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Progesterone Metabolism by the Filamentous Fungus *Cochliobolus lunatus*

Marko Vitas,[1] Kelvin Smith,[2] Damjana Rozman[1] and Radovan Komel[1]*

[1]*Institute of Biochemistry, Medical Faculty, Vrazov trg 2, 61000 Ljubljana, Slovenia and*
[2] *Institute of Biochemistry, Queen Mary and Westfield College, Mile End Road, London E1 4NS, England*

Studies of *Cochliobolus lunatus* m118 steroid metabolism by thin-layer chromatography, mass spectrometry and NMR spectroscopy revealed that the fungus hydroxylates progesterone at positions 7α, 11β and 14α, and oxidizes the 11β-hydroxy group to the ketone. The ^{1}H NMR spectra of two of the steroid metabolites, 11β,14α-dihydroxyprogesterone and 11-oxo-14α-hydroxyprogesterone, are reported for the first time. It is still not known if all the hydroxylation reactions are performed in *C. lunatus* by a single, non-specific, steroid hydroxylase, structurally different from the 11β-hydroxylase found in higher eucaryotes, or if different forms of the enzyme are involved.

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INTRODUCTION

Microbial bioconversion has replaced complex synthetic chemical routes for the stereochemical introduction of oxygen functionality into substrates in the large-scale commercial production of steroids because the microbial enzyme systems catalyse reactions with high regio- and stereospecificity. One of the biotechnologically important steroid-transforming reactions is 11β-hydroxylation of corticosteroid precursors catalysed by strains such as *C. lunatus* [1]. There have been several reports in the last few years concerning inducibility of the steroid 11β-hydroxylating system of this fungus, its response to putative inducers, as well as its ability to perform other steroid bioconversion reactions with different substrates [2–7]. A systematic kinetic and structural study of the metabolism of a single steroid used both as inducer and substrate, which would lead to a better understanding of the enzyme system(s) involved in 11β-hydroxylation and those of the various side reactions, has yet to be described.

MATERIALS AND METHODS

Strains and growth conditions

Cochliobolus lunatus m 118 was obtained from the strain collection of Friedrich Schiller University, (Jena, Germany). Media, growth conditions and storage of strains have been described previously [8].

*Correspondence to R. Komel.
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Bioconversion of progesterone

Bioconversion of progesterone was performed by modification of the method of Dermastia *et al.* [9]. Progesterone served as inducer and as the steroid substrate. 10 g (wet wt) of 24 to 48 h old mycelia were resuspended in 100 ml of phosphate buffer (0.75 mM Na_3PO_4, 0.21 mM EDTA, 0.04 mM glutathione, reduced, pH = 5.5) in 500 ml Erlenmayer flasks. Progesterone (Sigma), dissolved in dimethylformamide, was added to each flask as substrate (10 mg/100 ml). Flasks were incubated on a rotatory shaker at 180 rev/min at 28°C. When a sufficient amount of the desired product had been produced (see Results) reaction mixtures were extracted three times with 10 ml of chloroform. The chloroform fractions were collected and dried over anhydrous sodium sulphate for 12 h before evaporation under reduced vacuum.

Isolation of biotransformation products

Biotransformation products were re-dissolved in 5 ml of chloroform and transferred to test tubes. Chloroform was evaporated under N_2 over a warm water bath. Individual products were subsequently dried under vacuum for 12 h. Separation of the biotransformation products was performed on TLC plates (Kieselgel 60 F_{254}, Merck) developed three times with a solvent system of CH_3Cl–CH_3OH-H_2O (96:4:0.5, by vol). Products were isolated by cutting strips containing metabolite from TLC plates and then extracting three times with a mixture (10 ml) of cold chloroform and methanol (1:1). The resultant solutions were

filtered through Whatman No. 1 filter-paper and dried under vacuum. Each product was transferred into a test tube in 5 ml of chloroform and dried over a warm water bath and under N_2. Products were again dried in vacuum for 12 h. Product B (Table 1 and Fig. 2) was further purified on TLC; plates were developed twice in a solvent system of cyclohexane–ethyl acetate–ethanol (45:45:10, by vol). Isolation of product B from TLC plates was performed as described above. Authentic progesterone and 11β-hydroxyprogesterone (Sigma) were used as standards for the identification of metabolites. For quantitative determinations, TLC plates were scanned with a Camag TLC Scanner II at 254 nm UV.

