Side Chain Degradation and 20-($\alpha+\beta$)-Reduction of Progesterone by Cell Free Preparations of *Penicillium lilacinum* NRRL 895

KJELL CARLSTRÖM

Division of Applied Microbiology, Royal Institute of Technology, and the Department of Obstetrics and Gynaecology, Sabbatsberg Hospital, Karolinska Institutet, Stockholm, Sweden*

The known steroid transformations of *Penicillium lilacinum* include degradation of the pregnane side chain, lactonization of ring D, 11α-hydroxylation and 17β, 20α and 20β-oxidoreduction.[1,2] Lactonization of ring D and 17β-oxidoreduction have been carried out in cell free extracts.[3] The preparation of C_{17-20}-lyase and 20-reductase active cell free extracts from this fungus seems not to have been reported previously. This communication describes the side chain scission and 20α- and 20β-reduction of progesterone by such extracts from *P. lilacinum*.

Experimental. All reagents were obtained from commercial sources and were of reagent quality. The steroids were checked for purity by gas-liquid chromatography (GLC) and thin layer chromatography (TLC). Solvents were redistilled before use. Tris buffer contained 0.06 M Tris-HCl pH 7.2 in glass-redistilled water.

TLC systems. TLC I: Silica gel GF_{254} with ethyl acetate—benzene—hexane 5:4:4, double run. TLC II: Silica gel GF_{254} with ethyl acetate—cyclohexane—heptane 6:4:1, quadruple run. The steroids were detected in UV light.

GLC systems. The instrument used was a Perkin-Elmer F-11 Mk II with flame ionization detection, equipped with 2 m × 3 mm i.d. glass columns. GLC I: 2.5 % OV-17 on AW-DMCS Chromosorb W, 80—100 mesh. Column temperatures 250 and 260°C, N_2 carrier gas pressure 2.8 atm. GLC II: 1.5 % QF-1 on AW-DMCS Chromosorb W, 80—100 mesh. Column temperatures 200 and 210°C, N_2 carrier gas pressure 2.5 atm.

Gas chromatography—mass spectrometry (GC—MS) was performed in an LKB 9000 gas chromatograph—mass spectrometer. GLC system: 3 m × 3 mm i.d. glass column with 1.5 % SE-30 on Chromosorb W HP, 80—100 mesh. Column temperatures 230 and 250°C, carrier gas flow 35 ml/min. The energy of the bombarding electrons was 22.5 eV. Spectra were taken automatically in the *m/e* range 0—700 and processed in a computer system.[4]

Growth of the organism. Inoculum was prepared by incubating cells from an agar slant culture of *P. lilacinum* NRRL 895 (CSB) with 300 ml sterile Capek-Dox substrate in a 1000 ml cotton-plugged Erlenmeyer flask on a shaking table at 26°C for six days. From this culture ten similar flasks with the same substrate were inoculated and incubated in the same way for six days. After that 70 mg of progesterone in 2 ml of dimethyl formamide was added to each flask as inducer and the incubation continued for 14 h.[5] The cells were harvested, washed three times with distilled water and twice with Tris buffer and finally stored at −22°C.

Preparation of cell free extracts. All operations were carried out at +4°C. Frozen cells from one flask (16—19 g wet weight) were ground in a mortar with 45 ml reagent quality sand for 30 min. After that the proteins were extracted with three 15 ml portions of Tris buffer. Each portion was thoroughly mixed with the solids for 3 min, decanted and centrifugated at 2000 rpm for 5 min. Before the centrifugation of the second and third portions they were mixed with the precipitate from the centrifugation of the preceding fraction. The supernatants were combined and centrifugated at 6000 rpm for 15 min and after that centrifugated in a Beckman-Spinco Mod. L ultracentrifuge at 100 000 *g* for 60 min. The resulting supernatant was immediately used for the steroid transformations.

