → 3β-OH)	24		
7β-OH-3-CO						31	16β	52		
11α-OH-3-CO	4	12β, 15α	24	–	–	5	16β	45—30	16β(3-CO → 3β-OH)	11
							16α	14	7β,17α, 16-CO	4
							16β(3-CO → 3β-OH)	11—50		
11β-OH-3-CO						27	9α, 16β(11β-OH → 11-CO)	13	16β(3-CO → 3β-OH)	4
							16β	11	6α(3-CO → 3β-OH), 11β-OH → 11-CO)	2
							16β(11β-OH → 11-CO)	9	16α-(3-CO → 3β-OH, 11β-OH → 11-CO)	1
							16β-(3-CO → 3β-OH, 11β-OH → 11-CO)	9		

Table 3 (cont'd).

Substituents in substrate	*Calonetria decora* (Refs. 13, 19, 20, 30)					*Rhizopus nigricans* (Refs. 14, 22, 23)				
	recovered substrate, %	principal hydroxylation sites	Yield, %[a]	Secondary hydroxylation sites	Yield, %[a]	recovered substrate, %	principal hydroxylation sites	Yield, %[a]	secondary hydroxylation sites	Yield, %[a]
16β-OH-3-CO						0	11α	61—80	11α(3-CO → 3β-OH)	23—27
17β-OH-3-CO						8	11α	34	7β(3-CO → 3β-OH)	7
							6α	30	5α	6
17β-OMe-3-CO	16	12β, 15α(3-CO → 3β-OH)	8	–	–					
3β-OH-6-CO						0	16α	43	16β	18
17β-OH-6-CO						0	3β	23	5α,11α	7
							3α	21	3α,11α	7
							11α	11		
3β-OH-7-CO						0	16β	77		
17β-OH-7-CO						0	3α	52	4α	3
									3β	12
3β-OH-11-CO						0	16β	34	7β,16β	8
							16α	11	17β, 16-CO	7
							6α	9	16-CO	6
									5α, 16β	5
									7β, 17α	4
17β-OH-11-CO						26	3α	47	–	–
15α-OH-12-CO	0	6α	13	–	–	–	4α	25	–	–
14β-OH-15-CO	23	7β, 12β	34	7β, 12β	–					
12β, 14β(OH)$_2$-15-CO	0	7β	21							
3β-OH-16-CO						0	7α	90	–	–
3α-OH-17-CO		1β, 15α	25	–	–	7	7β	24		
		1β, 6α	37	–	–	–	11α	23	6α	5
3α-OMe-17-CO	3	1β, 6α	60	–	–					
3β-OH-17-CO	6	12β, 15α(3β-OH → 3-CO)	32			0	7β	48	7α	15
		12β, 15α	21						6α	12
6α-OH-17-CO						16	11α	58	3β	10
7α-OH-17-CO						10	3β	77		
17β-OH-17-CO						25	3β	57		
2,16-(CO)$_2$	–	12β-6-CO	27	6α,12β	12	0	7α(2-CO → 2α-OH)	55	7α	7
									6α(2-CO → 2α-OH)	5
1,17-(CO)$_2$	15	6α	33	6α, 19	25					
2,17-(CO)$_2$	15	6α	13			0	7α(2-CO → 2α-OH)	20	7β(2-CO → 2α-OH)	6
									6α(2-CO → 2α-OH)	2
3,6-(CO)$_2$						6	16β(3-CO → 3β-OH)	26	16β	9
							16α(3-CO → 3β-OH)	23		
							16α	23		
3,7-(CO)$_2$	26	12β(3-CO → 3β-OH)	19			4	16β	32	16β(3-CO → 3β-OH)	9
3,11-(CO)$_2$	27	15α(3-CO → 3β-OH)	23			4—19	16β	55—20	9α,16β	6
		15α	18				16β(3-CO → 3β-OH)	20	9α,16α	5
									16α(3-CO → 3β-OH)	4
									16α	2

Table 3 (cont'd).

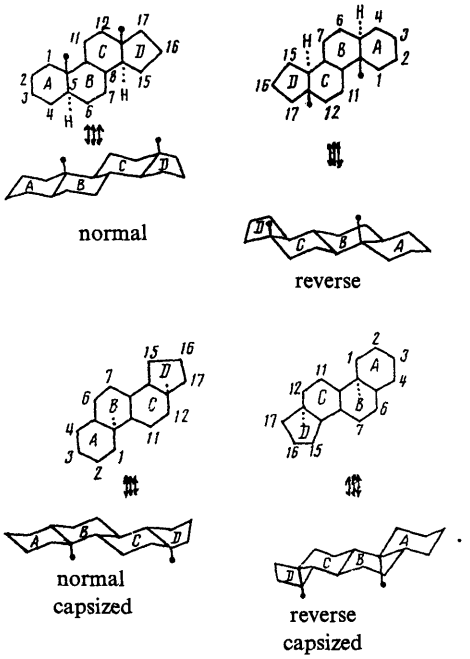
Substituents in substrate	*Calonetria decora* (Refs. 13, 19, 20, 30)					*Rhizopus nigricans* (Refs. 14, 22, 23)				
	recovered substrate, %	principal hydroxylation sites	Yield, %[a]	Secondary hydroxylation sites	Yield, %[a]	recovered substrate, %	principal hydroxylation sites	Yield, %[a]	secondary hydroxylation sites	Yield, %[a]
3,12-(CO)$_2$	10	15α	14							
3,16-(CO)$_2$	69	15α(3-CO → 3β-OH)	26	12β, 15α	11	0	7α(3-CO → 3β-OH)	50	-	-
3,17-(CO)$_2$	23	12β, 15α	5			32	11α	23	6α(3-CO → 3β-OH)	3
							6α	15	7β(3-CO → 3β-OH)	4
6,17-(CO)$_2$	27	1β	64—56							
7,17-(CO)$_2$	25	1β	55	-	-	0	3α	37	3α, 11α	5
							3β	7	3β(17-CO → 17β-OH)	4
									3α-(17-CO → 17β-OH)	6
11,16-(CO)$_2$ (EtOH, 6 j)						22	3β, 7β	24	3β	5
							3α	10		
11,17-(CO)$_2$	4	6α	32	-	-	21	3α(17-CO → 17β-OH)	36	4α(17-CO → 17β-OH)	15
1,17-(CO)$_2$-6α-OH	6—40	19	26—29	-	-					
								-	3α	14
3β, 6α-(OH)$_2$						6	16α	37	16β	17
3β, 6β-(OH)$_2$						17	16β	38	15α	17

Considerable progess in the understanding of the mechanism of the hydroxylation of steroids has been achieved as a result of studies by workers at Oxford University. They investigated the influence of the functional groups of the substrate on the type of hydroxylation by different fungal cultures. In an attempt to depart from the uniformity which had arisen in connection with the use of substrates of the pregnane series with oxygen-containing substituents at C(3) (mainly CO) and to find a general model of hydroxylation, the research group led by Jones[32] investigated a very wide set of 5α-*H*-steroids (more than 300) in which the position, nature, and number of the oxygen-containing substituents were varied systematically and additional structural factors (double bonds, ring junction, the presence of a heteroatom) were excluded beforehand. This giant study, in which more than 800 hydroxysteroids were obtained and whose importance it is difficult to overestimate, was carried out in 1969—1980.[13—38] Initially the familiar microorganisms characterised by both substrate specificity and substrate independence were included in the research and were then followed by new microorganisms, not investigated previously as steroid hydroxylating biocatalysts, up to the fungi mentioned above—the *Acromyrmex octospinosus* symbionts.[32]

The data in Tables 2 and 3 show that these studies replaced the occasional and few investigations of the transformations of 3,17-disubstituted 5α-*H*-androstanes by individual cultures.[54—56,64,70,77] The workers at Oxford University investigated the influence of a wide range of substituents in 5α-*H*-androstane on the hydroxylating capacity of *A.r.*, *P.u.*, *R.a.*, *R.c.*, *W.g.*, and *O.h.* with emphasis on *C.d.*, *R.n.*, *and A.o.* The results confirmed in most cases Brannon's hypothesis[4] that the hydroxylation of the steroid molecule in different positions is catalysed by the same non-specific enzyme. The enzyme—substrate interaction model, determining the mode of hydroxylation, which was constructed on the basis of the interpretation of the results of the hydroxylation of various substrates by *R.n.*,[22,23] does not conflict with this hypothesis either. The general interpretation of the hydroxylation mechanism proposed by Jones[78] not only explained satisfactorily the results of the oxidation of steroids by the cultures listed above but was also recognised and developed by other groups of investigators.[79—96]

The proposed model of the hydroxylation reaction postulates the existence on the surface of the enzyme globule of three active centres designed for two purposes, i.e. each centre-site can perform two functions—the function involving the binding of the enzyme to the substrate and the hydroxylation function (Fig. 1). According to this model, the sites have a triangular location, corresponding approximately to that occupied by the C(3), C(11), and C(17) atoms of the steroid ring. The way in which the enzyme—substrate complex is formed and hence the result of the hydroxylation depend on whether these centres of the steroid ring carry oxygen-containing substituents (how many and what kind); the binding of the steroid to the substrate can then take place in one of the possible orientations. It is believed that four orientations of the steroid in relation to the enzyme are theoretically possible: (a) normal; (b) reverse; (c) normal capsized; (d) reverse capsized. The last two are obtained by the rotation of the steroid molecule about the C(3)—C(17) axis by 180°. The normal and reverse orientations are preferable and virtually only these are considered in Jones's hydroxylation model (Scheme 5).

Scheme 5



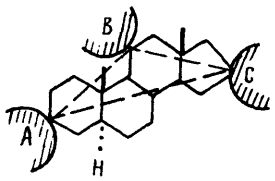


Figure 1. Jones's model of the enzyme–substrate interaction.[78]

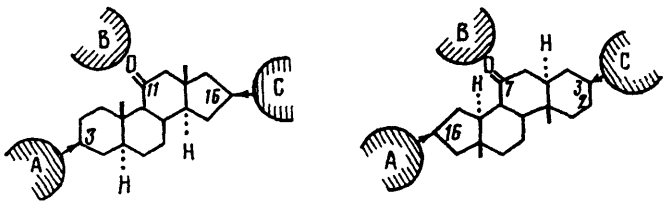
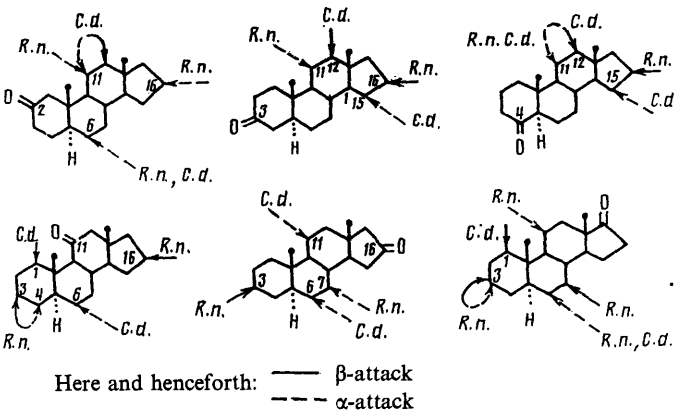


Figure 2. Site of hydroxylation of 11-oxo- and 7-oxo-5α-*H*-androstanes by a *D. r.* culture.[28]

According to this model, the binding of the enzyme to the steroid substrate occurs at two oxygen centres, which is satisfactorily confirmed by data for the transformation of the monoketones of the 5α-*H*-androstane series by different cultures. Virtually all the monoketones (1-, 2-, 3-, 4-, 6-, 7-, 11-, 12-, 15-, 16-, and 17-CO) are transformed either with much difficulty—with recovery of up to 90% of the unreacted substrate—or are not transformed at all. When transformation does occur, a considerable non-uniformity of the reaction products is observed. The high percentage of the recovery can be attributed to the low solubility of the monoketones compared with other steroids and hence with the greater difficulty of their permeation into the cell. However, the main explanation of the low reactivity the monoketones and the non-uniformity of the products obtained from them is apparently that the substrate molecule, fixed on the surface of the enzyme at only one site, rotates or oscillates about the binding centre, subjecting not all the predicted positions to the action of the enzyme. The monoketones are then as a rule dihydroxylated. Fig. 2 shows how this occurs for *D.r.*

