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Steroid Transformations by Species of *Cephalosporium* and Other Fungi[1]

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A total of 58 cultures, tentatively identified as species of the genus *Cephalosporium*, were screened in flask fermentations for their ability to effect conversions of progesterone (Δ^4-pregnene-3,20-dione) and Reichstein's Substance S (Δ^4-pregnene-17α,21-diol-3,20-dione). A large number of transformations were observed by means of a series of five paper chromatography systems rated for analysis of steroid compounds ranging in polarity from progesterone to polyhydroxylated steroids. Five different transformation products were selected for isolation and identification. For purposes of recovery, conversions were conducted under submerged conditions in either 4- or 200-liter fermentors in which the broth was agitated and aerated. The steroid substrate was dissolved in acetone and added aseptically to the growing culture in a final concentration of 0.025%. After the conversions were effected, the whole broth was extracted with chloroform, and the transformation products were recovered, either by direct crystallization from solvents or through the use of silica gel columns. It was determined that *C. ciferrii* 21C converted progesterone to Δ^4-androstene-3,17-dione. Kendall's Compound F (Δ^4-pregnene-11β,17α,21-triol-3,20-dione) was converted to its 20β-ol analogue by *Geotrichum* sp. 51C (during these studies, a number of cultures were taxonomically reclassified). *Cephalosporium* sp. 27C formed the Δ^1-analogue of Reichstein's Substance S, and *Cephalosporium sclerotigenum* 31C and *Verticillium aphidum* both converted Substance S to the 6β-hydroxy derivative. *Paecilomyces persicinus* 22C converted Substance S to a product believed to be a dihydroxylated derivative.

The first microbiological transformation of a steroid was reported nearly three decades ago by Mamoli and Vercellone (14). However, it was not until 1955 that a systematic study of steroid transformations by microorganisms belonging to the same genus was reported. Dulaney et al. (7) screened 475 species of *Aspergillus* and 476 species of *Penicillium* for steroid transforming activities. In 1958, Iizuka et al. (10) investigated the ability of 473 members of the genus *Aspergillus* to transform progesterone (Δ^4-pregnene-3,20-dione). Similarly, Kondo (13) screened 22 species of *Fusarium*, and Iizuka and Naito (11), employing progesterone and Reichstein's Substance S (Δ^4-pregnene-17α,21-diol-3,20-dione) as substrates, studied 162 species of *Pseudomonas* and 24 of *Micrococcus*. Vondrová and Hanč (24) reported in 1960 the transformation of progesterone by 76 species of actinomycetes. Čapek and Hanč (4, 5) investigated 98 strains of 24 species of *Fusarium* as well as 191 strains of 94 species of the genus *Penicillium*. In another study, Čapek et al. (6) indicated that 248 microorganisms of various genera, viz., *Fusarium*, *Actinomyces*, *Proactinomyces*, *Nocardia*, and *Mycobacterium*, were tested for their capacity to introduce a double bond into position 1,2 of the A ring of the pregnene skeleton. The latter investigators, however, were interested primarily in selecting an organism suitable for determining optimal conditions for dehydrogenation of cortisone in position 1,2, and they did not provide a record of the dehydrogenating activities of the various genera. On the other hand, Shirasaka, Ozaki, and Sugawara (19) catalogued the names of over 400 species and strains of bacteria and fungi (of about 700 studied) which were investigated, listing the transformation products obtained from each steroid

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substrate. In 1963, Vondrová and Čapek (25) suggested a broad taxonomy of actinomycetes on the basis of steroid transformations by 58 species and 157 strains of this group. Schuytema et al. (18) listed conversions of progesterone by 54 of 254 basidiomycetes examined.

