MICROBIAL TRANSFORMATION OF 17α-CYANOMETHYL-17-HYDROXY-4,9-ESTRADIEN-3-ONE (STS 557) AND 17α-CYANOMETHYL-19-NORTESTOSTERONE BY MYCOBACTERIUM SMEGMATIS

G. Hobe, R. Schön, C. Hörhold, M. Hübner, W. Schade and K. Schubert

Academy of Sciences of GDR, Research Center for Molecularbiology and Medicine, Central Institute of Microbiology and Experimental Therapy (Director: Prof. Dr. U. Taubeneck), Department of Steroid Biochemistry (Head: Prof. Dr. K. Schubert) GDR-6900 Jena

Received 1-4-82

ABSTRACT

Microbial transformation of the new progestagen STS 557 (17α-cyanomethyl-17-hydroxy-4,9-estradien-3-one) by Mycobacterium smegmatis yielded predominantly ring A-aromatized compounds: 17α-cyanomethyl-1,3,5(10),9(11)-estratetraene-3,17-diol, 17α-cyanomethyl-1,3,5(10)-estratriene-3,17-diol and the corresponding 3-methyl ethers. The analogous compound without the 9(10) double bond, 17α-cyanomethyl-19-nortestosterone, was transformed mainly to 5α-hydrogenated metabolites: 17α-cyanomethyl-17-hydroxy-5α-estran-3-one, 17α-cyanomethyl-17-hydroxy-5α-1-estren-3-one, 17α-cyanomethyl-5α-estrane-3α,17-diol, and 17α-cyanomethyl-5α-estrane-3β,17-diol. From these results, it is concluded that 4,9-dien-3-oxo compounds are not substrates for enzymatic 5α-hydrogenation.

INTRODUCTION

Mycobacterium smegmatis SG 98 is known to exhibit a variety of steroid transforming activities. Some of these are 1- and 4-dehydrogenation, 5α-hydrogenation of 4-en-3-oxo steroids, introduction of a 9-oxygen group in 1,4-dien-3-oxo steroids accompanied by the formation of

9,10-seco compounds and aromatization of ring A, and followed by a further degradation of the steroid skeleton (1 - 6).

This microorganism has been used for the preparation of hydrogenated metabolites of norgestrel and norethisterone (7). Recently, 5αH-metabolites of progestagens have become of interest because of their potential antiprogestagenic activities (8). Therefore, the attempt was made to obtain 5αH-metabolites of the new progestagen STS 557[+)] (17α-cyanomethyl-17-hydroxy-4,9-estradien-3-one) by incubating this compound and also its analogue without the 9(10)-double bond, 17α-cyanomethyl-19-nortestosterone, with M. smegmatis.

MATERIALS AND METHODS

STS 557 (I), ^{3}H-STS 557 (spec. activity ≈4 Ci/mmole (10), and 17α-cyanomethyl-19-nortestosterone (II) were provided by the Department of Steroid Synthesis (Head: Prof. Dr. K. Ponsold) of CIMET. 17α-Cyanomethyl-1,3,5(10), 9(11)-estratetraene-3,17-diol, 17α-cyanomethyl-1,3,5(10)-estratriene-3,17-diol and their 3-methyl ethers as also 17α-cyanomethyl-17-hydroxy-5α-estran-3-one, 17α-cyanomethyl-5α-estrane-3α,17-diol, 17α-cyanomethyl-5α-estrane-3ß,17-diol and 17α-cyanomethyl-17-hydroxy-5ß-estran-3-one were synthesized by one of the authors (M. H.); the syntheses are described elsewhere (11, 12).

[+)] STS 557 belongs to a series of steroid compounds (9) synthesized in the Department of Steroid Synthesis of the Central Institute of Microbiology and Experimental Therapy (CIMET), Jena/GDR.