The same model explains the principle of the dihydroxylation of the monoketones by the *C.d.* and *R.n.* fungi in the following way: the keto-group of the substrate in one (or several) of its orientations is bound to one of the binding centres of the enzyme, while the other two sites perform the function of hydroxylating those C–H bonds which are in vicinal positions in relation to them. For example, the 3- and 16-ketones are dehydroxylated by *R.n.* in the 11,16- and 3,7-positions as a consequence of the binding of the keto-groups to the enzyme at the centre A in the normal and reverse ways respectively (Fig. 3). The authors explain the formation of a multicomponent mixture of the 6,11-, 3,7-, and 3,11-dihydroxy-derivatives on transformation of 17-oxo-5α-*H*-androstane by the non-uniform binding in both ways. In this case, the formation of 6,11-dihydroxy-derivatives and also of 6α,16α- and 6α,11α-dihydroxy-products rather than the 11,16-dihydroxy-derivatives on transformation of 2-oxo-5α-*H*-androstane with the aid of *R.n.* cannot be readily explained.[22]

Scheme 6



One cannot rule out the possibility that the above variants of the dihydroxylation are a consequence of the successive monohydroxylation initially in the 11α- or 16α-position and then in the 6α-position; at any rate, it is known that 6-hydroxylase can be induced in *R.n.* by 11α-hydroxysteroids on incubation with Δ^4-3-oxosteroids.[2] It may be that the 16α-hydroxy-derivatives of 5α-*H*-steroids also induce the synthesis of a second enzyme. It is noteworthy that the high percentage of the recovery of the initial substrate (17-CO and 2-CO) shows that none of the available binding methods creates a situation favourable for hydroxylation (Scheme 7).

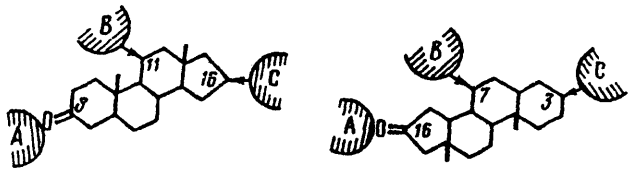
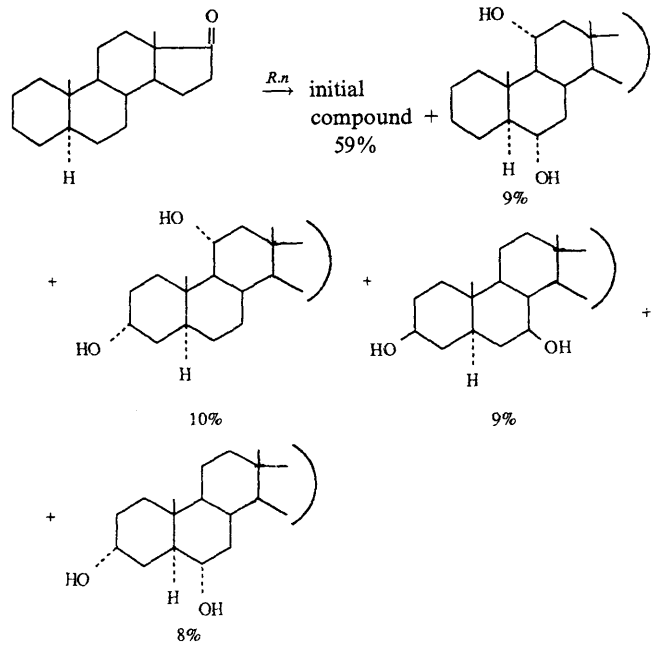


Figure 3. The site of hydroxylation of 3-oxo- and 16-oxo-5α-*H*-androstanes by the *C.d.* and *R.n.* fungi.[20,22,30]

Scheme 7



We shall compare these data (Scheme 6) with the results of the oxidation of the monoketone derivatives of 5α-*H*-androstanes by a *C.d.* culture. As a result, the *C.d.* culture introduces both equatorial groups at virtually the same distances from the oxygen atom of the substrate and the distance between the attacked carbon atoms is also approximately the same (1−6 = 3.9 Å; 6−11 = 4.4 Å; 11−15 = 4.5 Å). Evidently the oxidation of the monoketones by *R.n.* is not quite so specific because, together with equatorial substitution, there is also axial substitution and the distance between the attacked centres is not quite so uniform although it is in most cases approximately 5 Å (11−16, 3−17). Appreciable conversion of the monoketones into the main product on incubation with *C.d.* takes place only when the substituent is in the terminal rings. An interesting feature of *R.n.* in relation to steroids of the 5α-*H*-androstane series is the intense hydroxylation in the 16β-position (see Table 3 above), which is never manifested in the pregnane series.[35] *R.n.* is also distinguished by a low capacity for the oxidation of monoalcohols (1α-, 3β-, 6α-, 11α-, 17β-). After incubation with the above microorganism, 75−95% of the initial substrate remains,[22] whereas *C.d.* hydroxylates at least the 3β- and 17β-alcohols (the others have not been investigated) (Table 3). The most characteristic feature of the *C.d.* species, known as the 12β,15α-dihydroxylating culture for Δ^4-3-oxosteroids,[104] is also the 12β-,15α-dihydroxylation of 5α-*H*-androstanes with 3-CO, 3β-OH, and 3α-OH substituents as well as compounds with the 3β-OH-Δ^1-, 3-CO-Δ^1, or 17-acetate groups.[13,19,29] In contrast to *C.d.*, the *R.n.* and *A.o.* moulds do not hydroxylate 3- and 17-monoacetates of 5α-*H*-androstane.[29]

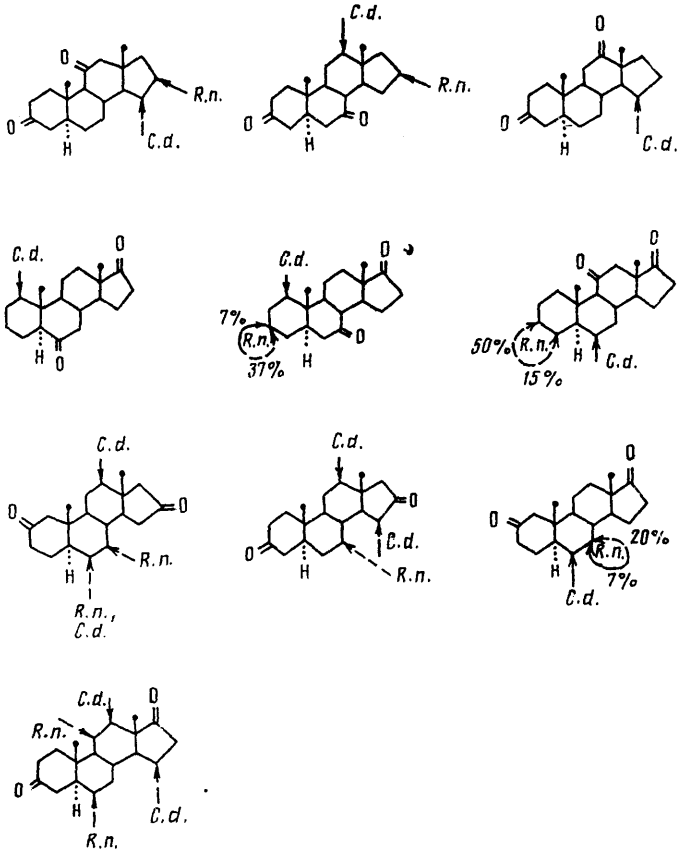
In the transformation of the monoketones with the aid of *D.r.*, the enzyme is bound to the substrate mainly with participation of the substituent at the centre of the molecule [C(7) and C(11)] with formation of 3β-,16β-dihydroxylated products (Table 2, Fig. 2). When androstanes with substituents in the terminal rings (in the 3-, 16-, and 17-positions) are used as the substrates, the recovery of the unreacted steroid is 50−90% and a complex mixture of unidentified products is formed.[28] The *W.g.*, *O.h.*, *D.c.*, *D.r.*, and *S.r.* cultures are inert in relation to the majority of monosubstituted (mainly CO-substituted) 5α-*H*-androstanes,[27] which agrees with the hypothesis of the involvement of two enzyme centres in the binding discussed here.

The *A.o.* culture does not transform the majority of monoketones. Exceptions are the 3- and 17-oxoandrostanes transformed respectively into 6β,11α-dihydroxy-derivatives with 44 and 35% conversions, 7β,11α-dihydroxy-derivatives (51 and 27%), and 11α-dihydroxy-derivatives (11 and 5%).[21] However, the formation of the dihydroxylated derivatives by the *A.o.* culture is due to the action of not just one enzyme, as for *C.d.*, but two steroid hydroxylases, the existence of which has been demonstrated in experiments with a cell-free extract.[75,76] It has been suggested that the second hydroxylase system in the *A.o.* cells is induced by the reaction product of the first enzyme, which is also the 11α-hydroxy-derivative. This leads to the formation of 6β,11α-dihydroxy-derivatives and sometimes the 6β-monohydroxy-derivatives.[28] The presence of the second oxygen-containing group (CO or OH) in the molecule in different positions facilitates the hydroxylation of 5α-*H*-androstanes with the aid of *A.o.*, but always in the 11α-position. Furthermore, whereas on incubation with monoketones the *A.o.* microorganism catalyses predominantly dihydroxylation, disubstituted androstanes are converted by this culture exclusively into 11α-monohydroxy-derivatives in 66−82% yield (Table 2). However, the functioning of two steroid hydroxylases in the *A.o.* cells and the site-specificity characteristic of this microorganism justify the assumption that it is unsuitable for the solution of the problem formulated. The research group therefore concentrated attention on *C.d.* and *R.n.*, which are capable of hydroxylating steroids in different positions depending on the structure of the substrate (Table 3), using the cultures indicated in Table 2.

Analysis of the results of the conversion of the monosubstituted 5α-*H*-androstanes has shown that the driving force determining the mode of hydroxylation is the specificity of the microorganism and the directing effect of the keto-group. The use of ketones and diketones substituted in different ways as substrates makes it possible, having increased the rate and efficiency of the transformation (by changing the hydrophilicity of the substrate, the solvent to be added to the reaction mixture, and the incubation time), to follow the competitive effect of the substituents (having restricted the monohydroxylation process as a result of the blocking effect of the second substituent) and, what is most important to discover the factors responsible for the mode of hydroxylation. It has been noted that polar groups in the terminal rings have a dominant effect on the hydroxylation process catalysed by the *C.d.* culture, as observed for monosubstituted androstanes. For example, the 3-oxo-group in 3,11-, 3,12-, and 3,7-diketones, which is common to these substrates, directs the hydroxylation to the *C* and *D* rings, while the 17-oxo-group in the 6,17-, 7,17-, and 11,17-diketones,

common to these substrates, directs the hydroxylation by the above microorganism to the *A* and *B* rings. The keto-groups in the middle *B* and *C* rings have a weak directing effect, blocking the hydroxylation in its own position and stimulating that in the neighbouring position or in a position close to it in space (Table 3, Scheme 8).