There are three references in the literature regarding the transformation of steroids by members of the genus *Cephalosporium*. Bodanszky, Kollonitsch, and Wix (2) reported that *C. subverticillatum* formed androstenedione (Δ^4-androstene-3,17-dione) from progesterone, which on prolonged incubation was further converted to Δ^4-testololactone. In 1959, Bernstein et al. (1) reported the conversion of Substance S to its 7α-hydroxy analogue by an unidentified species of *Cephalosporium*. Holmhund et al. (9) described the conversion of progesterone to testololactone, which was hydrolyzed in turn to testolic acid by *C. acremonium*. In view of the limited information on the transformation of steroids by *Cephalosporium*, and since a collection composed largely of cephalosporia was available, an investigation of steroid conversions by these fungi was undertaken.

MATERIALS AND METHODS

Organisms. The organisms employed in this study consisted of 58 cultures of filamentous fungi, most of which were of the genus *Cephalosporium*. There also were other deuteromycetes as well as a few ascomycetes and one unidentifiable basidiomycete. Identification of some of the cultures was done at the Centraalbureau voor Schimmelcultures, Baarn, Netherlands.

Media. All stock cultures were maintained on slants of Sabouraud Dextrose Agar (Difco). Cultures inoculated onto this medium were incubated at 28 C until luxuriant growth developed (1 to 2 weeks), and then were stored at 4 C until used.

Two media were employed for submerged growth. One of these was described by Shull and Routien (U.S. Patent 2,872,380, 1959), and is indicated here as medium 1A. This medium contains in 1 liter of tap water: 33.0 g of soybean meal and 1.43 g of KH_2PO_4. The other medium was Sabouraud Liquid Medium (Difco); it is labeled 1B. The latter medium was used exclusively in the preliminary screening studies. Media 1A and 1B were autoclaved at 121 C for time periods which varied with volume and with the medium used, i.e., medium 1A: 30 min for 100 ml, 45 min for 1 liter, 60 min for 2 liters or more; medium 1B: 15 min for 100 ml.

Sterility checks of all media were made by inoculating samples onto plates of Brain Heart Infusion (Difco) solidified with 1.5% agar. The plates were incubated at 37 C for 24 to 48 hr.

Fermentation technique: screening. Inoculum was prepared by washing fungal growth from slants with 5 ml of sterile distilled water. A 0.5-ml amount of the growth suspension was added to 100 ml of sterile medium 1B contained in 300-ml Erlenmeyer flasks. Flasks were incubated on a rotary shaker at 28 C for 72 hr. At the end of the 72 hr, 25 mg of steroid, dissolved in 2 ml of acetone and sterilized through a sterile Morton bacterial filter with an ultrafine fritted disc (Corning Glass Works, Corning, N.Y.), were added. The flasks were reincubated as described above for 96 hr. After this incubation period, the entire medium was extracted for paper chromatographic analysis as described below.

All cultures were screened on two steroid substrates, viz., progesterone and Reichstein's Substance S. The fermentation broths containing progesterone as substrate were chromatographed on systems A and B (see below), and those containing Substance S were chromatographed on system F. Systems C, D, and E were used primarily to compare the R_F values of known compounds with transformation products as an aid to identification.

Fermentation technique: reactor stage. Inoculum was prepared by adding 2.5 ml of growth suspension from a slant to 1 liter of sterile medium 1B contained in a 2.8-liter Fernbach flask. Flasks were incubated on a rotary shaker for 48 to 96 hr at 28 C. After this period of time, 100 ml of the resultant growth were added to 2 liters of sterile medium 1A contained in 4-liter Pyrex fermentors equipped for submerged aeration and agitation, as described by Shull and Kita (20). The inoculated vessels were incubated in a constant-temperature water bath at 28 C with aeration set at 0.5 volume per volume per min. Agitation was accomplished by stirring at 1,750 rev/min. Incubation proceeded until ample growth was obtained (usually 24 to 72 hr). At this point, 0.5 g of the sterile steroid substrate contained in 40 ml of acetone was added to the growth mixture. Incubation was maintained as before. This reactor stage was continued until the transformation of interest occurred. The reaction was followed by paper chromatographs of small portions taken periodically during the course of the fermentation.