Determination of structures of progesterone metabolites

The structures of progesterone metabolites were determined by NMR and mass spectrometry as described previously [8, 10].

RESULTS

Kinetics of progesterone metabolism by C. lunatus

Steroid 11β-hydroxylation is the main reaction of biotechnological importance carried out by *C. lunatus* during the first hour of incubation. After that time several side products appear. Figure 1 shows rapid progesterone metabolism. Approximately half the progesterone added to the medium is taken up by the cells and converted almost quantitatively into 11β-hydroxyprogesterone, making the 11β-hydroxylation reaction the sole reaction occurring early in progesterone transformation. After prolonged incubation the percentage of 11β-hydroxyprogesterone declined being replaced by the new metabolites A, B and C (Fig. 1).

Assignment of the structures of progesterone metabolites by mass spectroscopy

Mass spectra, obtained for metabolites A, B and C, were compared with the literature data [11]. The mass spectrum of compound A showed ion peaks at m/z 344(M^+), 326(M^+-H_2O), 311(M^+-H_2O-CH_3). The structure of an hydroxylated keto-progesterone with the molecular formula $C_{21}H_{28}O_4$ was predicted.

The mass spectrum of compound B showed ion peaks at m/z 328(M^+-H_2O), 310(M^+-2H_2O), 295(M + -2H_2O-CH_3) and 267(M^+-2H_2O-CH_3-CO). The ratio of intensity of the M + -H2O peak to the M^+ peak is much greater in a mass spectrum of compounds with axial hydroxyl groups [12]. Thus the fact that compound B exhibited two strong peaks at 328 and 310 but no M^+ peak suggests that this compound has two axial hydroxyl groups. Substance B was predicted to be a dihydroxyprogesterone with the molecular formula $C_{21}H_{30}O_4$.

In the spectrum of compound C, ion peaks at m/z M^+(346), 328(M^+-H_2O), 310(M^+-2H_2O), 295(M^+-2H_2O-CH_3) and 285 were found. This compound was also predicted to be a dihydroxyprogesterone ($C_{21}H_{30}O_4$).

Determination of the position of substituents on the progesterone ring by ¹H NMR spectroscopy

The three hydroxylated progesterones, 11-oxo-14α-hydroxyprogesterone [compound A, Fig. 2(B)] 7α,14α-dihydroxy-progesterone [compound B, Fig. 2(C)] and 11β,14α-dihydroxyprogesterone [compound C, Fig. 2(D)], were readily identified from their NMR spectra (Fig. 3). All retained the characteristic signals for 4-H (singlet or narrow doublet at 5.70–5.82), 18-H_3 (singlet at 0.74–1.03), 19-H_3 (singlet 1.23–1.48) and

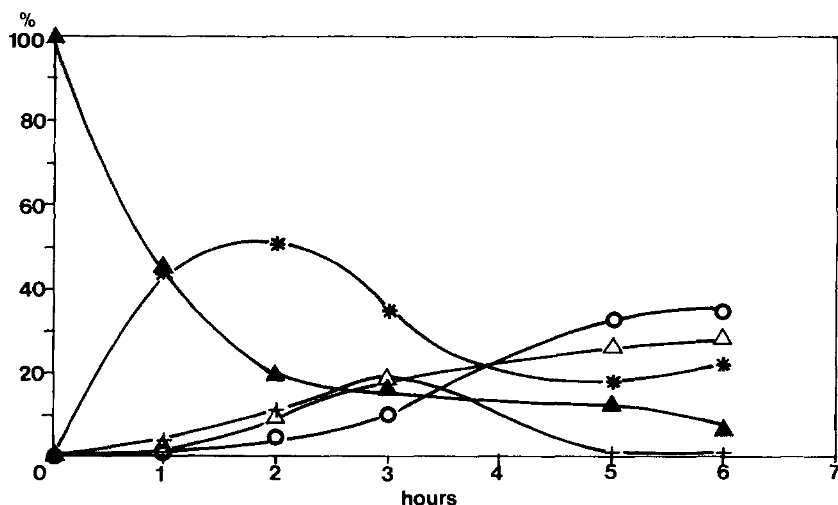

Fig. 1. Kinetics of progesterone biotransformation by *C. lunatus*. ▲, Progesterone; *, 11β-hydroxyprogesterone; ○, 11β, 14α-dihydroxyprogesterone; △, 7α,14α-dihydroxyprogesterone; +, 11-keto,14α-hydroxyprogesterone.