Scheme 8



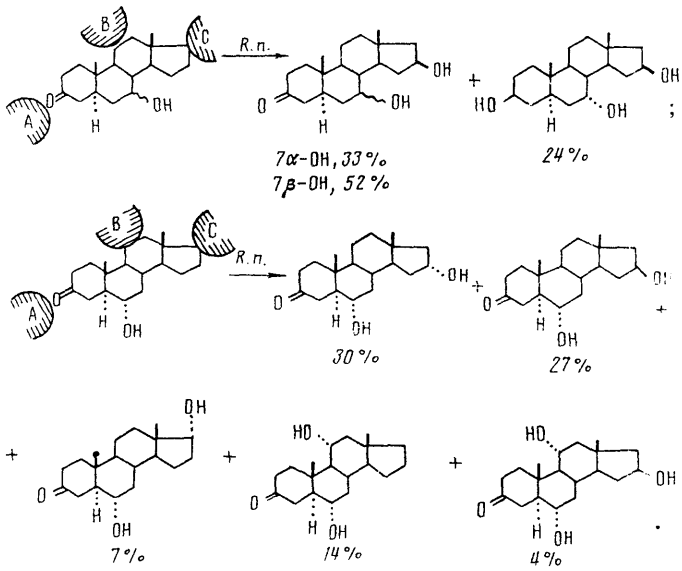
Naturally the competition between the keto-groups located in the terminal rings (the last four examples in Scheme 8) is reflected in the dihydroxylation, the mode of which is determined by the directing effect of both keto-groups. The hydroxy-groups in the main products formed as a rule assume the equatorial orientation and are at a distance of 3.5–4.5 Å from the nearest oxygen-carrying carbon atom and at a distance of 6.3–8 Å from either C(3) (substrate with a substituent in the *A* ring) or from C(17) (substrate substituted in the *D* ring). The directing force of the principal directing substituents has been estimated as follows in the analysis of the results of the oxidation reaction: 3-CO-Δ^4 (and less distinctly 5α- and 3β-OR, R = H, Me) > 17-CO > 3α-OR (R = H, Me); 3-CO-Δ^4 > 17β-OH, etc., i.e. the nature of the oxygen group is less important than its steric orientation relative to the steroid nucleus as a whole. This is an extremely important conclusion, which makes it possible to eliminate the established view, which is apparently false as shown using the *A.o.* culture, that the presence of the Δ^4-3-oxo-group, for example, in the steroid molecule is necessary for its successful microbiological hydroxylation.

According to the hydroxylation model discussed, the substrates with two oxygen-containing groups are oriented at the enzyme molecule in such a way that the maximum possible hydrophobic binding between both substituents and the corresponding enzyme centres is ensured. In this case the third catalytic centre is responsible for the hydroxylation of the nearest carbon atom.

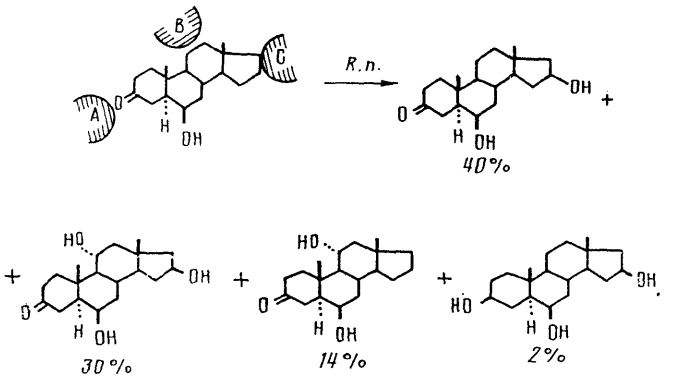
The *R.n.* enzymes hydroxylate terminally substituted substrates oriented in the normal manner in the 11-position, while those oriented in the reverse manner are hydroxylated in the 6- or 7-position. Thus, among the four terminally substituted diketones subjected to oxidation, three substrates (2,16-, 2,17-, and 3,16-) are hydroxylated only in the reverse manner (Table 3, Scheme 8).[23] The models described show that, for the first two steroids, binding in the normal manner does not permit contact between the steroid and the enzyme site B, while in the 3,16-diketone the geometrical disposition of the centres is favourable for both types of binding. The reverse type of hydroxylation observed for the 3,16-diketone indicates the preferential binding of the 16-oxo-group to the site A. Although the 3,17-diketone is in fact hydroxylated in the normal manner, the degree of conversion (32% of the starting material) and the yield of the 11α-hydroxy-derivative (23%) are low and the 6α-hydroxy-derivative is formed. It is thus legitimate to extend what has been stated above for the 17-monoketone to the 3,17-diketone. A striking result is the quantitative 11α-hydroxylation (~100%) of the 16β-hydroxy-3-ketone and the 7α-hydroxylation (90%) of the 3β-hydroxy-16-ketone bearing in mind that the corresponding monoalcohols are not transformed by the *R.n.*[23] In combination with the 3-oxo- and 16-oxo-groups, these alcohols probably constitute the optimum model for the normal and reverse hydroxylation respectively. The 17-oxo-group combined with the 3β-hydroxy group exhibits preferential hydroxylation in one particular (reverse) way (48% 7β-OH and 15% 7α-OH); on the other hand, the presence of the 3α-hydroxy-group promotes the formation of products of hydroxylation in both ways (23% of 11α-OH and 5% of 6α-OH). The presence of the 17β-hydroxy-group in 17β-hydroxy-5α-androstan-3-one not only facilitates the hydroxylation by *R.n.*, but also makes it selective. The asymmetric disposition of the substituents at C(17) probably interferes with the optimum binding of the substrate to the enzyme in this case also.

The results of the oxidation, catalysed by *R.n.*, of virtually all the ketones and ketols investigated, in which one oxygen atom is in the terminal ring and the other is in the middle ring, can be interpreted satisfactorily from the standpoint of the hypothesis of three two-target sites. As a rule, substrates with the substituent in the *A* ring are hydroxylated preferentially in the *D* ring and conversely the substrate substituted in the *D* ring is hydroxylated in the *A* ring, i.e. here too, as in the case where *C.d.* is used, the dominant influence of the terminal substituents is observed. These substituents are bound to the site A of the enzyme usually in the normal or reverse manner and are hydroxylated at the site C. On the other hand, the substituents in the central rings exert only an auxiliary influence, discussed above for *C.d.* (Scheme 9). The minor hydroxylation products can probably arise when a hydroxy-group enters positions adjoining the main hydroxylation site (for example the 4- or 17-position). One cannot fail to notice that monohydroxylation is more characteristic of disubstituted compounds even if the type of binding of the substrate molecule favours the entry of a second group. Thus in the examples presented in Scheme 10, the epimeric 7-hydroxy-3-ketones are not hydroxylated at the site *B* at all, while the epimeric 6-hydroxy-3-ketones are only partly hydroxylated at this site (Table 3).

Scheme 9

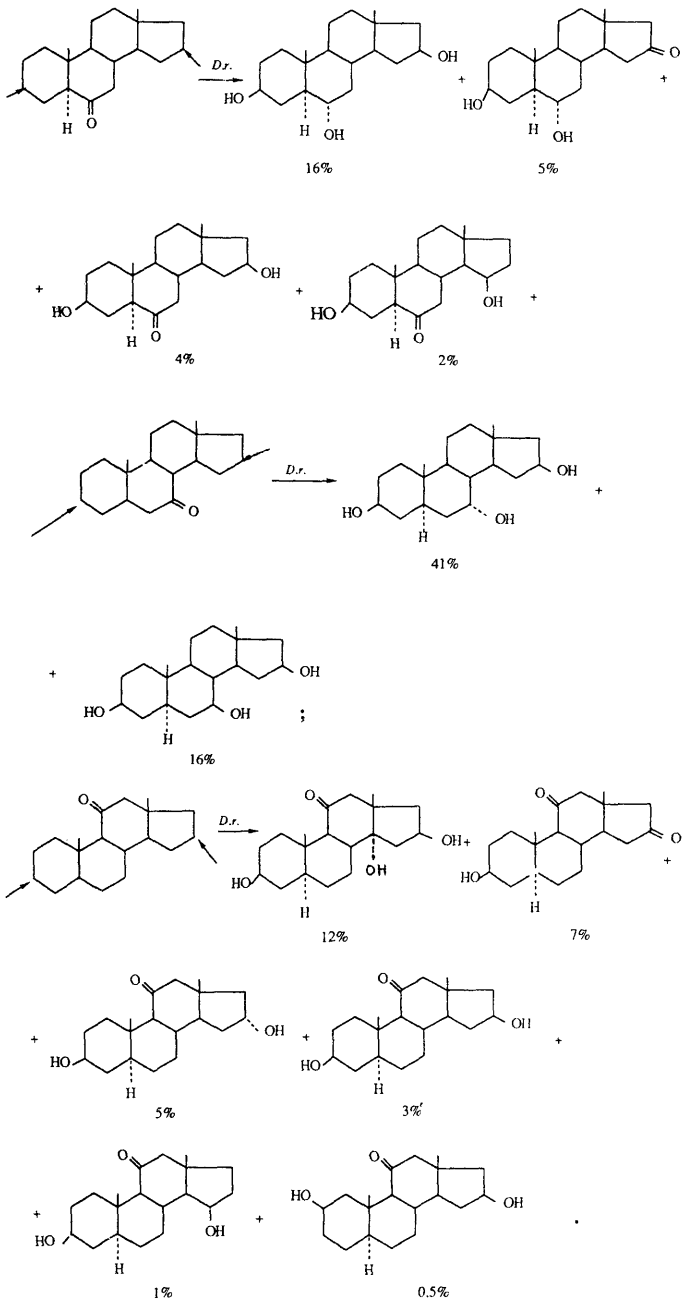


Scheme 10



In the authors' version, the proposed dehydroxylation model concentrates attention on the regiospecificity of the reactions but completely ignores their stereochemical aspects: the stereospecificity of the reactions or its absence and the influence of the stereochemistry of the substituent in the substrate. After two years, McCrindle et al.,[79] who analysed the hydroxylation of norcaranes catalysed by *R.n.* from the standpoint of Jones's hypothesis of the formation of a temporary enzyme–substrate complex, proposed a modification, which takes into account the three-dimensional nature of the steroid molecule and the protein globule. Their idea was to draw an imaginary plane through the steroid substrate molecule in such a way that the sites A and B are below it (or are coplanar with it), while the site C is above it. It is assumed that the site A binds preferentially the oxygen atoms below this plane and that hydroxylation takes place in the α-region. The site B imposes a similar requirement on the binding to the enzyme, but hydroxylation can occur both in the α-region (axial or equatorial) or in the β-region (only equatorial). The site C is bound preferentially to the enzyme above this plane and directs hydroxylation preferentially to the β-position. From the author's point of view, all the results for the stereochemistry of the hydroxylation of steroids catalysed by *R.n.* and their own data on the hydroxylation of norcaranes fit satisfactorily within the framework of this hypothesis.[79]