Microbial transformation: Mycobacterium smegmatis SG 98 was obtained from the Type Culture Collection of CIMET Jena. The microorganism was cultivated in Sauton culture medium for 4 days at 37 °C on a gyratory shaker. The cells were harvested by centrifugation, washed once with the original volume of 0.03 M phosphate buffer (pH 7) and resuspended in the same buffer (10 g wet cells in 100 ml buffer in each flask).

5 mg steroid, in the case of STS 557 with addition of 20 µCi of the radioactive compound, were added to each flask and incubated at 28 ° for 24 h and 72 h respectively, under aerobic conditions. The culture medium was extracted 3x with 2 volumes of $CHCl_3$. The extract was evaporated in vacuo.

For defatting, the residue was distributed between n-hexane and methanol/water (9:1) according to Freudenthal et al. (13). The methanol solution was evaporated, and the residue subjected to thin layer chromatography.

Thin layer chromatography (TLC): Silica gel plates GF 254 (Merck, Darmstadt), 0.25 mm, 10 x 20 cm were used. Ascending solvent systems: benzene/ether 1:2 (= A) and 1:3 (= B); $CHCl_3$/ether 87:13 (= C) and 8:2 (= D); $CHCl_3$/EtOAc/MeOH 100:40:2 (= E); $CHCl_3$/acetone 93:7 (= F); benzene/EtOAc 8:2 (= G); cyclohexane/ether 2:8 (= H).

Substances on the chromatograms were localized by radioscanning (windowless methane flow counter VEB Vakutronik Dresden) when using ^{3}H-STS 557 as substrate, or by their UV absorption at 254 nm and by staining of aliquot strips of the chromatogram with phosphotungstic acid (15 % in MeOH, 120 °). TLC was repeated in different systems until uniformity of a compound was evidenced.

Isolated metabolites were characterized by UV, IR and mass spectrometry, by measuring the circular dichroism, and by comparison with authentic reference compounds. Ultraviolet spectra were measured in ethanol with a Specord UV VIS (VEB Carl Zeiss Jena) and infrared spectra (KBr) with a Perkin Elmer 325 spectrophotometer. Mass spectra were recorded with a Jeol mass spectrometer JMS-D 100, and CD data were obtained in ethanol with a circulardichrograph Cary Model 60. Melting points were determined on a Boetius melting point apparatus.

Oxidation of 3-hydroxy to 3-oxo compounds was performed with CrO_3 in acetone according to Jones (14).

RESULTS

Transformation of 17α-cyanomethyl-17-hydroxy-4,9-estradien-3-one (STS 557)

STS 557 was transformed by Mycobacterium smegmatis predominantly (> 80 %) to compounds less polar than the starting material in TLC (system A). By TLC in different solvent systems, two metabolites, III and IV, were isolated; also, a non-uniform zone x occurred which could not be separated by TLC. The quantitative composition of the metabolite mixture changed depending on the incubation time. After 24 h, III and IV were present in nearly the same amounts; with longer incubation time, III decreased and zone x increased.

Metabolite III (identified as 17α-cyanomethyl-1,3,5(10),9(11)-estratetraene-3,17-diol):

TLC: system A R_F 0.59 (R_F^I 0.18); system C 2x R_F 0.30; system D R_F 0.23, 2x R_F 0.36.

UV: λ_{max} 265 (273) nm. IR: 2220 cm^{-1} (CN)

MS: m/z 309.1753 (M^+); calcd. for $C_{20}H_{23}NO_2$ 309.1729

Metabolite IV (identified as 17α-cyanomethyl-1,3,5(10)-estratriene-3,17-diol:

TLC: system A R_F 0.59; system C 2x R_F 0.22; system D 2x R_F 0.27. UV: λ_{max} 280 (287) nm. IR: 2244 cm^{-1} (CN).

MS: m/z 311.1885 (M^+); calcd. for $C_{20}H_{25}NO_2$ 311.1885.