Conversions also were conducted in 200-liter stainless-steel tanks which were operated in essentially the same manner as the 4-liter fermentors, with the exception that sterilization and incubation temperatures were attained by means of an internal coil. In addition, agitation was maintained at 300 rev/min. Up to 25 g of steroid was used as substrate for transformation.

Recovery. All broths and samples, whether for paper chromatographic analysis or for isolation of transformation products, were extracted in the same manner. Each portion of broth was extracted three times with 0.5 volume of chloroform. The three chloroform extracts were combined and concentrated to a residue. The residue was taken up in methanol, and all methanol-insoluble substances were filtered off and discarded (chromatography showed no steroids present in these insoluble substances). For recovery purposes, the methanol-soluble substances were taken to dryness, and were dissolved in a minimum of methylene chloride. The clear methylene chloride solutions were placed on a silica gel column where indicated; otherwise steroids were crystallized and then recrystallized from ethyl acetate or from methanol to which an excess of water

was added. When used, the silica gel column was prepared by the method described by Shull, Kita, and Davisson (21), which is essentially the technique first reported by Katzenellenbogen, Dobriner, and Kritchevsky (12). The column was eluted by adding increasing quantities of 3A ethyl alcohol to methylene chloride (usually from 1 to 5%, v/v). All recovered crystals were dried in vacuo (0.1 mm) at 78 C for 18 to 24 hr.

Paper chromatography. Six paper chromatographic systems were used in these studies. Systems A and B were those of Zaffaroni (26) and Zaffaroni et al. (27). Systems C, D, and E corresponded, respectively, to systems A, B-1, and B-3 of Bush (3), and system F corresponded to system 6 of Pachet (16).

Detection and partial identification of steroids on paper chromatograms were accomplished by various techniques, viz., the scanning technique of Haines and Drake (8) to reveal α,β-unsaturated ketosteroids; the sodium hydroxide fluorescent technique of Bush (3) to assist in identification of the Δ^1-analogues of Δ^4-3-ketosteroids; the method of Oertel (15), which employs 2,3,5-triphenyltetrazolium chloride to aid in the detection of α-ketol chains; and the Zimmermann reaction described by Savard (17) to indicate the presence of Δ^4-3,17-diketosteroids.

Melting points. Most melting points were determined by capillary tubes in the Hoover Capillary Melting Point Apparatus (Arthur H. Thomas Co., Philadelphia, Pa.). The melting point of compound VIa (see below) was measured in the Fisher-Johns Melting Apparatus (Fisher Scientific Co., Pittsburgh, Pa.). All melting points reported are uncorrected.

Absorption spectra. The infrared spectra of the transformation products were determined on a Baird Recording Spectral Photometer. Steroids were incorporated in a potassium bromide pellet (2.0%). The ultraviolet spectra were obtained by use of a Cary Model 11 Recording Spectral Photometer with methanol as the steroid solvent.

Optical rotations. The values reported are for methanol solutions at 23 to 25 C, unless stated otherwise.

RESULTS

Screening. Chromatography of the reaction broths revealed a number of transformations present. The transformations detected by chromatography are summarized in Table 1.

In these initial studies, the isolation and identification of all products from each culture were not undertaken. Rather, a few representative cultures were selected for further study on the basis of three criteria; viz., the products formed by each culture should be different from each other (or else the cultures should be different from each other); the yields should be adequate for recovery; there should be a minimum of lesser by-products.

It will be noted (Table 1) that progesterone was converted to a product with an R_F similar to androstenedione by *C. acremonium* 38C and 39C, *C. ciferrii* 21 C, and *Cephalosporium* spp. 26C, 27C, and 41C, as well as by *Verticillium malthousei.* Since all of these cultures produced about the same amount of conversion product, *C. ciferrii* 21C was selected arbitrarily for isolation studies.

Cephalosporium sp. 27C and *Colletotrichum gloeosporioides* converted Substance S to a slightly more polar product with an R_F similar to its Δ^1-analogue (i.e., Δ^1-S). The transformation product of each culture also had the same R_F as authentic Δ^1-S on chromatography in system E and gave a negative sodium hydroxide reaction. Recovery of the transformation compound was conducted with *Cephalosporium* sp. 27C, since it yielded a large quantity of product.