Scheme 11

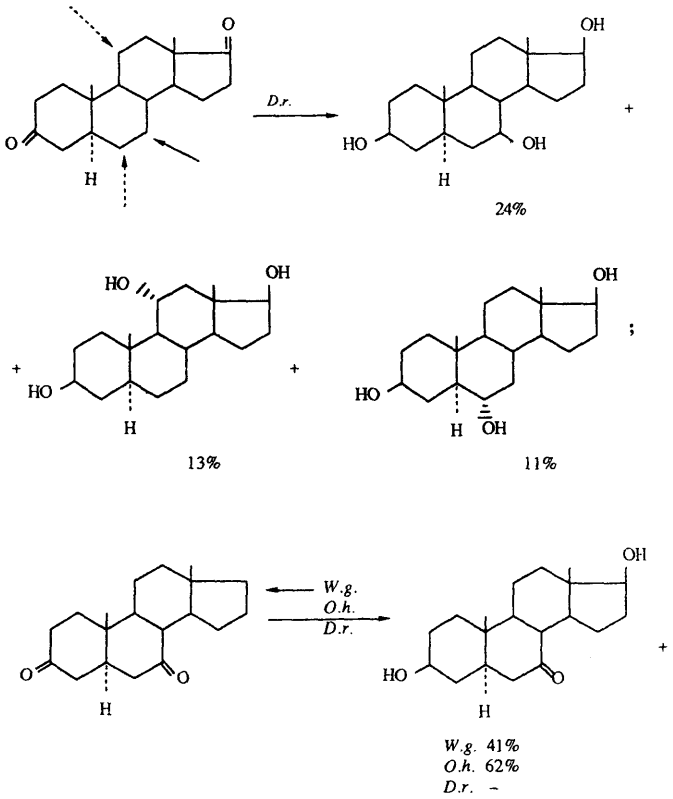


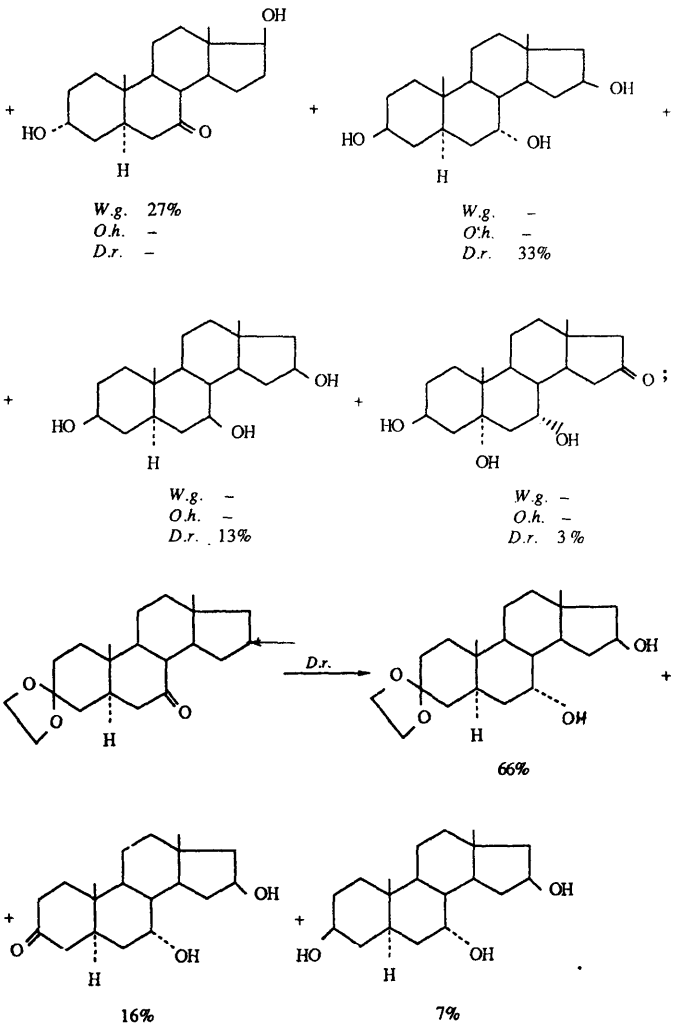
The results of the transformation of 5α-androstanes by *W.g.*, *O.h.*, and *D.r.* cultures, where the majority of monosubstituted, mainly with CO, 5α-androstanes are inert in the presence of *W.g.* and *O.h.*, are satisfactorily consistent with the proposed hypothesis. The *D.r.* enzyme system exhibits a surprising contrast between the slow hydroxylation of substrates having a carbonyl group in the terminal ring and the higher rates of conversion of substrates having this

group in the central ring. Thus 5α-androstanes with 3-CO, 16-CO, and 17-CO groups are transformed by *D.r.* only to the extent of 10–40%, forming a complex mixture of unidentified products, while their 6-CO, 7-CO, and 11-CO analogues are oxidised with 80–90% conversion at the terminal rings. The hydroxylation reactions of the 11- and 7-ketones catalysed by the *D.r.* culture are distinguished by their specificity: the former afford several hydroxylation products, while the 7-ketones are hydroxylated more unambiguously (Scheme 11, Table 2).

We shall consider in relation to these cultures (*W.g.*, *D.r.*, *O.h.*) how the results obtained with their aid fit within the framework of Jones's model. The *O.h.* enzyme system proved to be inert in relation to the terminally substituted androstanes with two oxygen-containing groups. On the other hand, the *W.g.* and *D.r.* cultures effectively hydroxylate these substrates (Table 2).[27,28] 5α-Androstanes in which one keto-group is in the terminal ring (2-, 3-, or 17-) and the other is in the middle ring (7- or 11-) are hydroxylated readily and always in the terminal ring regardless of the microorganism used, the replacement of the keto-group by a hydroxy-group having an insignificant influence on the hydroxylation reaction. The clear tendency towards preferential attack on specific positions in the steroid nucleus (usually 3-, 7-, and 16- or neighbouring positions) is characteristic of all three cultures (Scheme 12).

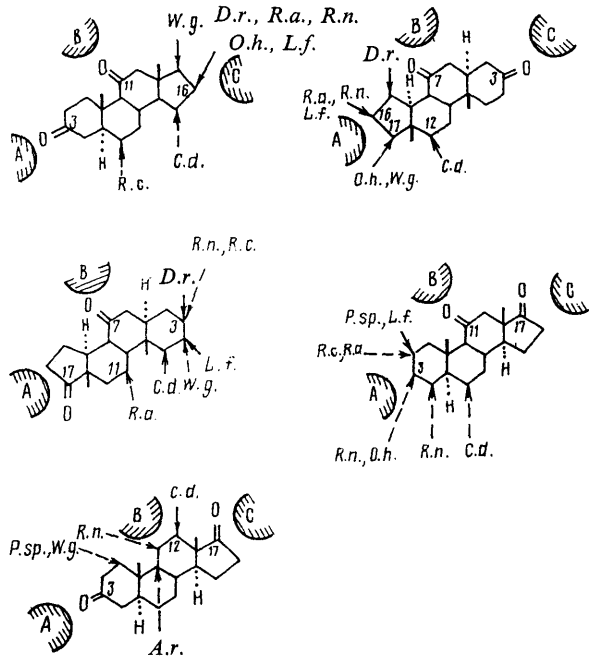
Scheme 12





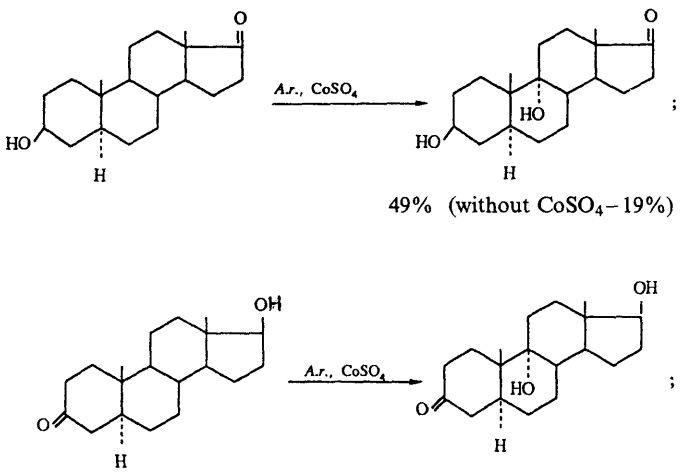
The data discussed above concerning the oxidation of 5α-androstanes and included in Tables 2 and 3 (the results of transformations catalysed by fungi of unidentified genus are not considered) are summarised in Scheme 13. In most cases, these results fit within the framework of the Jones–McCrindle model of enzyme–substrate specificity. Thus, on binding to the sites A and B or B and C, hydroxylation takes place at the sites C and A respectively [the 3,11-$(CO)_2$, 3,7-$(CO)_2$, 7,17-$(CO)_2$, and 11,17-$(CO)_2$ structures], while on binding to the sites A and C, all fungi (it must be emphasised that these represent different classes), introduce an axial hydroxy-group (with the exception of *C.d.*) in the positions within the molecule oriented towards the site B. At the same time, one of the binding sites can effect hydroxylation and in such a case dihydroxylated products are formed. On the other hand, if this does not occur, then monohydroxylated products are formed, which is observed on transformation of disubstituted androstanes in the vast majority of cases. The reactions of androstane substrates take place similarly when keto-groups are replaced by hydroxy-groups and also for 3- and 17-monoacetals and 3,17-diacetals and 2α,3α-epoxides, but not 2β,3β-, 16α,17α-, and 16β,17β-epoxides.

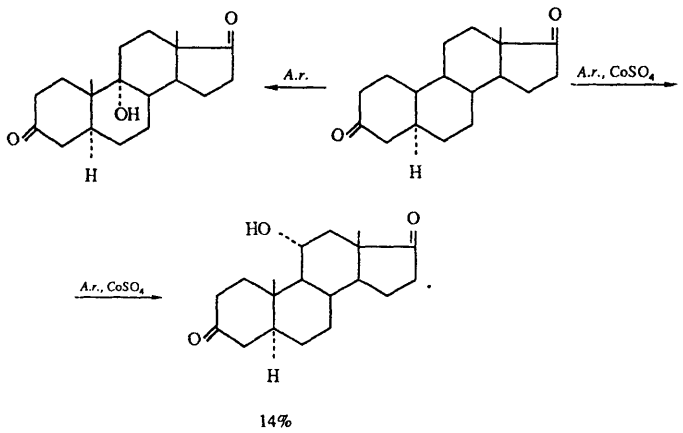
Scheme 13



The results of hydroxylation catalysed by the *A.r.* microorganism, which introduces an 11α- or 9α-hydroxy-group into the 3,17-diketone depending on the presence of divalent cobalt in the medium, are not susceptible to explanation.[33] If the substrates are 3β- or 17β-keto-alcohols, then the hydroxy-group is introduced in the 9α-position regardless of the presence of cobalt. However, in its absence the yield of the 9α-hydroxy-products is smaller by a factor of 2. The exceptional nature of these data consists also in the fact that heavy metal salts, including cobalt salts, fully inhibit the activity of 9α-hydroxylases of bacterial origin[2] (Scheme 14).

Scheme 14

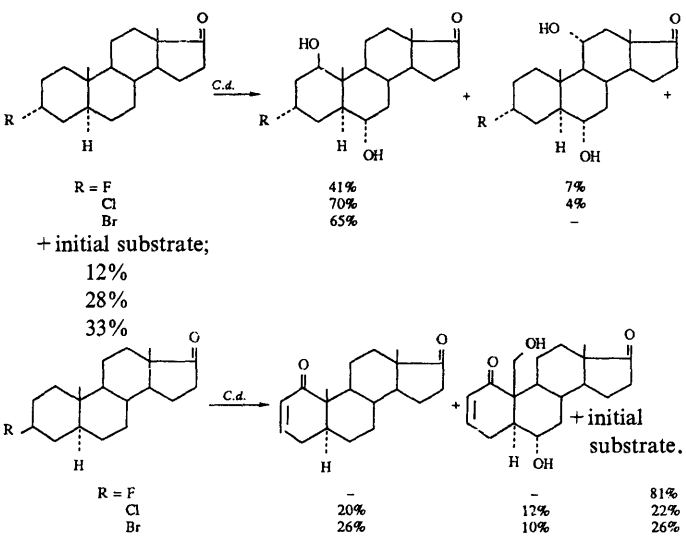




IV. Transformations of halo-5α-*H*-androstanes

In order to elucidate the influence of halo-substituents on the hydroxylation of 5α-*H*-androstanes (3-, 16-, and 17-oxo-derivatives), a number of monohalo-substituted (3-, 6-, and 17-) and *gem*-difluoro-substituted (6-, 7-, 12-, 16-, 17-) substrates were incubated under standard conditions with *C.d.*, *R.n.*, and *A.o.* micro-organisms[34,36] and the results were compared with those obtained for related unsubstituted 5α-*H*-androstanes. It was found that the catalytic properties of the biocatalysts are determined by the halogen atom: its nature, position, and configuration. This is illustrated by the results of the hydroxylation of 3α- and 3β-halo-5α-*H*-androstan-17-ones (Scheme 15).