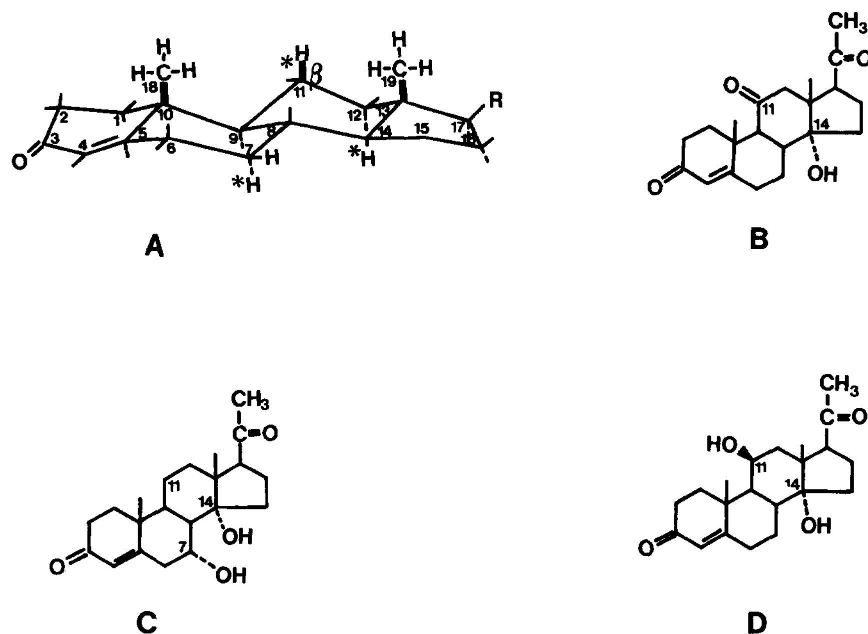

Fig. 2. Structure formulae of the steroid ring and of progesterone metabolites, produced by bioconversion reaction with the filamentous fungus *Cochliobolus lunatus*. (A) Structure of progesterone in the "chair" conformation. The 11β, 7α and 14α sites, attacked by the fungal enzyme(s), are marked with an asterisk (*). The 11β, 18 and 19 positions, recognized by the higher eucaryotic 11β-hydroxylase, are marked with a bold line (—). R = -$COCH_3$. (B) Structure of 11-oxo-14α-hydroxyprogesterone (compound A). (C) Structure of 7α,14α-dihydroxyprogesterone (compound B). (D) Structure of 11β,14α-dihydroxyprogesterone (compound C).

21-H_3 (singlet at 2.12–2.14) showing retention of the fundamental pregnane structure during incubation. The positions of hydroxylations, and of other substitutions, were also determined from the distinctive features of the individual spectra using the "fingerprinting" technique (Table 1) as described by Smith *et al.* [10, 13] and from the data of Kirk *et al.* [14]. NMR reference data for authentic progesterone derivatives, used for identification of *C. lunatus* metabolites, are listed in parentheses.

DISCUSSION

In this paper we describe for the first time the kinetics of *C. lunatus* m118 progesterone metabolism and the structures of the metabolites produced. With the aid of ^{1}H NMR and mass spectroscopy we established that secondary hydroxylation reactions, which lowered the levels of 11β-hydroxyprogesterone, occurred at positions 7α and 14α. Oxidation of the secondary 11β alcohol to a keto group also occurred. The ^{1}H NMR data for two

Table 1. ^{1}H NMR chemical shifts for progesterone transformation products from C. lunatus