Other chromatograms from the screening studies showed that 17 cultures converted Substance S to a more polar substance with an R_F essentially identical to that of its 20-hydroxy analogue. All of these spots on the chromatograms gave a negative tetrazolium reaction, which is considered presumptive evidence for the 20-hydroxy analogue. *Geotrichum* sp. 51C was selected for further study in view of the large amounts of compound produced with no other by-products.

It was determined by chromatography that two cultures, *C. sclerotigenum* 31C and *V. aphidum* each transformed Substance S to a compound (with identical R_F values) more polar to 6β-hydroxy-S. Since the cultures were of different genera, it was decided to isolate the more polar material produced by each culture, in spite of the likelihood that the compounds were identical. The most polar of all transformation substances detected on the chromatograms was produced by *Paecilomyces persicinus* 22C from Substance S. Though only a small amount of this material was produced, we decided to attempt its recovery.

Isolation and identification of Δ^4-androstene-3,17-dione. C. ciferrii 21C was grown for 60 hr in a 4-liter pot. A 1-g amount of progesterone was added aseptically to the pot, and the fermentation was allowed to proceed for 48 hr. After 48-hr reaction, paper chromatography on system C with androstenedione and progesterone as controls showed only one spot present with an R_F identical to androstenedione. This spot also gave the characteristic purplish color with the Zimmermann test. The steroidal material was recovered as crystals from the chloroform extract of the broth and was purified via a series of recrystallizations from various solvents. The final crop of crystals weighed 213 mg after drying. These were labeled I. Constants of I: melting point, 168 to 170 C; ultraviolet absorption maxi-

TABLE 1. *Survey of steroid transformations*

Name of organism	Transformation[a]	Name of organism	Transformation[a]
Basidiomycete, unidentifiable	D	*Cephalosporium* sp. 41C	A
Beauveria bassiana		*Cephalosporium* sp. 42C	
Cephalosporium acremonium 38C	A	*Cephalosporium* sp. 43C	D
C. acremonium 39C	A	*Cephalosporium* sp. 45C	
C. acremonium 56C		*Cephalosporium* sp. 46C	D
C. alvaradi	B	*Cephalosporium* sp. 48C	B
C. apii		*Cephalosporium* sp. 54C	D
C. chrysogenum	D	*Cephalosporium* sp. 55C	
C. ciferrii 14C	B	*Colletotrichum gloeosporioides*	B, C, D
C. ciferrii 21C	A[b]	*Emericellopsis salmosynnemata*	
C. ciferrii 24C	B	*E. terricola*	
C. gramineum	B	*Fusarium moniliforme*	D
C. gregatum		*F. solani*	B, D
C. kiliense		*Geotrichum* sp. 51C	D, E[b]
C. longisporum	B	*Gliocladium roseum* 9C	
C. mycophilum	D	*G. roseum* 36C	
C. roseo-griseum	B, E	*G. roseum* 50C	F
C. sclerotigenum 31C	B, F[b]	*Paecilomyces javanicus*	
C. sclerotigenum 32C	B, F	*P. persicinus* 5C	B, D
C. sclerotigenum 33C		*P. persicinus* 10C	B
C. sclerotigenum 34C		*P. persicinus* 15C	B, D
Cephalosporium sp. 7C	D	*P. persicinus* 20C	B
Cephalosporium sp. 11C	B, D	*P. persicinus* 22C	B, G[b]
Cephalosporium sp. 12C	B, D	*P. persicinus* 25C	B
Cephalosporium sp. 17C	B, D	*P. persicinus* 49C	B, F
Cephalosporium sp. 26C	A	*Paecilomyces* sp.	
Cephalosporium sp. 27C	A, C[b]	*Verticillium aphidum*	B, F[b]
Cephalosporium sp. 35C	B	*V. malthousei*	A, B
Cephalosporium sp. 37C		*Zythia* sp.	B, D

[a] Letters denote transformations as determined by paper chromatography. Absence of a letter is indicative of no chromatographically detectable transformation. Conversions are as follows: A = progesterone to androstenedione; B = progesterone to more polar, but unknown, products; C = Reichstein's Substance S to its Δ^1-analogue; D = Substance S to its 20-ol analogue; E = Kendall's Compound F to its 20β-ol analogue; F = Substance S to its 6β-hydroxy analogue; G = Substance S to a more polar, unidentified product.

