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TRANSFORMATION OF PROGESTERONE BY *FUSARIUM ARGILLACEUM*

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SUMMARY

Progesterone was converted by *Fusarium argillaceum* into testosterone, 4-androstene-3,17-dione, 5α-androstane-3,17-dione, 15α-hydroxyprogesterone, 15α-hydroxytestosterone, 15α-hydroxy-4-androstene-3,17-dione and 16α-hydroxy-4-androstene-3,17-dione, as revealed by a thin-layer chromatographic method. The metabolites were isolated and identified. A scheme is presented describing the probable metabolism.

INTRODUCTION

IN THE COURSE of our studies of *Fusaria* which transform progesterone[1], we observed that *F. argillaceum* forms metabolites not formed by other tested members of the group *Fusaria*. These metabolites were revealed by thin-layer chromatography and attracted our attention because they gave with sulphuric acid a characteristic blue color, a phenomenon which is rather uncommon. The aim of the present study was to identify these metabolites and to elucidate the metabolism of progesterone by *F. argillaceum*.

EXPERIMENTAL

Melting points were determined on a Kofler block and are uncorrected. Optical rotations were taken in a 1 dm cell at 20°C. Infrared spectra were recorded with a Perkin–Elmer Model 521 spectrophotometer in KBr. Ultraviolet spectra were determined with a Unicam spectrophotometer in ethanol. Mass spectra were recorded with a CEC 21–110 C mass spectrometer by direct insertion of the sample into the ion source maintained at 200°C. Electron energy 70 eV and ionising current 100 μA were used.

Thin-layer chromatography was done either on 0·25 or 1·5 mm thick silicagel G or silicagel GF_{254} chromatoplates using cyclohexane–ethyl acetate (1 : 2, v/v) as the developing phase and 50 per cent sulphuric acid for detection as previously described[2].

Fusarium argillaceum was isolated and identified at the Microbiological Department of the Chemical Institute Boris Kidrič. The mycelium is floculous and of a dirty white color. Chlamydospores are terminal, round to ovoid. Conidia are spindle to sickle shaped, narrowing towards their ends. Three-septated macroconidia predominate.

Incubation

F. argillaceum spores were maintained at 28°C on potato-glucose-agar slants. The cultures were grown in 500 ml flasks containing 100 ml of the medium composed of 0·005% glucose, 0·6% corn steep liquor (50% dry wt.) and 1·5% peptone. Before sterilisation, the pH was adjusted to 6·5 with diluted alkali.

After 48 h of incubation at 28° on a reciprocal shaker, 20 mg of progesterone dissolved in 1 ml of acetone, was added to each flask and the incubation continued for 24 h or longer.

Isolation and identification of metabolites

The pooled broths were filtered to remove mycelium and the filtrates extracted with methylene dichloride. The combined extracts were washed with water, dried over sodium sulfate and evaporated under reduced pressure. The oily residue was dissolved in methanol, filtered and the solvent removed. The residue was then chromatographed on 80 g of neutral alumina (Merck, grade I) and eluted with benzene–ether (9 : 1, v/v) and ether–methanol (97 : 3, v/v). The collected fractions were tested by t.l.c. and appropriate fractions were combined and further purified by preparative t.l.c. and recrystallisation.

RESULTS

Transformation of progesterone

When progesterone (850 mg) was incubated for 24 h with *F. argillaceum*, seven metabolites could be isolated and identified. The R_F value (0·57) of the main metabolite (340 mg), the color of its spot in day and U.V. light as well as the I.R. spectrum were identical with those of *androstenedione*.* In addition, its m.p. 172–173°C (cryst. from benzene–ether 9 : 1, v/v), λ_{max}^{EtOH} 239 nm and $(\alpha)_D + 207°$ ($CHCl_3$) were in agreement with the data from the literature [3, 4], which requires: m.p. 170–172°C; $(\alpha)_D + 197°$ ($CHCl_3$); λ_{max}^{EtOH} 239 nm.

Traces of a substance with the R_F value 0·44 were eluted just after the main product. When the incubation was interrupted after 5 h, more of the latter substance (0·5 mg) was obtained. With respect to the R_F value, color in day and U.V. light, and mass spectrum (M^+ 288 and prominent peaks at m/e 246 and 124), the substance did not differ from *testosterone*.

The substance (1 mg) with the highest mobility (R_F 0·68) showed a green color in daylight and a blue fluorescence both characteristic of *5α-androstanedione* [5]. The I.R. spectrum showed the presence of a 3-keto group at 1724 cm^{-1}, and of a 17-keto group at 1736 cm^{-1}, but no double bond absorption. Moreover, the I.R. spectrum was superimposable on that of an authentic sample of 5α-androstanedione and different from that of 5β-androstanedione [6]. Its m.p. 127–130°C (cryst. from hexane) and molecular weight 288 (M^+ 288) were also in agreement with the structure of 5α-androstanedione.