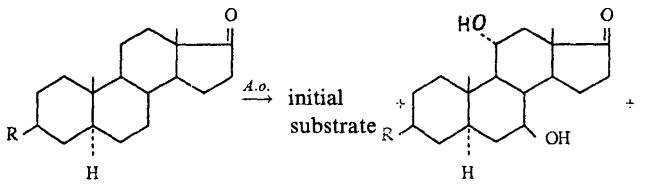
Scheme 15



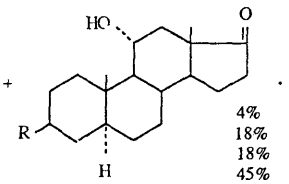
Another activity sequence in the halogenation of substrates has been observed for 17α-halogenated 5α-*H*-androstan-3-ones: in this case, the chloro-substituted derivatives are inactive in the presence of the *C.d.* microorganisms, while the fluoro-substituted derivatives undergo 12β,15α-dihydroxylation [like the unsubstituted 3-oxo-5α-*H*-androstanes (Table 3)]. A clear and very useful difference exists between the fluoro-ketones, on the one hand, and the chloro- and

bromo-ketones, on the other, in the hydroxylation of 3- and 17-oxoandrostanes catalysed by the *A.o.* microorganism. For example, 3α-chloro- and 3α-bromo-derivatives do not react, whereas the 3α-fluoroketone is effectively hydroxylated in the 11α-position by this culture. An extremely important conclusion, reached in a study of a wide range of fluorinated 5α-androstanes, is that the fluorine atom, which is remote from the site of attack specific to each 5α-androstanone (and microorganism), facilitates the hydroxylation process without altering its specificity. This can be clearly traced for the 11α-hydroxylating *A.o.* culture, which, in contrast to *C.d.* and *R.n.*, exhibits a low substrate specificity. The results presented in Scheme 16 demonstrate not only a clearly expressed induction of the hydroxylation process by the 3-fluoro-substituent but also the possibility of specific 11α-hydroxylation of 17-oxo-5α-androstanes by this culture (c.f. Table 3).

Scheme 16

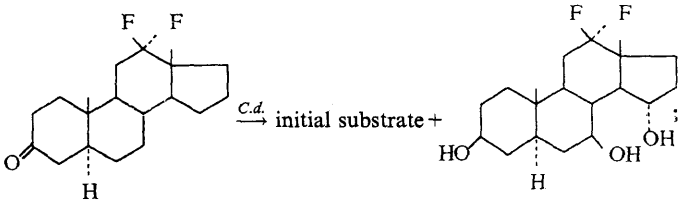


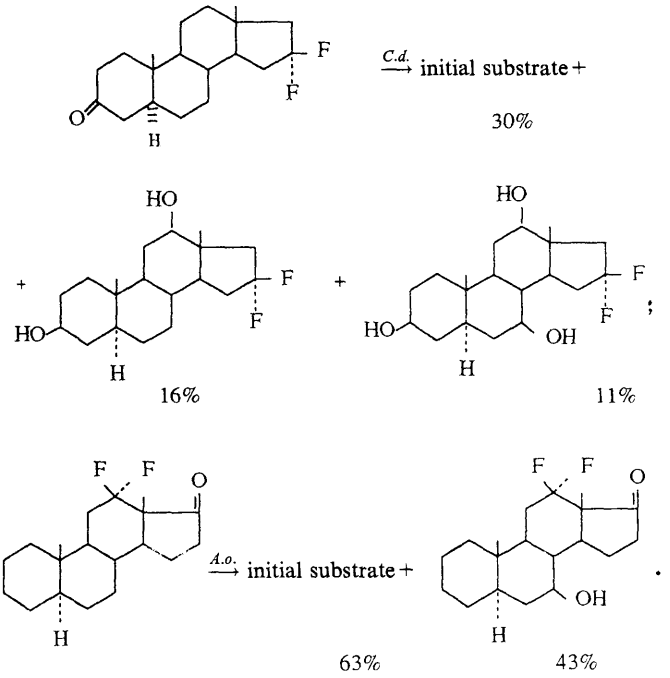
R = H	67%	34%
R = 3α-F	18%	41%
R = 3β-F	18%	40%
R = 3, 3-F_2	28%	–



On the other hand, a chlorine atom close to the standard points of attack as a rule blocks the hydroxylation process in these standard or neighbouring positions or directs it along the "reverse" pathway. For example, the 12β,15α-dihydroxylation by the *C.d.* culture, which is characteristic of 3-oxo-5α-*H*-androstanes, has not been observed in reactions with 12,12-difluoro- and 16,16-difluoro-3-ketones, where the main targets of attack are respectively the 7β,15α- and 7β,12β-atoms. A striking example of such displacement of the site attacked is provided by the result of the hydroxylation of 12,12-difluoro-17-oxoandrostanes to give the 7β-hydroxy-derivative. This result is particularly striking in the sense that the presence of the 11α-hydroxy-group is essential for the 6β- or 7α-hydroxylation by this enzyme, while monohydroxylation always takes place only in the 11α-position[76] (Scheme 17).

Scheme 17





V. Hydroxylation of 5α-*H*-*D*-homoandrostanes

In order to discover the additional factors determining the enzyme–substrate interaction, research groups in Venezuela and Great Britain[89–96] analysed the influence of the formation of *A*- and *D*-homo-derivatives in the series of 5α-*H*-androstanes on hydroxylation by three cultures—*A.o.*, *R.n.*,[80,81] and *C.el.* Comparison of the results with those for the transformation of similarly substituted 5α-*H*-androstanes permitted the conclusion that, firstly, the *A.o.* culture exhibits a narrow site-specificity also in relation to the *D*-homosteroids, hydroxylating the latter only in the 11α-position (Table 4). Secondly, compounds of this series do not induce the synthesis of a second hydroxylase, which is additionally confirmed by the absence of the hydroxylation of *D*-homo-5α-androstanes having two oxygen-containing substituents in the 3,11-positions. It is noteworthy that the same compounds are not hydroxylated by the *R.n.* culture either, which is evidence in support of the hypothesis put forward previously that two hydroxylases can also function in the *R.n.* cells but they are not quite so specific as in the *A.o.* culture. However, according to the authors,[80,81] in the main the results of the hydroxylation of *D*-homo-5α-androstanes by the *R.n.* culture fit satisfactorily within the framework of Jones's mechanism, from the standpoint of which virtually all the data obtained can be accounted for. In this series, the normal mode of hydroxylation is rarely observed and virtually all the substrates investigated are hydroxylated in the reverse manner, the reaction being accompanied to a large extent by the reduction of the keto-group. An exception is *D*-homo-5α-*H*-androstane-3,17-dione, which is hydroxylated to some extent in the normal way in the 11α-position. However, since only the 3-oxo-group is reduced to the 3β-hydroxy-group, favourably oriented for binding in the reverse manner, the process begins to proceed precisely in this direction with formation of 7β-hydroxy-derivatives. On the other hand, the 3,17*a*-*D*-homoketone (Table 4) is not hydroxylated even in the

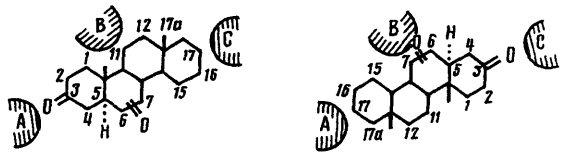
Table 4. Hydroxylation of *D*-homo-5α-*H*-androstanes by different cultures.

Substituents	Culture	Initial compound,%	Principal hydroxylation sites	Yield, %[a]	Secondary hydroxylation sites	Yield, %[a]	Refs.
2,17a-$(CO)_2$	*R.n.*	14	6α and 7α	20	2-CO → 2α-OH	14	[81]
3,6-$(CO)_2$	*R.n.*	47	17aα(3-CO → 3β-OH)	17	3-CO → 3α-OH	6	[81]
3,7-$(CO)_2$	*R.n.*	7	17aα	31	–	–	[81]
3,17-$(CO)_2$	*R.n.*	–	7β(3-CO → 3β-OH)	20	3-CO → 3α-OH	45	[81]
			11α(3-CO → 3α-OH)	29			
3,17a-$(CO)_2$	*R.n.*	35	–	–	3-CO → 3α-OH	19	[81]
11α-OH, 3-CO	*R.n.*	39	–	–	3-CO → 3β-OH	11	[81]
3β-OH, 17a-CO	*R.n.*	0	7β(17-CO → 17α-OH)	18	17-CO → 17α-OH	19	[81]
7β-OH, 17a-CO	*R.n.*	50	7β	47	17a-CO → 17α-OH	10	[81]
3α-OH, 17a-CO	*R.n.*	50	7 products				[81]
6-CO	*A.o.*	90			7β-OH → 7-CO	21	[80]
2,17a-$(CO)_2$	*A.o.*	61	11α	65	–		[80]
3,6-$(CO)_2$	*A.o.*	50	11α	29	–		[80]
3,7-$(CO)_2$	*A.o.*	24	11α	20	–		[80]
3,11-$(CO)_2$	*R.n., A.o.*	10	not isolated				[80]
3,17-$(CO)_2$	*A.o.*	24	11α	79	–		[80]
3,17a-$(CO)_2$	*A.o*	0	11α	73	–		[80]
11α-OH, 3-CO	*A.o*	85	not isolated				[80]
3β-OH, 17a-CO	*A.o*	38	11α	46	3β-OH → 3-CO	41	[80]
3β-OAc, 17a-CO	*A.o*	–	11α	–			[94, 95]
3α-OH, 17a-CO	*A.o.*	33	11α	48	–		[80]
3,17-$(CO)_2$	*C.el*	–	7α, 9α	35	7α	2	[94, 95]
			9α, 14α	20	3-CO → 3β-OH		
3,17a-$(CO)_2$	*C.el*	–	7α, 14α	33.5	7α	2	[94, 95]
					1β, 3β, 7-CO	1.5	
					1β, 7α	3.2	
					1β, 7β	1.2	
3β-OAc, 17-CO	*C.el.*	–	9α, 14α	36	–	–	[95]
3β-OAc, 17a-CO	*C.el.*	–	7β(3β-OAc → 3β-OH)	29	1β, 7-CO	4	
			7α(3β-OAc → 3β-OH)	9	1β, 6β	4	
					7-CO	0.6	
3β-OH, 17a-CO	*C.el.*	–	1β, 7-CO	4	–	–	[95]
			1β, 7β	4	–	–	
			1β, 7α	10	–	–	
17-CO	*C.d.*	–	6α, 17α	10	–	–	[19]
			7β, 12β, 15α	11	–	–	
			1β, 7β, 15α	6	–	–	
17aβ-OH, 17aα-Me-3,17-$(CO)_2$	*C.cl.*	–	11β	2	11β(3-CO → 3β-OH)	8	[117]
			6α	4	6α(3-CO → 3β-OH)	3	

reverse manner, because its 3α-hydroxy-derivative, having a hydroxy-group with an unfavourable orientation for binding to the site C, is formed initially. It is hydroxylated with the formation of a multicomponent mixture of unidentified products. The latter result probably justifies a general conclusion concerning the hydroxylation of steroids. If the structure of the substrate does not promote effective binding to the enzyme, then the latter is achieved with participation of molecules oriented in different ways, which leads to a non-uniform hydroxylation process or reduction of the keto-groups.

In contrast to 5α-androstanes, the 3,6- and 3,7-diketones of which are hydroxylated by *R.n.* in 16α- and 16β-positions, the corresponding homoanalogues form 17*a*α-hydroxy-products.[81] The authors suggest that the above *D*-homosteroids are hydroxylated by the reverse procedure at the site A, the formation of 16α-hydroxy-5α-androstane-3,6-dione is a consequence of the same hydroxylation pathway, while the 16β-epimer is formed in the normal way by transformation at the site C, provided, of course, that the "twisted" forms of the molecules do not participate in the process (Scheme 18).