Transformation of progesterone. The reaction mixture contained 1.0 ml 100 000 *g* supernatant and cofactors added in Tris buffer according to Table 1. The final volume of each sample was 1.2 ml. To each sample 0.319 μmol of progesterone in 10 μl of ethanol was added and incubation took place in duplicates in 10 ml wide-necked bottles on a shaking table at 26°C. After 90 min three drops of 1 M HCl were added and the steroids were extracted with 4 ml of chloroform. The chloroform layer was dried over Na_2SO_4 and analyzed. Blanks without added progesterone were prepared and treated in the same way. They contained minor amounts of testosterone coming from the inducer and correction for this was made when the samples were analyzed.

Quantitative analysis. One half of each sample was acetylated with acetic anhydride—

* Present address.

Table 1. Results from incubations of progesterone with cell free extracts from *P. lilacinum* in the presence of various cofactors. Experimental conditions are given in the text.

Cofactors added (1.13×10^{-3} M of each)	Mol % steroid				
	Progesterone	20($\alpha+\beta$)-Hydroxy-4-pregnene-3-one	Testosterone	4-Androstene-3,17-dione	Compound T
None	47.7	trace	46.3	trace	6.0
NADPH	17.7	6.1	66.7	trace	9.5
NADPH+NAD	20.6	trace	17.3	38.4	23.7
NADH	24.2	10.3	31.9	27.0	6.6

Table 2. t_R- and R_F-Values. t_R-Values are related to 5α-cholestane. Conditions are given in the text.

Compound	t_R, GLC I	t_R, GLC II	t_R, GC−MS	R_F, TLC I	R_F, TLC II
Progesterone	2.14			0.50	
20α-Hydroxy-4-pregnene-3-one	2.22			0.32	0.52
20α-Acetoxy-4-pregnene-3-one	2.79	7.96	1.30	0.65	
20α-Trimethylsilyloxy-4-pregnene-3-one	1.78	4.02	1.21		
20β-Hydroxy-4-pregnene-3-one	2.00			0.34	0.57
20β-Acetoxy-4-pregnene-3-one	2.63	7.61	1.23	0.67	
20β-Trimethylsilyloxy-4-pregnene-3-one	1.73	3.59	1.13		
Testosterone	1.39			0.28	0.45
Testosterone acetate	1.70	4.60	0.78	0.60	
Testosterone silyl ether	0.95	1.92	0.67		
4-Androstene-3,17-dione	1.41	4.73	0.56	0.38	
Compound T	4.60	28.00	1.23	0.12	

pyridine 5:1 and subjected to GLC in system I with 5α-cholestane as internal standard. The "compound T" was estimated at 260°C due to its long retention time, the other steroids at 250°C.

Identifications. The steroid metabolites were identified by comparing their chromatographic properties and mass spectra with those of known reference compounds. The chromatographic properties are given in Table 2.

R_F-values. Each sample was subjected to qualitative TLC in system I before and after acetylation.

Relative retention times (t_R) and mass spectra. t_R-Values in system I for the compounds in each sample were obtained during the quantitative analysis. For identification of the individual metabolites the steroids were isolated from the acetylated and the free samples by TLC in system I. Testosterone, 20($\alpha+\beta$)-hydroxy-4-pregnene-3-one (both epimers together) and compound T were isolated from the pooled free samples from the incubations with NADPH and NADH, testosterone acetate, 20($\alpha+\beta$)-acetoxy-4-pregnene-3-one (all three acetates together) and

compound T from the corresponding acetylated samples. 4-Androstene-3,17-dione and compound T were isolated from the pooled samples from the NADPH + NAD incubations. The free testosterone and 20($\alpha+\beta$)-hydroxy-4-pregnene-3-one fractions were converted to trimethylsilyl (silyl) ethers. All isolated fractions were subjected to qualitative GLC in systems I and II and to GC-MS analysis.

Results and discussion. The results from the incubations are given in Table 1. It was not possible to estimate the individual 20-epimers in the quantitative GLC due to the relatively small amounts and insufficient resolution of the two peaks. However, the 20-epimer fractions isolated by TLC contained sufficient amounts of substance to give clearly separated but not completely resolved peaks in GLC system II and in the GC – MS. The 20-alcohols could also be separated in TLC system II and they were after that subjected to qualitative GLC in systems I and II. The ratio between the two 20-epimers was approximately 1 : 1.