[b] Transformation product isolated and described in this paper.

mum at 239.5 mμ; ϵ = 16,190; $[\alpha]_D$ = 191.7° (*c*, 1.112 in chloroform). Calculated for $C_{19}H_{26}O_2$ (286.40); C, 79.68%; H, 9.15%. Found: C, 79.69%; H, 9.22%. The infrared curve of I corresponded with authentic androstenedione.

Isolation and identification of $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,20-dione. Cephalosporium sp. 27C was inoculated into two pots as described for *C. ciferrii* 21C above, except that after 48 hr of growth 0.5 g of Substance S was added aseptically to each pot. After 40 hr of reaction, paper chromatography indicated only one compound present with an R_F identical to authentic Δ^1-S. This transformation compound also gave a negative sodium hydroxide reaction characteristic of the Δ^1-analogue. The broths were combined and extracted with chloroform. Crystals were obtained, which upon repeated recrystallizations were purified sufficiently for analysis. A total of 159 mg was obtained. These were labeled II. Constants of II: melting point, 230 to 233 C; ultraviolet absorption maximum at 244 mμ; ϵ = 15,270; $[\alpha]_D$ = 78° (*c*, 1.108). Calculated for $C_{21}H_{28}O_4$ (344.43): C, 73.22%; H, 8.20%. Found: C, 72.88%; H, 8.24%. The infrared curve of II was comparable to authentic $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,20-dione.

Since enough material was at hand, the acetate of II was prepared essentially in accord with the method described by Bernstein et al. (1). A 95-mg amount of II was dissolved in 1.025 ml of pyridine-acetic anhydride (1.000:0.025) and kept at room temperature overnight. Approximately 5.0 ml of cold distilled water was added, and the crystalline precipitate which formed was collected and washed several times with cold distilled water. A total of 80 mg was recovered. These crystals were recrystallized several times from ethyl acetate, and the final crop was dried in vacuo. A final yield of 28 mg was obtained.

This material was labeled IIa. Constants of IIa: melting point, 221 to 223.5 C; ultraviolet absorption maximum at 245 mμ; ϵ = 15,900; $[\alpha]_D$ = 101° (*c*, 1.116). Calculated for $C_{23}H_{30}O_5$ (386.47): C, 71.48%; H, 7.80%. Found: C, 71.07%; H, 7.81%. The infrared curves of IIa and authentic $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,20-dione 21 acetate were comparable. The characterization of IIa provides added proof of the identity of II.

Isolation and identification of Δ^4-pregnene-11β,17α,20β,21-tetraol-3-one. It was determined in shake flasks that *Geotrichum* sp. 51C could form the presumed 20-hydroxy derivative of Kendall's Compound F (Δ^4-pregnene-11β, 17α, 21-triol-3,20-dione), as well as of Reichstein's Substance S. Since some 20β-ol-F was available as a control, an attempt was made to produce and recover this compound. Three fermentation pots were set up in the usual manner and inoculated with *Geotrichum* sp. 51C. After 48 hr of growth, 0.5 g of Compound F was added aseptically to each pot. At 72 hr after the addition of the steroid substrate, no Compound F remained in any of the broths. However, a compound with an R_F identical to authentic 20β-ol-F, negative to tetrazolium (presumptive reduction of 20-keto to 20-ol), was present. The whole broths from the three pots were combined and extracted with chloroform. Analytically pure crystals (50 mg) finally were obtained after a succession of recrystallizations. These were labeled III. Constants of III: melting point, 122.5 to 125 C; ultraviolet absorption maximum at 242 mμ; ϵ = 14,050; $[\alpha]_D$ = 90° (*c*, 1.029). Calculated for $C_{21}H_{32}O_5$ (364.47): C, 69.20%; H, 8.85%. Found: C, 69.54%; H, 9.04%. The infrared curves of III and authentic 20β-ol-F exhibited excellent correspondence.