Scheme 18

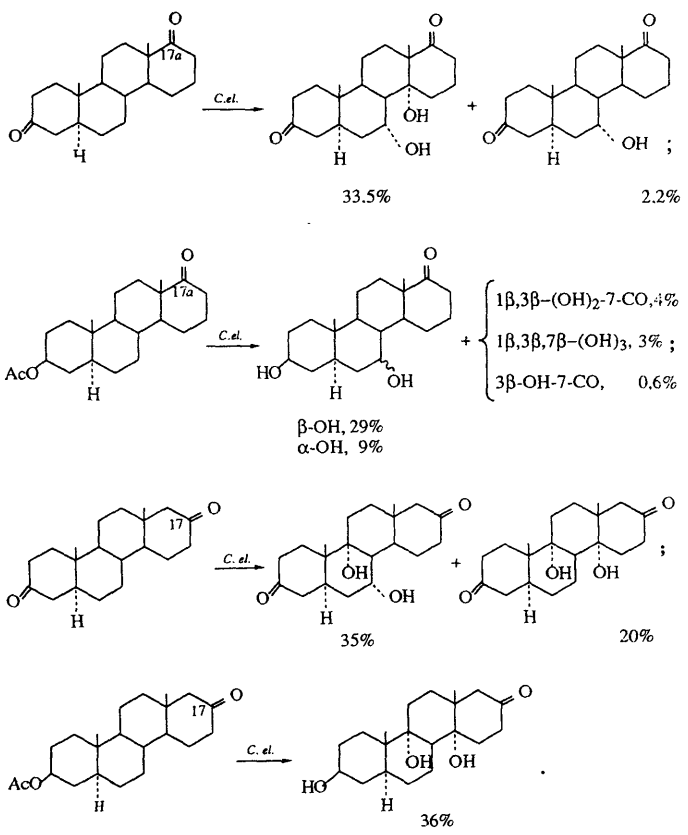


normal reverse

Unfortunately there have been no quite so systematic studies on the hydroxylation of *D*-homo-5α-*H*-androstanes by other cultures. However, one should note the different behaviour of the dihydroxylating *C.d.* culture in relation to 5α-*H*-androstan-17-one (Table 3) and its *D*-homoanalogue (Table 4): the 1β,6α-dihydroxylation in the former case and preferential trihydroxylation (7β,12β,15α- and 1β,7β,15α-) in the latter.

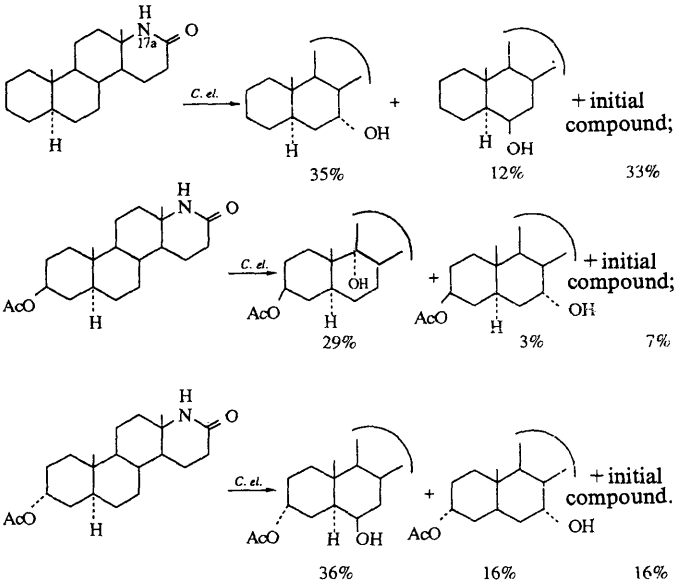
The existing, albeit few, results for the hydroxylation by the *C.el* culture of two pairs of *D*-homo-5α-*H*-androstanes 3,17-disubstituted by oxygen-containing groups (Table 4) and their comparison with data for the oxidation by the same culture of the corresponding 5α-*H*-androstane analogues (Table 2) can also be interpreted within the framework of the hydroxylation model mentioned above. The striking change, particularly notable in the series of 17*a*-*D*-homoanalogues, in the site at which *D*-homo-5α-*H*-androstan-17 (or 17*a*)-ones are hydroxylated on replacement of the 3-oxo-group by the 3β-acetate group, hydrolysing during the reaction, is noteworthy (Table 4, Scheme 19).

Scheme 19



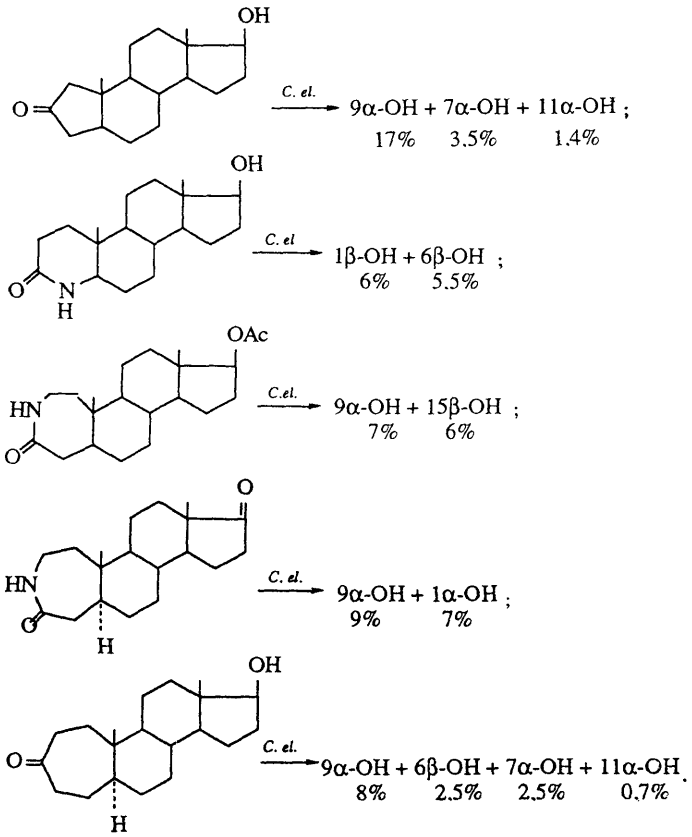
These data demonstrate convincingly also a significant dependence of the specificity of the hydroxylation on the position of the keto-group in the *D* ring. Thus, whereas the main product of the incubation of 3β-acetoxy-17*a*-*D*-homoketone is the 7β-hydroxy-derivative, the transformation of its 17-oxo-analogue affords preferentially the 9α,14α-dihydroxy-derivative. We may note that the introduction of a nitrogen atom in the 17*a*-position in *D*-homo-5α-*H*-androstan-17-one probably inhibits the 14α-hydroxylation process catalysed by this culture. Thus, only monohydroxylation, preferentially in the 9α-position, has been observed for 3β-acetoxy-17*a*-aza-*D*-homo-5α-*H*-androstan-17-one (Scheme 20), in contrast to its carbo-analogue (Scheme 19).[90,92]

Scheme 20



The influence of substitution at C(3) on the specificity of hydroxylation can be clearly traced in relation to the transformation of 17*a*-aza-5α-*H*-androstanes by the *C.el* culture. A change in the structure of the *A* ring of 5α-*H*-androstanes introduces no less significant changes into the specificity of the hydroxylation of the structure. In a series of studies by Crabb and co-workers,[86–96] the influence of the *A* and *D* rings on the hydroxylation by the *C.el.* culture was investigated. Analysis of these data did not lead to the discovery of definite correlations. One can only note that the *A*-nor-, *A*-homo-, and aza-*A*-homo-5α-*H*-androstanes, whilst exhibiting a tendency towards monohydroxylation (preferentially in the 9α-position), are nevertheless transformed with a low degree of conversion regardless of the substitution in the *D*-ring (CO or OH group) (Scheme 21). In contrast to *C.el.*, the *A.o.* culture hydroxylates *A*-homo-3,17-diketone at the site typical for this micro-organism, forming the 11α-hydroxy-product in 58% yield.[93]

Scheme 21



VI. Hydroxylation of 5α-*H*-pregnanes

The virtually specific use of 5α-*H*-pregnanes in the synthesis of corticosteroids probably determined the choice of the hydroxylating microorganism and substrates, the range of which is extremely limited compared with 5α-*H*-androstanes. In the series of 5α-*H*-pregnanes, studies have been made on cultures known to possess the ability to hydroxylate Δ^4-3-oxosteroids in the following positions: 11β-(*Curvularia, Cunninghamella, Tieghemella*), 11α-(*Rhizopus, Aspergillus*), and 12β-(*Calonectria decora*).[2,6,11] Among the 5α-*H*-pregnanes, the 9α-hydroxy-derivatives have also been obtained,[118–122] but this type of transformation should be considered separately, because it is effected by bacteria and, in contrast to hydroxylation by fungi, takes place as a stage in the microbiological cleavage of the steroid molecule after the introduction of the Δ^4-3-oxo-group.[2,6–9]

The introduction of a hydroxy-group in the C(9)-, C(11α)-, and C(12)-positions ensures their subsequent transformation into the 11β-hydroxy-derivatives by chemical methods. On the basis of the same practical considerations, 3β-hydroxy- and most frequently 3-oxo-5α-*H*-pregnanes have been mainly investigated as substrates. Studies on compounds of this series are not quite so fundamental as those on 5α-*H*-androstanes and the results summarised in Table 5, do not reveal a rigorous correlation in the enzyme–substrate interaction. However, a few systematic studies on the substrate specificity of a number of cultures are of undoubted interest.

Continuing the studies on the enzyme–substrate interaction, Jones et al.[35] turned their attention to compounds of the pregnane series assuming undoubtedly that, when the side chain is capable of rotation, certain corrections may be applied to the model constructed on the basis of data for the androstane series. They investigated the set of microorganisms most effective in the transformation of 5α-*H*-androstanes: *A.o.*, three Rhizopus species, *C.d.*, and *D.r.*[35] Unfortunately, not having at their disposal such a variable set of substrates as in the androstane series, the authors were forced to restrict their studies to 20-oxo-5α-*H*-pregnanes substituted in the 3-, 7-, and 11-positions by oxygen-containing groups (Table 5). Owing to the lack of quantitative data on the transformation of substituted 20-oxopregnanes by the *A.o.* culture, only a qualitative analysis of the results is possible. 5α-*H*-Pregnane is hardly transformed by *A.o.* or a complex mixture of unidentified products is formed. On incubation with 3,20-dioxopregnane, the transformation proceeds via the pathway usual for this microorganism with formation of the 11α-hydroxy-product. However, on incubation with its 3β-hydroxy-analogue, hydroxylation in the 1β-position, atypical for *A.o.*, is observed after the introduction of the 11α-hydroxy-group.

After prolonged incubation (6 days), 3β-hydroxy-5α-*H*-pregnan-20-one affords a mixture of the 1β,11α-dihydroxy- and 11α-monohydroxy-derivatives, the dihydroxy product being formed not synchronously but consecutively via the 11α-monohydroxy-product (Table 5). It has been noted[25] that 1β,11α-dihydroxylation is characteristic of 16β-methyl-Δ^{16}- and 16α-,17α-epoxy-analogues of 3β-hydroxy-5α-*H*-pregnan-20-one, but is not observed in the case of their 17α-hydroxy- and 16-methyl-Δ^{16}-derivatives. The behaviour of the 2,20- and 3,20-diketones[25] derived from 5α-*H*-pregnane contrasts sharply with that of the 3β-hydroxy-analogues, affording exclusively the products of 11α-monohydroxylation. Here it is noteworthy that the corresponding Δ^4-3-oxo-analogues are converted quantitatively into the 6β,11α-dihydroxy-derivatives.

A remarkable result has been achieved by hydroxylating the steroid alkaloids funtumidine (3α-NH_2) and funtumine (3α-NH_2, 20α-OH), which are hydroxylated by *A.o.* in the 12β- and 11α-positions.[98] It follows from the results that the nature and stereochemistry of the substitution at C(3) affect extremely significantly the hydroxylation of 5α-*H*-pregnanes by the *A.o.* microorganism.