The chromatographic properties, UV, IR and mass spectra of III and IV were found to be identical with those of authentic 17α-cyanomethyl-1,3,5(10),9(11)-estratetraene-3,17-diol and 17α-cyanomethyl-1,3,5(10)-estratriene-3,17-diol, respectively (11).

Zone x had the same chromatographic properties as the 3-methyl ethers of III and IV which could not be separated by TLC: System A R_F 0.48 (R_F^{III} 0.37); system C R_F 0.38; system D R_F 0.32; UV: λ_{max} 265 (274) nm. IR: 2242 cm^{-1} (CN). MS: m/z 323.1916 (M^+), calcd. for $C_{21}H_{25}NO_2$ (III-3-methyl ether) 323.1885; m/z 223.1095, calcd. for $C_{16}H_{15}O$ (M^+ - D ring - 2H) 223.1123.

In addition to the molecular ion peak of III at m/z 323, a smaller peak at m/z 325 was present, indicating that obviously IV-3-methyl ether has also been formed.

Separation of zone x could finally be achieved by means of HPLC. A self-assembled liquid chromatograph of Fa. Knauer (Bad Homburg/FRG) was used: Column length 25 cm, inner diameter 4.6 mm; stationary phase 10 μ Lichrosorb RP 18 (Merck, Darmstadt), mobile phase CH_3OH-H_2O (80:20); 40 bar, 1 ml/min; Knauer spectrophotometer type 85.02. Retention times of the III- and IV-methyl ethers were 8.1 and 8.4 minutes, respectively. On the basis of the peaks of UV detection (215 nm) and the different molar extinctions coefficients, a ratio of III- and IV-

methyl ether of about 9:1 was estimated following an incubation time of 72 h.

Transformation of 17α-cyanomethyl-19-nortestosterone (II)

Like STS 557, compound II was extensively transformed by M. smegmatis. However, ring A aromatized compounds, IV and its 3-methyl ether, constituted only a small part in the metabolite mixture (< 20 %). Four other metabolites were isolated: V, VI, VII and VIII. The masses determined by MS indicated the introduction of 2 hydrogen atoms into V, and of 4 hydrogen atoms into VI and VII, whereas VIII had the same elemental composition as the parent compound II.

Metabolite V (identified as 17α-cyanomethyl-17-hydroxy-5α-estran-3-one):

TLC: system C R_F 0.43 (R_F^{II} 0.27); system E R_F 0.38 (R_F^{II} 0.30); system F 2x 0.46; system G R_F 0.22

UV: negative. IR: 2220 cm^{-1}(CN), 1710 cm^{-1} (3-ketone).

MS: m/z 315.2207 (M^+); calcd. for $C_{20}H_{29}NO_2$ 315.2198; m/z 297.2102 ($M^+ - H_2O$); calcd. for $C_{20}H_{27}NO$ 297.2093; m/z 274.1937 ($M^+ - CH_3CN$); calcd. for $C_{18}H_{26}O_2$ 274.1933.

CD: $\Delta\varepsilon\lambda$ 240 nm (0); 260 nm (0.18); 280 nm (0.81); 285 nm (0.95); 290 nm (1.02); 295 nm (0.98); 300 nm (0.83); 320 nm (o.06); 330 nm (0).

M.P.: 178 - 180 ^{o} (recrystall. from acetone/H_2O).

V was shown to be identical with authentic 17α-cyanomethyl-17-hydroxy-5α-estran-3-one obtained by reduction of II with $NaBH_4$ in pyridine (12).

Metabolite VI (identified as 17α-cyanomethyl-5α-estrane-3α,17-diol):

TLC: system A 2x R_F 0.49; system E 2x R_F 0.50;

UV: negative; IR: 2242 cm^{-1} (CN)

MS: m/z 317.2381 (M^+), calcd. for $C_{20}H_{31}NO_2$ 317.2355;

m/z 299.2276, calcd. for $C_{20}H_{29}NO$ ($M^+ - H_2O$) 299.2249.