Isolation and identification of Δ^4-pregnene-6β,17α,21-triol-3,20-dione. Four fermentation flasks, each containing a 4-day growth of *C. sclerotigenum* 31C, were inoculated into a stainless-steel fermentation tank containing 90 liters of sterile medium 1A. After 24 hr of growth, 25 g of Substance S was added aseptically. The progress of the conversion was followed by paper chromatography. At 43 hr after the addition of substrate, the compound of interest was produced in sufficient quantity for recovery.

The whole broth was extracted in the usual manner with chloroform. The chloroform was concentrated to a residue which was taken up in methylene chloride and placed on a column of silica gel. Column fractions, on evaporation of solvent, yielded the material of interest in crystalline form. These fractions were pooled, and 153 mg of analytically pure crystals was eventually obtained. The compound was labeled IV.

Paper chromatography of IV on system F revealed that this compound had an R_F identical with an authentic sample of Δ^4-pregnene-6β,17α, 21-triol-3,21-dione (6β-hydroxy-S). Authentic 6β-hydroxy-S and IV were both scanner-positive, sodium hydroxide-positive, and tetrazolium-positive. Constants of IV: melting point, 221 to 223 C; ultraviolet absorption maximum at 236 mμ; ϵ = 14,240; $[\alpha]_D$ = 57° (*c*, 1.151). Calculated for $C_{21}H_{30}O_5$ (362.47): C, 69.59%; H, 8.34%. Found: C, 69.27%; H, 8.27%. The infrared curve of IV was comparable to the infrared curve of authentic 6β-hydroxy-S.

The 21 monoacetate of IV, labeled IVa, was formed essentially by the method described above. Constants of IVa: melting point, 253 to 255 C; ultraviolet absorption maximum at 236 mμ; ϵ = 13,172; $[\alpha]_D$ = 70° (*c*, 0.623). Calculated for $C_{23}H_{32}O_6$ (404.51): C, 68.29%; H, 7.97%. Found: C, 68.06%; H, 8.09%. There was no authentic Δ^4-pregnene-6β, 17α,21-triol-3,20-dione 21 acetate available for comparison of infrared curves, but the constants of IVa correspond well with this compound according to the published data (22).

A crystalline compound, V, was isolated in essentially the same manner as IV. Compound V was a conversion product of Substance S, effected by *V. aphidum*. It displayed an R_F on paper chromatography (system F) identical to IV and authentic 6β-hydroxy-S. It was also scanner-positive, sodium hydroxide-positive, and tetrazolium-positive. Constants of V: melting point, 222 to 224 C; ultraviolet absorption maximum at 235.5 mμ; ϵ = 14,120; $[\alpha]_D$ = 55° (*c*, 0.585). Calculated for $C_{21}H_{30}O_5$ (362.47): C, 69.59%; H, 8.34%. Found: C, 69.42%; H, 8.28%. The infrared curve of V was identical to that of authentic 6β-hydroxy-S.

Isolation of an unidentified steroid. In the screening studies, *Paecilomyces persicinus* 22C was found to produce two conversion products more polar than the substrate, Reichstein's Substance S. The product with the lesser polarity had an R_F similar, though not identical, to the products of *C. sclerotigenum* 31C and *Verticillium aphidum*, which proved to be the 6β-hydroxy analogue of Substance S. It was decided to attempt the production and isolation of the more polar of the two conversion products formed by *P. persicinus* 22C. No attempt was made to isolate both transformation products, since these were produced in very small quantities, and, as the microbiological conversions proceeded, the more polar compound increased, apparently at the expense of the compound of lesser polarity.