Compound	Chemical shifts 4-H	17-H	18-H	19-H	21-H	C*H*OH
A						
11-oxo-14α-	5.75	3.41	0.74	1.41	2.12	—
hydroxyprog.	(5.76)	(3.45)	(0.77)	(1.44)	(2.13)	—
B						
7α-14α-di-	5.82	3.23	0.77	1.23	2.14	4.34*
hydroxyprog.	(5.81)	(3.22)	(0.77)	(1.22)	(2.13)	(4.31)
C						
11β-14α-di-	5.70	3.20	1.03	1.48	2.14	4.43+
hydroxyprog.	(5.69)	(3.12)	(1.03)	(1.46)	(2.14)	(4.42)

δ, relative to Me_4Si. Chemical shifts calculated from data for progesterone and individual group increments are shown in parentheses. *7β-H; +11α-H.

of the steroids, i.e. that of 11β,14α-dihydroxyprogesterone [Fig. 3(C)] and of 11-oxo-14α-hydroxyprogesterone [Fig. 3(C)], are presented here for the first time.

Although there were some reports of steroid transformations by *C. lunatus* in the past, the enzymes, involved in hydroxylation(s), have not yet been purified to homogeneity. However, a *C. lunatus* microsomal cytochrome *P*-450 monooxygenase system that catalyses steroid 11β-hydroxylation has recently been described by Jaenig [15] suggesting that such hydroxylations might be catalysed by a cytochrome *P*-450-based reaction mechanism identical to that already characterized in corticosteroid biosynthesis in higher eucaryotes [16].

It is not known if a single steroid hydroxylase is involved in the progesterone hydroxylation reactions carried out by *C. lunatus* m118. The report of Zuidweg [17], who was unable to separate the 11β- and 14α-hydroxylating activities in cell-free preparations from the anamorph strain *Curvularia lunata*, opens the question of there being a single non-specific enzyme also in *Cochliobolus lunatus*. The positions of hydroxy group substituents suggest there is a strong tendency for *C. lunatus* to hydroxylate the central region of progesterone molecule (Fig. 2). A keto group at position 3 helps