With sulphuric acid, metabolites with the R_F values of 0·26 (1 mg) and 0·22 (75 mg), showed a characteristic color, blue in daylight and red in U.V. light. This fact together with the rather low R_F values pointed to the probable presence of a hydroxyl group in ring C or D [7]. By comparing their R_F values with those of authentic samples (of 14α-, 15α-, 15β- and 16α- hydroxyandrostenediones), the substance with the R_F value of 0·26 was tentatively identified as *16α-hydroxyandrostenedione* and that with R_F 0·22 as *15α-hydroxyandrostenedione*. The latter had a m.p. 197–199°C (cryst. from benzene), λ_{max}^{EtOH} 240 nm and $(\alpha)_D + 229°$ ($CHCl_3$). γ_{max}^{KBr} 1·736 (17 C=O), 1·675 (3 C=O), 1·626 cm^{-1} (C=C). Literature [8, 9] requires: m.p. 194–196°C; 190–198°C; λ_{max}^{EtOH} 240 nm; $(\alpha)_D + 217°$ ($CHCl_3$).

**Trivial names:* Androstenedione: 4-androstene-3, 17-dione; 5α-androstanedione: 5α-androstane-3, 17-dione; 5β-androstanedione: 5β-androstane-3,17-dione.

The mass spectrum of the latter substance (M^+ 302, m/e 284 ($M-H_2O$), 260, 124) was found to have all prominent peaks with the same relative intensities as the mass spectrum of 15α-hydroxyandrostenedione determined by Zaretskii, Wulfson, Zaikin, Kogan, Voishvillo, Torgov[10]. It was easily acetylated with acetic anhydride–pyridine giving a monoacetate with the required molecular weight 344 (M^+ 344). The substance tentatively identified as *16α-hydroxyandrostenedione* showed absorption at λ_{max}^{EtOH} 240 nm as well as corresponding absorption bands in the I.R. spectrum at 1·675 cm^{-1} and 1·625 cm^{-1} confirming an unchanged 4-ene-3-ketone configuration. The band at 1·740 cm^{-1}, typical of 5-ring ketones, indicated that the progesterone side chain was split off and a 17-keto compound was formed. The band at 3·400 cm^{-1} was due to the introduction of a hydroxyl group into the molecule. The substance gave a red color with triphenyltetrazolium chloride, which proved the presence of an alpha ketol group.

The mass spectra of the isolated 16α-hydroxyandrostenedione and of an authentic 16α-hydroxyandrostenedione were identical, both having a more intense M^+ than $M-H_2O$ ion. The claim of Zaretskii *et al.*[10] that the configuration of the corresponding alcohol in the monohydroxy steroid series may be determined on the basis of its mass spectrum even if the second epimer is not available, was questioned by Grostic and Rinehart[11] in cases where only the mass spectrum of one isomer is available. Since in our case the second isomer, 16β-hydroxyandrostenedione, was not available, the benzoates of our substance and that of the authentic 16α-hydroxyandrostenedione were prepared and their mass spectra compared with that of the authentic 16β-hydroxyandrostenedione benzoate. The two 16α-hydroxyandrostenedione benzoates gave superimposable mass spectra which differed from that of 16β-hydroxyandrostenedione benzoate in the ratio of $M^+/M-C_6H_5CO$ peaks. In this way the structure of the substance with R_F 0·26 was established as 16α-hydroxyandrostenedione even though only one milligramme of the substance was available.

On thin-layer chromatograms, 15α-hydroxyandrostenedione was closely followed by a substance with almost the same mobility. Differentiation, however, was possible since with sulphuric acid the latter substance gave a yellow color in daylight and green in U.V. light. This metabolite was probably *15α-hydroxyprogesterone* since its R_F value and color reaction were identical with those of an authentic sample of 15α-hydroxyprogesterone.

The metabolite (14 mg) with the R_F value of 0·06, brown in daylight and yellow–orange in U.V. light, had a molecular weight 304 (M^+ 304), λ_{max}^{EtOH} 243 nm and $(\alpha)_D + 137°$ (MeOH) and a m.p. 101–102°C/160–162°C. The I.R. spectrum 1·675 and 1·625 cm^{-1} showed the presence of a 3-keto-4-ene group. The above properties were in agreement with the data reported for 15α-hydroxytestosterone by Tamm, Gubler, Juhasz, Weis-Berg and Zürcher[9], who obtained it as a metabolite of testosterone by *Fusarium lini*. Acetylation of the above metabolite with acetic anhydride-pyridine yielded a diacetate (M^+ 388) which melted at 149–153°C and whose $(\alpha)_D$ was + 106° (MeOH), confirming the assignment as *15α-hydroxytestosterone*. Literature[9] requires: m.p. 153°C; $(\alpha)_D + 108°$ (MeOH).