The growth and reactive phases of *P. persicinus* 22C were set up as described for *C. sclerotigenum* 31C. At 40 hr after the addition of 25 g of Sub-

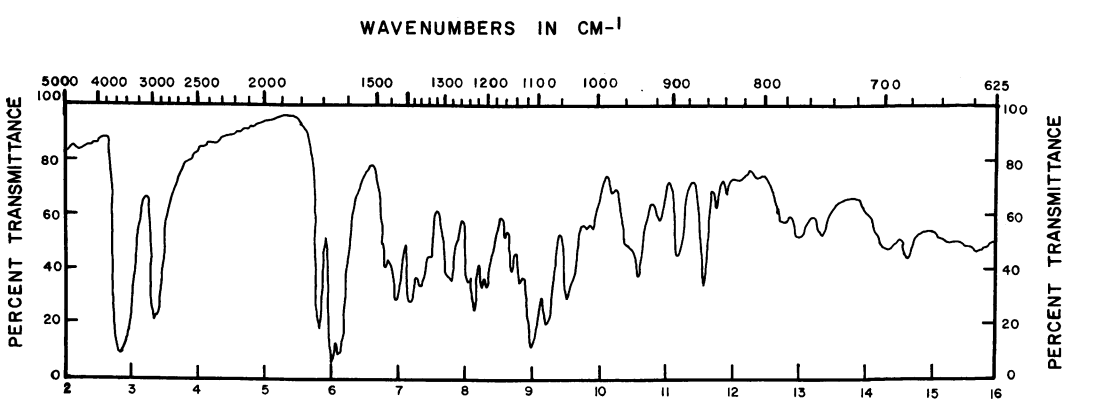

FIG. 1. *Infrared absorption spectrum of compound VIa.*

TABLE 2. *Summary of transformations from which products were isolated and identification attempted*

Name of organism	Substrate	Transformation product
Cephalosporium ciferrii 21C	Progesterone	Androstenedione
C. sclerotigenum 31C	Substance S	6β-Hydroxy-S
Cephalosporium sp. 27C	Substance S	Δ^1-S
Geotrichum sp. 51C	Compound F	20β-ol-F
Paecilomyces persicinus 22C	Substance S	Unidentified
Verticillium aphidum	Substance S	6β-Hydroxy-S

stance S, paper chromatography (system F) of a sample of broth revealed only a trace of both substrate and the compound of lesser polarity, as well as a small, but significant, amount of the more polar product. The fermentation was terminated at 42 hr by the addition of chloroform. The whole broth was extracted with chloroform. The extracted material was chromatographed twice on silica gel columns from which a small quantity of the crystals of the material of greater polarity was finally recovered. Recrystallization yielded a material labeled VI with the following constants: melting point, 174 to 179 C; ultraviolet absorption maximum at 242 mμ; $[\alpha]_D$ = 121° (*c*, 0.756). The compound was recrystallized once again to yield a few milligrams of material labeled VIa. Constants of VIa: melting point, 185.5 to 189 C. Found: C, 67.29%; H, 8.33%. Figure 1 is an infrared curve of VIa. Efforts to identify this steroid have been unsuccessful to date. Since VIa is not as pure as desirable for analytical purposes, it is planned to collect additional quantities of this material for identification.

Table 2 lists those microorganisms and their respective transformation reactions for which products were isolated and identification was attempted.

DISCUSSION

The techniques employed for testing the ability of microorganisms to convert steroids in submerged culture vary somewhat from laboratory to laboratory. However, all such techniques represent compromises of one sort or another. For example, one may use a number of basically different media for each strain tested under varying conditions of *p*H, temperature, agitation, aeration, etc. The choice of substrate often is dictated by such uncontrollable factors as cost and availability. The time of addition of the substrate, as well as the length of time in contact with the organism, can be investigated for periods extending from less than 1 hr to more than 96 hr. In addition, the limits which are set for recovery and identification of a given conversion are governed by the amount of substrate used and the yield obtained. The foregoing is an indication that a particular screening procedure is restricted with respect to its efficacy in disclosing the steroid-transforming capabilities of a given microorganism.