The *C.d.* microorganism dihydroxylates 3-oxo- and 3β-hydroxy-20-oxo-5α-*H*-pregnane in the 12β,15α-positions. When the C(12) atom is blocked by the 11α-hydroxy-group, the attack is directed exclusively to C(15) and a similarity is thus manifested with the behaviour of this culture in the 5α-*H*-androstane series. The stability of this type of specificity of hydroxylation has been observed for various substrates of the pregnane series regardless of the presence or absence of the Δ^4- and Δ^5-double bonds and even the junction between the *A* and *B* rings.[100,102] The ability of the *C.d.* culture, indicated in Scheme 22, to effect, together with hydroxylation, the isomerisation of 3-hydroxy-Δ^5-analogues to the Δ^4-3-oxo-derivatives is striking.

Table 5. Hydroxylation of substituted 5α-*H*-pregnan-20-ones by mould fungi.

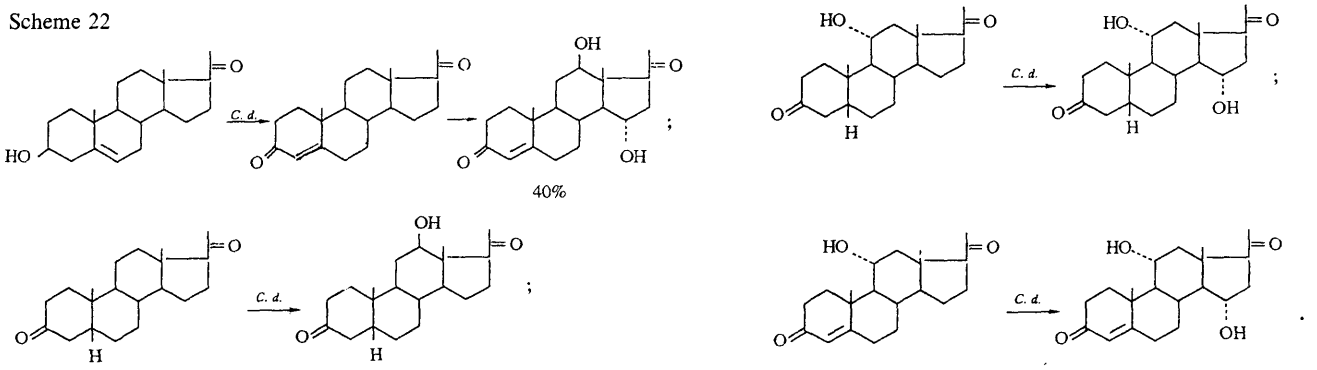
Substituent	Micro-organism	Initial compound, %	Principal hydroxylation site	Yield, %	Secondary hydroxylation site	Yield, %[a]	Refs.
–	*L.f.*	19	not isolated	–			[37]
–	*A.o.*	91	not transformed	–			[25]
2-CO	*A.o.*	0	11α	50	–	–	[25]
3-CO	*A.o.*	28	11α	38	11α, 17β	9	[25]
3-CO	*C.d.*	–	12β, 15α	40	–	–	[100, 102]
3-CO	*D.r.*	0	7α(3-CO → 3β-OH)	4	3-CO → 3β-OH	5	[35]
			7β(3-CO → 3β-OH)	5			
3-CO	*R.a.*	14	11α	12	7β, 11α(3-CO → 3β-OH)	7	[35]
3-CO	*R.c.*	7	11α	9	11α, 17-CO(3-CO → 3β-OH)	4	[35]
			11α, 17β	4	–	–	
			11α, 17-CO	7	11α(3-CO → 3β-OH)	3	
3-CO	*A.t.*	17	11β	10		5	[4]
3-CO	*S.c.*	–	11α	–			[63]
3-CO	*C.sp.*	–	9α	–			[67]
3-CO	*R.n.*	25	11α	22	7β, 11α(3-CO → 3-OH)	7	[35]
					11α(3-CO → 3β-OH)	5	
					3-CO → 3β-OH	3	
3-CO	*R.n.*	60	11α	40			[112]
7-CO	*C.d.*	45*	12β	18			[35]
7-CO	*C.d.*	10**	1α, 12β	33	1-CO, 12β	21	[35]
7-CO	*D.r.*	30	3β(7-CO → 7α-OH)	13	3β(7-CO) → 7β-OH)	3	[35]
7-CO	*R.n.*	21	3α, 11α	10			[35]
			3β, 11α	14			
			4α, 11α	12			
3-CO-11α-OH	*C.d.*	–	15α	–			[100]
11-CO	*D.r.*	10	3β	35			[35]
11-CO	*R.n.*	57	not isolated				[35]
11-CO	*C.d.*	58	not isolated				[35]
3β-OH	*R.n.*	12*	7β	15	7β, 12β	12	[35]
					11α	7	
3β-OH	*R.n.*	10**	7β, 12	22	7β, 11α	10	[35]
					7β	8	
					11α	5	
3β-OH	*R.a.*	14	11α, 7-CO	16	7β	11	[54]
3β-OH	*R.c.*	5	7β	11	11α	4	[35]
3β-OH	*A.o.*	3	1β, 11α	57	1β, 11α(3β-OH → 3-CO)	8	[17, 25]
3β-OH	*A.n.*	–	1β, 11α	–			[38]
3β-OH	*D.r.*	17	7α	9			[35]
7α-OH	*C.d.*	26	12β	17			[35]
7α-OH	*D.r.*	9	3β	27			[35]
7α-OH	*R.n.*	53	3β	24			[35]
11α-OH	*R.n.*	58	not isolated				[35]
11α-OH	*C.d.* *D.r.*	10	3β	35			[35]
11β-OH	*C.d.* *D.r.* *R.n.*	64—71	not isolated				[35]
3β-OH-Δ^{16}	*C.d.*	21	not isolated				[35]
3β-OH-Δ^{16}	*D.r.*	8	14α, 15β	21			[35]
3β-OH-Δ^{16}	*R.n.*	16	7α, 15α	12			[35]
			7α	13			
3β-OH-11-CO	*P.f.*	–	7-CO, 15β	12			[64]

Table 5 (cont'd).

Substituent	Micro-organism	Initial com-pound, %	Principal hydroxylation site	Yield, %	Secondary hydroxylation site	Yield, %*	Refs.
3β-11β-$(OH)_2$	*D.v.*	–	16α	–			[64]
3β, 21-$(OH)_2$	*R.a.*	–	7β	–			[69]
3β-OH-5, 6α-O	*C.l.*	–	11β	24—45			[105, 106]
3β, 21-$(OH)_2$-5, 6α-O	*C.l.*	–	11β	16			[105]
3β-OH-21-OAc-5, 6α-O	*C.l.*	–	11β	50			[105, 106]
3β, 17β-$(OH)_2$-5, 6α-O	*C.l.*	–	11β	20			[105]
3β, 17α, 21-$(OH)_3$-5, 6α-O	*C.l.*	–	11β	16			[105]
3β, 17α-$(OH)_2$-21-OAc-5, 6-O	*C.l.*	–	11β	28			[105]
3β, 17α, 20α-$(OH)_3$-5, 6α-O	*C.l.*	–	11β	33			[105]
3β, 20β-$(OH)_2$-5, 6α-O	*C.l.*	–	11β	52			[105]
3β-OH-16α-Me-5, 6α-O	*C.l.*	–	11β	45			[106]
3β, 20α-$(OH)_2$-5, 6α-O	*C.l.*	–	11β	8			[105]
3β, 20β, 21-$(OH)_3$-5, 6α-O	*C.l.*	–	11β	10			[105]
3β-OH-21-OAc-16α-Me-5, 6α-O	*C.l.*	–	11β	55			[105]
3β-OAc-21-OAc-16α-Me-5, 6α-O	*C.l.*	–	11β	36			[105]
3β,21-$(OH)_2$-5, 6α-O-17α, 20-O	*C.l.*	–	11β	28			[105]
3β-OH-5, 6α-O-16, 7α-O	*C.l.*	–	11β	45			[105, 106]
3β, 17β-$(OH)_2$-5, 6α-O	*C.l.*	–	11β	31—45			[105, 106]
3β-OH-16, 17α-O	*A.o.*	–	1β, 11α				[38]
3β, 21-$(OH)_2$-5α-Br-6β-F-16α-Me	*C.l.*	–	11β	45	3β-OH-5α-Br-6α-F-Δ^4-3-oxo-6α-F		[68]
3α-NH_2	*A.o.*	–	12β	6.7	11α	8.5	[98]
3-CO-11α-OH	*C.d.*	–	15α	60			[100, 102]
3-CO-16, 17α-O	*R.n.*	–	11α	60			[111]
3-CO-21-OH-16β-Alk	*Gi.c.*	–	11α	–			[58]
3-CO-21-OH-16α-тр.Bu	*Ph.*sp.	–	11β	–			[60]
			11α	–			
3-CO-17α, 21-$(OH)_2$	*C.l.*	–	11β, 14α	50—53			[107]
3-CO-17α, 21-$(OH)_2$	*C.bl.*	–	11α	70—75			[107]
3-CO-17α, 21-$(OH)_2$	*T.o.*	–	11α	40—45	11β	5	[107]
3-CO-16α, 17α-21-$(OH)_3$	*C.l.*	–	11β	38—42			[107]
»	*T.o.*	–	11α	30	11β	5	[107]
»	*C.bl.*	–	11α	60—65	11β	5	[107]
3-CO-21-OH-16, 17α-O_2CMe_2	*C.l.*	–	11β	10—12			[107]
»	*T.o.*	–	11β	40—45	11α-OH	5—8	[107]
»	*C.bl.*	–	11α	30—35			[107]
			11β	10—12	11-CO	5	

Notation. Incubation of the steroid dissolved in ethanol for 24 h (*), 6 days (**), 2 days (+), and 4 days (++).

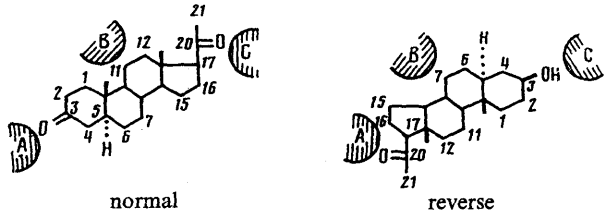
Scheme 22



A surprising feature of *C.d.* is the 11α-hydroxylation of 3α,6α-dihydroxy-substituted derivatives, uncharacteristic of this microorganism, which is manifested only in the 5β-*H*-pregnane series.[101] Earlier data indicating 15β-hydroxylation by the *C.d.* culture[103] must be regarded as erroneous.