Metabolite VII (identified as 17α-cyanomethyl-5α-estrane-3β,17-diol):

TLC: system A 2x R_F 0.40; system E 2x R_F 0.43

UV: negative; IR: 2242 cm^{-1} (CN), triplett between 1000 and 1100 cm^{-1}.

MS: m/z 317.2396 (M^+), calcd. for $C_{20}H_{31}NO_2$ 317.2355;

m/z 299.2279, calcd. for $C_{20}H_{29}NO$ ($M^+ - H_2O$) 299.2249.

The chromatographic properties, IR and mass spectra of VI and VII were found to be identical with those of authentic 17α-cyanomethyl-5α-estrane-3α,17-diol and 17α-cyanomethyl-5α-estrane-3β,17-diol, respectively (12). Jones' oxidation of VI and VII yielded the same product being identical with V (17α-cyanomethyl-17-hydroxy-5α-estran-3-one).

Metabolite VIII (tentatively identified as 17α-cyanomethyl-17-hydroxy-5α-1-estren-3-one):

TLC: system C R_F 0.35; system D R_F 0.34;

system F 2x R_F 0.37 UV: 230 nm

IR: 2220 cm^{-1}; 1670 cm^{-1} (Δ^1-3-CO).

MS: m/z 313.2069 (M^+), calcd. for $C_{20}H_{27}NO_2$ 313.2042.

By incubation with Mycobacterium smegmatis, VIII was obtained from V as evidenced by UV, IR and mass spectrum.

DISCUSSION

In recent time, increasing interest has been paid to 5αH-metabolites of 4-en-3-oxo steroids, as some of these show interesting biological effects. For obtaining 5αH-metabolites of the new progestagen STS 557, we used Mycobacterium smegmatis, as this microorganism has been shown to transform 19-nortestosterone and its 17α-alkyl derivatives stereospecifically to 5αH-compounds (7). The pronounced hydrogenation ability of M. smegmatis was also demonstrated by hydrogenating 4-en-3-oxo compounds with unnatural configuration such as ent-19-nortestosterone (4) and 9ß,10α-retrosteroids (7).

The transformation of 17α-cyanomethyl-19-nortestosterone proceeded as expected. Hydrogenation of the 4-double bond and reduction of the 3-oxo group dominated, resulting in the formation of dihydro and tetrahydro derivatives of II. The 5α-configuration of metabolites V, VI, and VII was evidenced by measuring the circular dichroism of V, and transforming VI and VII into V by Jones' oxidation. The 1-dehydrogenase activity of the microorganism was demonstrated by the formation of the 1-en-3-oxo compound VIII, in which a 5αH-configuration like in V, VI, and VII can be assumed very probably, and

of the ring A-aromatized metabolite IV. These results indicate that 1-dehydrogenation occurs both in ring A-saturated compounds and in those with a 4-en-3-oxo structure. The formation of methyl ethers in compounds with phenolic hydroxy groups by M. smegmatis has been demonstrated already by other investigations (7, 15).

It is noteworthy that the corresponding 5ßH-3-oxo compound of II, 17α-cyanomethyl-17-hydroxy-5ß-estran-3-one, is transformed by M. smegmatis into the 5αH-3-oxo compound with intermediary formation of II. Thus, the 5ßH-configuration of steroids is obviously a less stable form for the microorganism and is transformed via 4-dehydrogenation to the more stable 5αH-compound.