The literature contains examples substantiating the fact that the steroid-transforming capacities of microorganisms are affected by the type of

medium employed (23). This might explain the production of a transformation more polar than 6β-hydroxy-S by *C. sclerotigenum* 31C and *V. aphidum* when grown in shake flasks containing medium 1B, but not when grown in 4-liter pots containing medium 1A. It should be noted that the method of fermentation (shake flasks versus pots) also might play a part.

It was indicated earlier that only three cephalosporia were reported to effect microbiological transformations of steroids. Bodanszky et al. (2) found that *C. subverticillatum* converted progesterone to androstenedione which, on prolonged incubation, was transformed to Δ^4-testololactone. Bernstein et al. (1) reported the conversion of Reichstein's Substance S to a 7α-hydroxy analogue by an unidentified species of the genus *Cephalosporium*. In 1963, Holmhund et al. (9) described the transformation of androstenedione to testolic acid by *C. acremonium*. To this list are added *C. sclerotigenum* and *Cephalosporium* sp. 27C, which, respectively, converted Reichstein's Substance S to the 6β-hydroxy and Δ^1-analogue. On the basis of paper chromatography, *C. roseogriseum* is a candidate for 6β-hydroxylation of Substance S.

Chromatographic techniques also offer presumptive evidence for reduction of the 20-keto group of Substance S by nine cephalosporia, as well as the conversion of progesterone to androstenedione by six cephalosporia (in the case of *C. ciferrii* 21C, the transformation product, androstenedione, was isolated and identified). Thirteen cephalosporia were found to convert progesterone to more polar, though unidentified, products. In all, 25 (>70%) of the 35 cephalosporia proved active. The range of transformations effected by the cephalosporia attest to the versatility of their enzyme systems. However, no clear pattern of activity of taxonomic value emerges from these data.

In addition to the cephalosporia noted above, 6β-hydroxylation of Substance S was effected by *V. aphidum*, and (presumptively) by *Gliocladium roseum* 50C and *Paecilomyces* sp. 49C. No references in the literature have been uncovered regarding 6β-hydroxylation by these organisms.

A survey of the literature also failed to turn up any reports relating to reduction of the 20-keto group of Reichstein's Substance S and Kendall's Compound F by species of *Geotrichum*. In addition to *Geotrichum* sp. 51C and nine cephalosporia (see above), the 20-keto group of Substance S was reduced by seven other organisms including species of *Colletotrichum*, *Fusarium*, *Paecilomyces*, *Zythia*, and an unidentified basidiomycete. Apparently, enzyme systems responsible for reduction of the 20-keto group in Substance S are fairly widespread in fungi.

Of the 58 microorganisms studied, two dehydrogenated Substance S to form the Δ^1-analogue. These were: *Cephalosporium* sp. 27C and *Colletotrichum gloeosporioides*. No reports in the literature of such activity by these organisms were found.

Four of the transformation products isolated in these studies are known compounds produced by organisms not previously reported to effect such transformations. Only compound VIa, produced by *P. persicinus* 22C, remains an unidentified steroid. This compound proved to be tetrazolium-positive and sodium hydroxide-negative, ruling out the possibility of its being either a 20-hydroxy derivative or the Δ^1-analogue. As a matter of fact, the observation was made during the fermentation that *P. persicinus* 22C converted Substance S to two quite polar compounds, the more polar of which was isolated as compound VIa. Furthermore, it was apparent during the fermentation that, as the compound of lesser polarity decreased, the more polar compound increased in quantity. In other words, the more polar compound appeared to be a probable transformation of the less polar compound.

Since dihydroxylation of steroid substrates is not uncommon (23), it is quite conceivable that compound VIa is just such a dihydroxylated derivative. Some support for this hypothesis derives from the following data: calculated for $C_{21}H_{30}O_6$ (378.47): C, 66.64%; H, 7.99%. Found: C, 67.29%; H, 8.33%. Elucidation of structure awaits isolation of additional material.

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