DISCUSSION

The results obtained show that *F. argillaceum* can effect several different transformations in the progesterone molecule as shown in Fig. 1: it can split

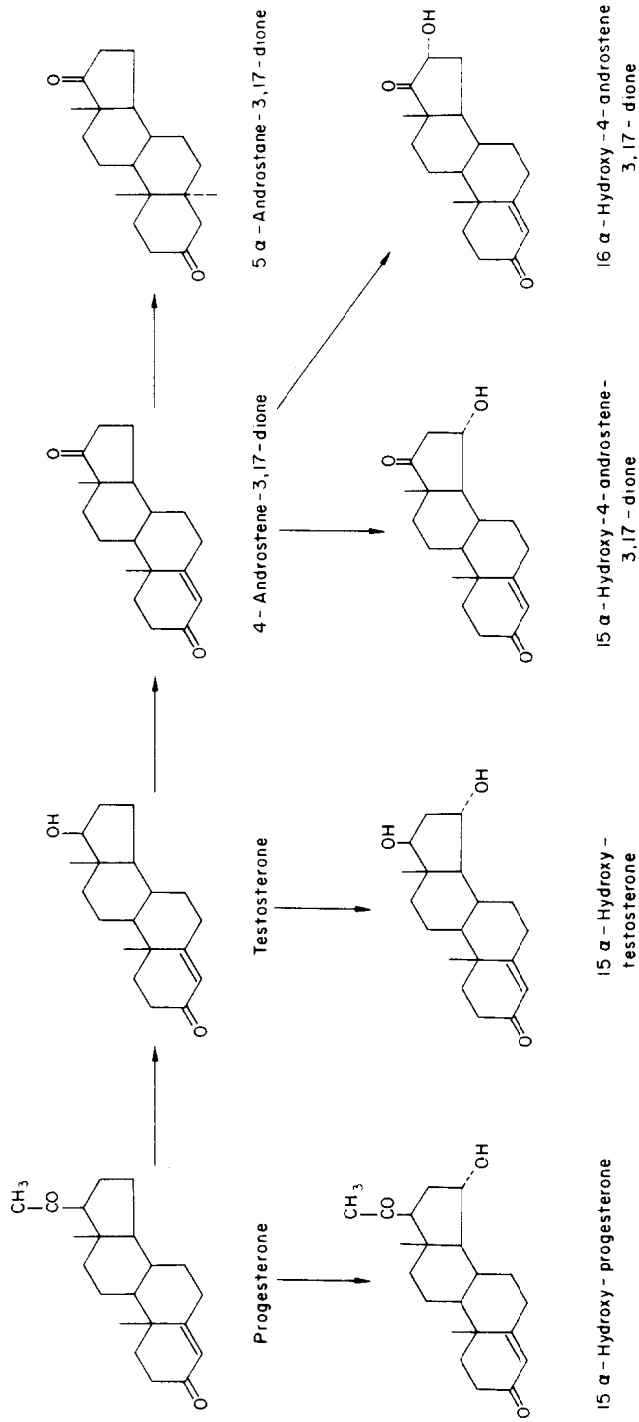

Fig. 1. Transformation of progesterone by *Fusarium argillaceum*.

off the side chain, hydroxylate at C-15α and to a smaller extent at C-16α, and hydrogenate the 4:5 double bond to C-5α. Although these types of microbial transformations are known to occur very often separately, their simultaneous actions are less common; thus, for example, side chain splitting with simultaneous 6-hydroxylation is described only for *Gliocladium catenulatum*[12] and 11-hydroxylation for *Beauveria bassiana*[13, 14], *Aspergillus tamarii*[15], *Aspergillus ochraceus*[16] and *Fusarium javanicum*[17, 18]. It is also known that *Fusarium lini*[9] effects a 15α-hydroxylation with the simultaneous hydrogenation of the 4:5 double bond of progesterone giving a 5α compound, but without side chain splitting. Therefore, *F. argillaceum* appears to be a unique example of a microorganism in which such different enzyme systems are combined.

On the other hand, a further transformation of 5α-androstanedione to androsterone as described by Hörhold, Böhme and Schubert[19] for *Mycobacterium smegmatis*, was not observed even though thin-layer chromatography using androsterone for comparison was employed.

When the course of the transformation of progesterone by *F. argillaceum* was followed by thin-layer chromatographic analyses of the broth at short time intervals, the first detectable metabolite after 2 h of incubation was shown to be testosterone. After 3 h, 15α-hydroxyprogesterone and 15α-hydroxytestosterone also began to appear. The main product, androstenedione, began to appear after 4 h of incubation, and one hour later its hydroxylated derivative, 15α-hydroxyandrostenedione. At the same time the presence of 5α-androstanedione was also noticed. These results indicate that the degradation of progesterone by *F. argillaceum* starts with the degradation of the side chain giving rise to the formation of testosterone and androstenedione.

Several possible pathways of side chain degradation of progesterone are known to occur in microorganisms and higher animals as reported in detail by Singh and Rakhit[20]. The above results suggest that the oxidative cleavage proceeds by a pathway similar to the non-enzymatic Baeyer–Villiger oxidation of ketones found to occur by Singh and Rakhit[20] for *Streptomyxa affinis* and by Tan and Smith for *Aspergillus ochraceus*[16].

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