With STS 557 as substrate, formation of ring A-aromatized compounds dominated, whereas conversion to 5α-hydrogenated metabolites could not be established. The absence of 5α-metabolites indicates that the 9(10)-double bond has an inhibiting effect on enzymatic 5α-hydrogenation and favours ring A aromatization. Formation of metabolite III can be explained by introduction of a 1-double bond, followed by spontaneous aromatization of ring A with shifting of the double bond from 9(10) to 9(11). The mechanism of the conversion of STS 557 to metabolite IV is unclear. Both compounds have the same elemental composition $C_{20}H_{25}NO_2$, and only shifting of the double bond from 9(10) to 1(10) would be necessary for the rearrangement, but spontaneous transformation of STS 557 to IV

has never been observed. If metabolite III would be an intermediate for the formation of metabolite IV, subsequent hydrogenation of the 9(11)-double bond would be necessary. However, when incubating III with M. smegmatis, formation of IV could not be established. Thus, the mechanism of transformation remains till now unknown. In Fig. 1, the pathways of transformation of STS 557 and II by M. smegmatis are summarized.

Raynaud et al. (16) investigated the in vitro metabolism of another 4,9-dien-3-oxo compound, the progestagen R 2453 (17-methyl-19-norpregna-4,9-diene-3,20-dione) by guinea pig liver. As principal pathways of biotransformation, aromatization of ring A and hydroxylation of R 2453 have been found. No hydrogenation of the 4,9-diene system could be established. These findings also support the assumption that 4,9-dien-3-oxo compounds are not substrates for enzymatic 5α-hydrogenation.

NOMENCLATURE

Trivial name		
19-Nortestosterone	=	17β-Hydroxy-4-estren-3-one
Norethisterone	=	17α-Ethynyl-17-hydroxy-4-estren-3-one
Norgestrel	=	17α-Ethynyl-18-methyl-17-hydroxy-4-estren-3-one

ACKNOWLEDGEMENTS

The authors are indebted to Dr. G. Luck (Department of Biochemistry, CIMET) for the CD measurement of metabolite V, and to Chem. Ing. K. Wehrberger for carrying out the separation of the methyl ethers of III and IV by HPLC.

This work was supported in part by a grant from the WHO Expanded Programme of Research, Development and Research Training in Human Reproduction (78 100).

REFERENCES

1. Schubert, K., Böhme, K.H., and Kühn, Ch., Dt. Akad. Wiss. 1, 517 (1959).
2. Schubert, K., Böhme, K.H., and Hörhold, C., Ztschr. f. Naturforsch. 16b, 595 (1961).
3. Schubert, K., Schlegel, J., Böhme, K.H., and Hörhold, C., Biochim. Biophys. Acta 144, 132 (1967).
4. Schubert, K., Hobe, G., Steroids 14, 297 (1969).
5. Schubert, K., Ritter, F., Sorkina, T., Böhme, K.H., and Hörhold, C., J. Steroid Biochem. 1, 1 (1969).
6. Schubert, K., Ritter, F., Sorkina, T., Böhme, K.H., and Hörhold, C., Steroid Biochem. 6, 1501 (1975).
7. Hobe, G., unpublished
8. Reel, J.R., Humphrey, R.R., Yu-Hsin Shih, Windsor, B.L., Sakowski, R., Creger, P.L., and Edgreen, R.A., Fertil. Steril. 31, 552 (1979).
9. Ponsold, K., Pharmazie 34, 312 (1979).
10. Wagner, H., Römer, J., Hübner, M., and Ponsold, K., J. Lab. Comp. Radiopharm. XVII, 317 (1980).
11. Hübner, M., Füssel, J., Ponsold, K., Pharmazie (in press).
12. Hübner, M., Menzenbach, B., Röhrig, H., and Ponsold, K., Pharmazie (in press).
13. Freudenthal, R.J., Cook, C.E., Rosenfeld, R., and Wall, M.E., J. Steroid Biochem. 2, 77 (1971).
14. Djerassi, C., Engle, R.R., and Bowers, A., J. Org. Chem. 21, 1547 (1956).
15. Schubert, K., Phai, Le-Dinh, Kaufmann, G., and Knöll, R., Acta Biol. Med. Germ. 34, 167 (1975).
16. Raynaud, J.P., Cousty, C., and Salmon, J., J. Steroid Biochem. 5, 224 (1974).