TLC R_F-values and GLC t_R-values for the metabolites formed from progesterone were identical to those of the authentic reference compounds. Mass spectra of the metabolites closely corresponded to those of the reference compounds. For the hydroxy steroids formed, acetate as well as silyl ether derivatives gave spectra identical to those from the corresponding derivatives of the reference standards. The spectrum of the silyl ether of the 20α-hydroxy-4-pregnene-3-one isolated from the reaction mixture showed an additional peak at m/e 460. This peak comes from the molecular ion of the enol-bis-silyl ether derivative of the 20β-epimer. When both epimers are mixed, this enol-bis-silyl ether derivative will cause a minor interference with the 20α-mono-silyl ether in the GLC system used.

Compound T was tentatively identified as testololactone from its mass spectrum and chromatographic properties. It was resistant to acetylation. However, authentic testololactone for comparison was not available.

Addition of NADPH stimulated the transformation of progesterone, but the crude cell free extract seems to contain sufficient amounts of endogenous cofactors for a certain degree of lyase activity. As expected addition of NAD together with the NADPH increased the ketone : alcohol ratio markedly. Addition of NADH caused a minor increase in the lyase activity and in the 20-reduction. Somewhat surprisingly the addition of NADH also caused an increase in the 17-ketone: 17β-alcohol ratio.

Microbial reduction of 20-ketosteroids mostly yields 20β-alcohols, but 20α-reduction as well as the formation of both epimers has also been reported.[1,2,6,7] When progesterone was incubated with intact cells of *P. lilacinum*, 20β-hydroxy-4-pregnene-3-one was reported as the sole reduced metabolite.[8] However, 17α-hydroxyprogesterone is reduced to a mixture of 20α- and β-alcohols by some strains of this fungus as well as of *Cladosporium resinae*, *Cylindrocarpon radicicola*, *Aspergillus flavus*, and *Aspergillus oryzae*.[2] The reduction of 20-ketosteroids by bacteria and actinomycetes has been extensively studied by Soviet workers.[6,7,9] Their results indicate that the direction and the degree of the 20-reduction are influenced by the chemical structure of the individual 20-ketosteroid, the strain of the microbial species used and the growth phase of the culture.

Acknowledgement. The GC – MS analysis was carried out at the Department of Chemistry I, Karolinska Institutet, Stockholm, Sweden.

1. Charney, W. and Herzog, H. L. *Microbial Transformations of Steroids. A Handbook*, Academic, New York and London 1967.
2. Nakano, H., Sato, H. and Tamaoki, B. *Biochim. Biophys. Acta* **164** (1968) 585.
3. Prairie, R. L. and Talalay, P. *Biochemistry* **2** (1963) 203.
4. Reimendal, R. and Sjövall, J. *Anal. Chem.* **44** (1972) 21.
5. Carlström, K. *Acta Chem. Scand.* **24** (1970) 1759.
6. Kogan, L. M. and Yelin, E. A. *Abh. deut. Akad. Wiss. Berlin. Kl. Med.* **2** (1968) 195.
7. Krasnova, L. A., Sokolova, L. V., Ryzhkova, V. M. and Suvorov, N. N. *Prikl. Biokhim. Mikrobiol.* **5** (1969) 260; *Chem. Abstr.* **71** (1969) 29268.
8. Sebek, O. K., Reineke, L. M. and Peterson, D. H. *J. Bacteriol.* **83** (1962) 1327.
9. Kupletskaya, M. B., Sokolova, L. V., Kovylkina, N. F., Suvorov, N. N. and Skryabin, G. K. *Izv. Akad. Nauk SSSR Ser. Biol.* **3** (1969) 406; *Chem. Abstr.* **71** (1969) 19721.

Received March 28, 1972.