Three species of the Rhizopus bread fungi, namely *R.a.*, *R.c.*, and *R.n.*, have been investigated as regards the hydroxylation of 5α-*H*-pregnanes. The differences between these cultures are similar to those observed for 5α-*H*-androstanes[23,24] but are less appreciable. A tendency towards the degradation of the side chain is observed in the transformation effected by *R.n.* Analysis of the results presented in Table 5 indicates a distinct tendency of this microorganism to effect the preferential 11α-hydroxylation of 3-ketones derived from 5α-pregnane and the 7β-hydroxylation of 3β-hydroxy-5α-*H*-pregnanes: a deviation is observed only for the 3β-hydroxy-Δ^{16}-analogue, which is attacked by hydroxylase not only in the C(7) position (from the α-region) but also in the allylic 15-position. The hydroxylation in the 12β-position on incubation with 3β-hydroxy-pregnanolone is atypical for *R.n.*[35] Murray and Peterson[55] pointed out in an earlier patent that *R.n.* hydroxylates this compound, as well as its 3,20-dioxo-analogue, in the 11α-position. The general similarity in the differentiated behaviour of 3-oxo- and 3β-hydroxy-derivatives of 5α-*H*-pregnanes and 5α-*H*-androstan-17(or 16)-ones indicates probably the possibility of using the enzyme–substrate interaction model in the 5α-*H*-pregnane series, taking into account, naturally, the stereochemical possibilities of the side chain and the conditions in the binding of the 20-oxo-group (Scheme 23):

Scheme 23



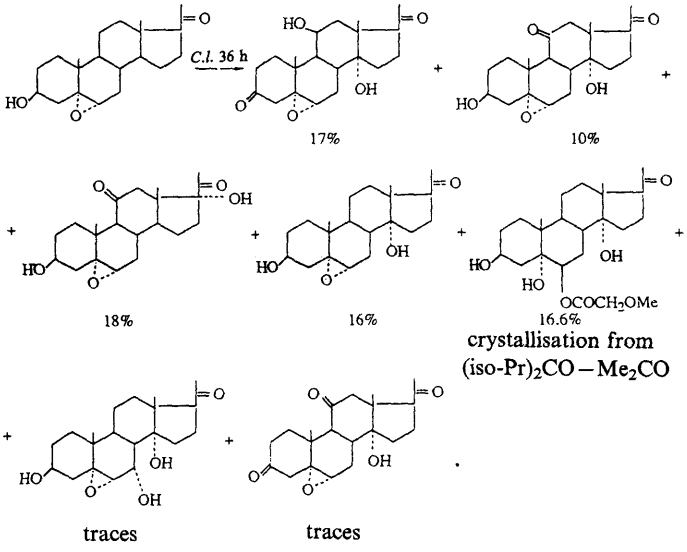
normal reverse

In constrast to 5α-androstanes, which are hydroxylated in the 9α-position by *C.el.*, *D.c.*, *A.r.*, *W.g.*, and *R.a.* cultures (Tables 2 and 4), only the *Circinella* sp. strain introduces the 9α-hydroxy-group into 5α-*H*-pregnanes.[67] This property is also manifested in relation to Δ^4-3-oxopregnane.[123] The series of data presented in Table 5 for transformations involving the 11α-position in 3β-hydroxy-5α,6α-disubstituted pregnanes, effected by the *C.l.* microorganism considered in the preceding context, shows that, when hydroxylation at C(7), (5,6-α-epoxy- or 6β-fluoro) is blocked, the reaction takes place exclusively at the C(11) atom. The same result casts doubt on the official view that the hydroxylation of 3β-hydroxy-derivatives of pregnanes unsaturated in the *A* ring is impossible.

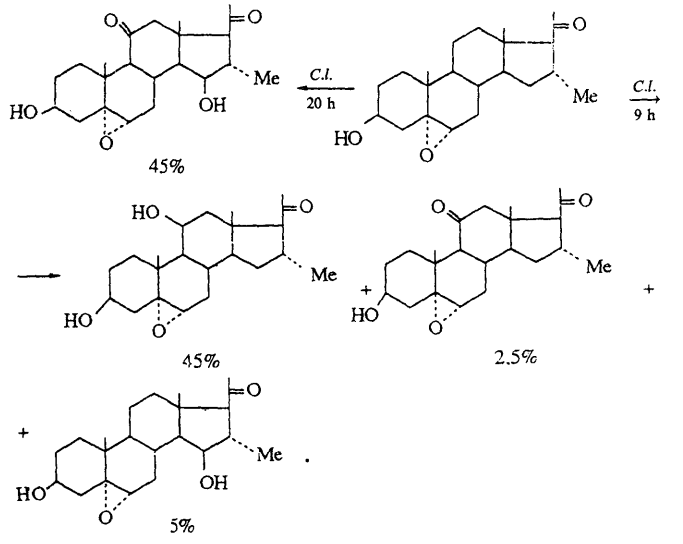
All the results presented in Table 5 concerning the transformation of 5,6-epoxides by *C.l.* are based on data obtained after brief incubation designed to achieve the maximum yield of the target product — 5,6α-epoxy-11β-hydroxy-5-pregnanones. Subsequent studies showed that this reaction proceeds with parallel formation of 14α-hydroxy-derivatives[104] and that prolonged incubation leads to a range of products[105] in which the hydroxylation site is now determined by the 11β- and 11α-hydroxy-groups and the 11-oxo-group (invariably formed by the subsequent oxidation of the 11β-hydroxy-group) already present in the molecule.[2,6]

For example, Scheme 24 presents the mixture of products obtained on transformation of 5,6α-epoxy-3β-hydroxy-5-pregnan-20-one by *C.l.* over a period of 36 h, in which the formation of 7α-hydroxy- and 17α-hydroxy-derivatives can be regarded, according to the authors, as a consequence of the directing effect of the 14α-hydroxy- and 11-oxo-groups introduced into the molecule beforehand,[105] while the formation of the 11,14α-disubstituted derivatives may be regarded as a consequence of the mutual influence of these groups. We shall show in relation to yet another example that the transformation time is an extremely important parameter in reactions of this type.[105] Here too the authors assume that the final oxidation product — the 15β-hydroxy-11-oxo-derivative — is formed as a result of the subsequent oxidation of both minor products (Scheme 25).

Scheme 24

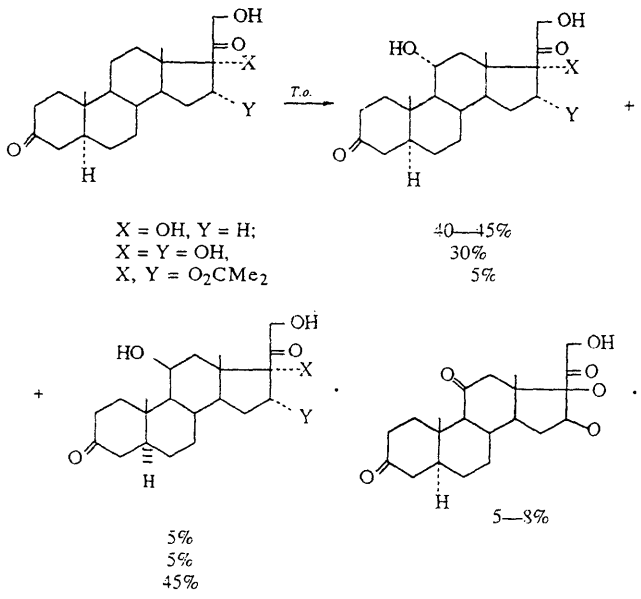


Scheme 25



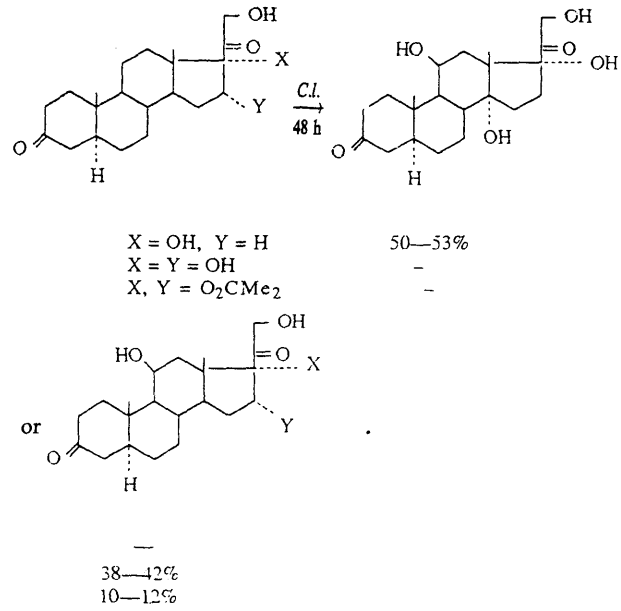
The *D* ring-substituted compounds are effectively transformed by *C.bl.* The 17α-hydroxy-, 16α,17α-dihydroxy-, and 16α,17α-dioxyisopropylidene-3,20-dioxo-5α-pregnanes are substituted preferentially in the 11α-position, the presence of an oxygen-containing 16α-substituent in the α-region of the steroid molecule stimulating the competitive 11β-hydroxylation (Table 5). The *T.o.* culture behaves similarly, but in this case the 16α,17α-acetonide group blocks 11α-hydroxylation almost completely, promoting 11β-hydroxylation (Scheme 26).

Scheme 26



All three types of substrate illustrated in Scheme 27 are hydroxylated by *C.l.* in the 11β-position, whereas in the case of the 16-unsubstituted compound (X = OH, Y = H) the subsequent 14α-hydroxylation is observed, being probably inhibited in the last two cases by the oxygen atom at C(16). Thin-layer chromatographic analysis of the reaction mixture after incubation of 17α,21-dihydroxy-5α-pregnane-3,20-dione for 24 h demonstrates the presence of 3–5% of the initial substrate, 15 and 20% respectively of the 14α- and 11β-monohydroxy-derivatives, and 30–35% of the 11β-,14α-dihydroxy-derivative, from which it follows that the 11β- and 14α-hydroxylation reactions take place consecutively. One must not fail to note that *C.l.* transforms cortexolone into a mixture of 11β- and 14α-monohydroxy-derivatives (67 and 15% respectively).[107] The authors conclude from this that 5α-*H*-steroids are sterically less hindered than their Δ^4-3-oxo-analogues. Because of this, the introduction of a hydroxy-group in one of the positions does not rule out the possibility of their reorientation relative to the enzyme, which leads to the formation of 11β,14α-dihydroxy-compounds. The authors therefore believe[107] that 16α,17α-dihydroxy-5α-pregnan-3-one is more susceptible to transformations by *C.bl.* and *T.o.*, affording 11α-hydroxy-groups in 65 and 30% yields respectively, while its Δ^4-3-oxo-analogue is not transformed by any of the cultures discussed.

Scheme 27



We do not find this view convincing; an explanation of the observed facts should probably be sought in the general model of the substrate–enzyme interaction involving pregnanes, in which the oxygen-containing functional group in the *D*-ring (its nature, position and stereochemistry) should not only participate but should also compete with the 20-oxo-group for geometrically or stereochemically favourable type of binding.

Analysing the data in Table 5 from the standpoint of Jones's model of the enzyme–substrate interaction, one may note that on the whole they agree with the postulate that three binding and simultaneously hydroxylating sites function on the surface of the enzyme. Indeed the pregnane monoketones, which can combine with the enzyme via only one centre, are hardly transformed, while in the presence of two terminal oxygen-containing groups in the steroid molecule hydroxylation at the site B takes place. In the presence of a second oxygen-containing substituent at the site B, hydroxylation can occur at the site A provided that it is not bound to a steroid molecule. When there are three oxygen-containing substituents, corresponding to the sites A, B, and C, in this case the transformation products can either not be isolated or, as happens when 11α-hydroxy-3,20-dioxopregnane is acted upon by *C.d.*, hydroxylation is apparently catalysed by one of the binding sites (Scheme 23).[102]

One should add to the foregoing that the transformation of 5α-*H*-pregnanes has also been achieved with the aid of streptomycetes of unidentified species and *S. roseochromogenes*, which introduce the 16-hydroxy-group into 3-oxo- and 3β-hydroxy-5α-*H*-pregnanes,[108,109] as in the case of compounds with the Δ^4-3-oxo-group. The mechanism of the hydroxylation of the *D* ring by these representatives of the Schizomycetes class as well as 9α-hydroxylation by *Rhodococcus*[118–122] probably differs from the mechanism proposed by Jones and co-workers for fungi.

Analysis of the data described makes it possible to trace the dependence of the specific capacity of microorganisms for hydroxylating 5α-*H*-steroids on the type of steroid and on the nature of the substituents in it (oxo-, hydroxy-, and acetoxy-groups), their

positions and stereochemistry, and their number. This makes it possible to achieve specific hydroxylation of steroids in a particular position within the molecule. Numerous examples, summarised in the tables, show that the saturation of the rings of the steroid molecule is not an obstacle to effective hydroxylation. This means that there is a possibility of altering the sequence in which 5α-*H*-steroids are converted into biologically active preparations, involving their initial hydroxylation and subsequent dehydrogenation of the *A* ring. The reverse and generally accepted sequence, involving the hydroxylation of the Δ^4-3-*H*-oxo-(or $\Delta^{1,4}$-3-oxo)-derivatives obtained initially from the 5α-*H*-steroids, is the subject of the next part of the review, in which emphasis is laid on the authors' own studies on the use of the *Rhodococcus* culture which catalyses both these processes.

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