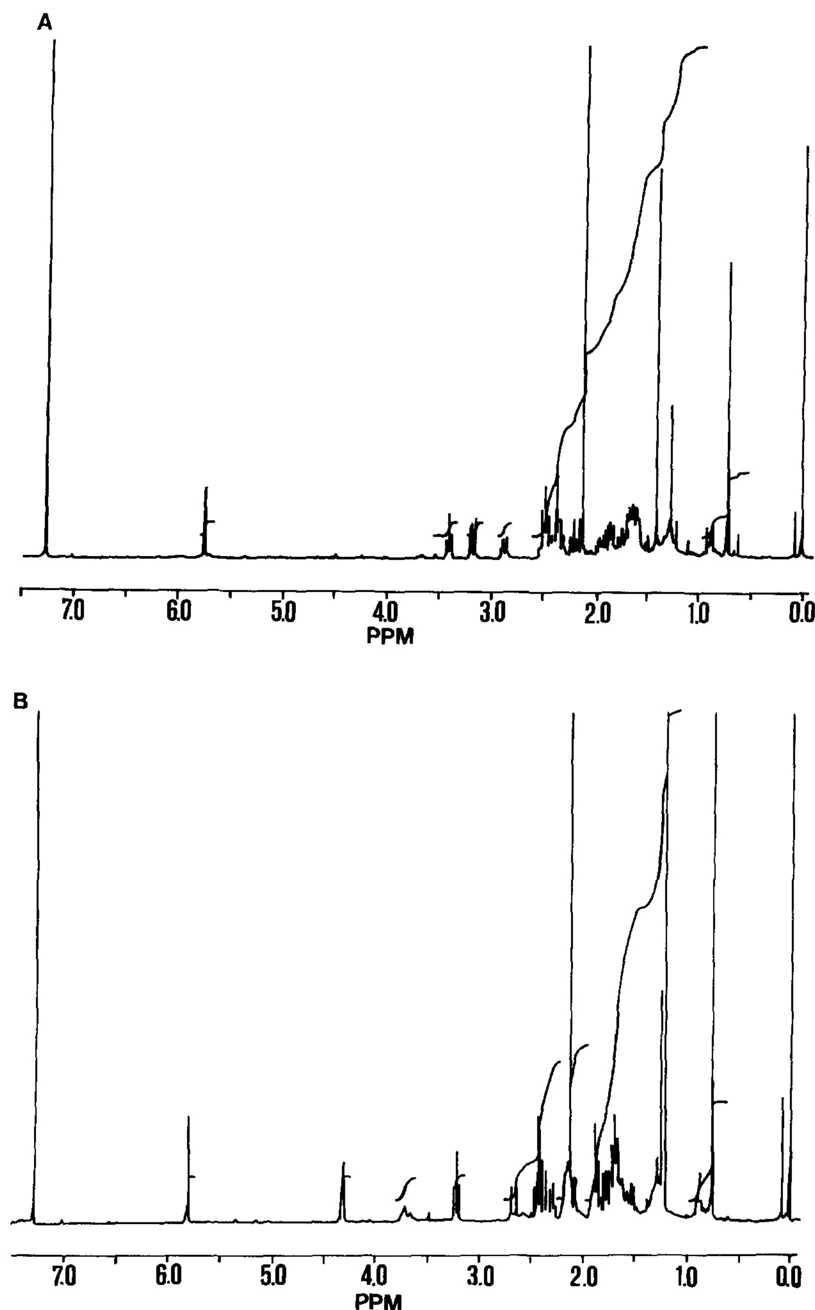

Fig. 3—*caption opposite.*

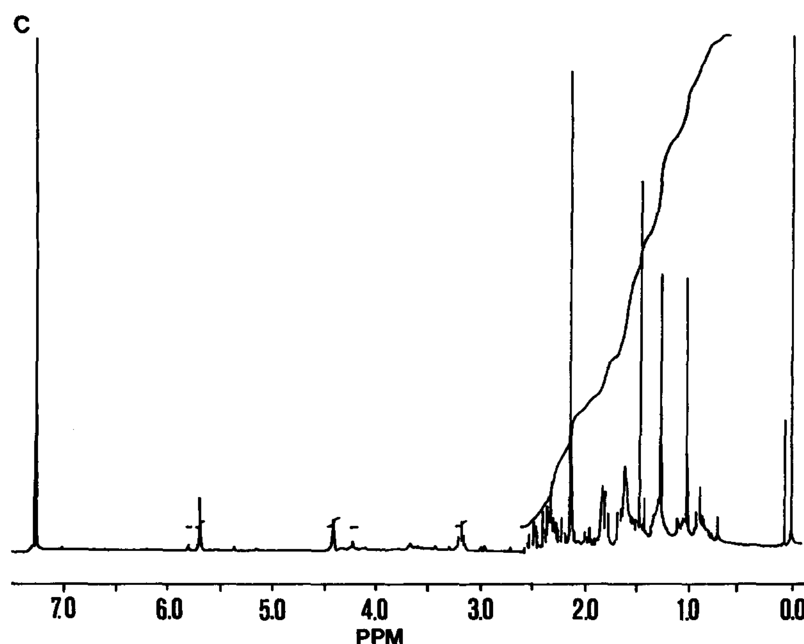

Fig. 3. 400 MHz ^{1}H NMR spectra of *C. lunatus* progesterone transformation metabolites. Spectrum A is 11-oxo-14α-hydroxyprogesterone. Spectrum B is 7α, 14α-dihydroxyprogesterone. Spectrum C is 11β, 14α-dihydroxyprogesterone.

orient the steroid substrate in the active site of the hydroxylase enzyme [18], but the substrate could be loosely bound and rotation of the substrate molecule around the long axis of the steroid ring might be permitted (capsizing). In this case the 11β position would correspond to the 7α site in the capsized orientation and the 14α site would be in the close spatial proximity necessary for hydroxylation. If this theory is true non-specificity of *C. lunatus* hydroxylation would be readily explainable. The microbial steroid 11β-hydroxylase cytochrome *P*-450 would be different from its non-specific mitochondrial adrenocorticoid counterpart of higher eucaryotes which has been shown to hydroxylate positions 11β, 18 and 19, as well catalysing oxidations of the 11β- and 18-hydroxy groups [19–21]. Here, the 11β-hydrogen as well as the 18- and 19-methyls, lie on the same side of the steroid ring [Fig. 2(A)].

Nevertheless, our results do not exclude the possibility that more than one steroid hydroxylase exists in *C. lunatus* if the capsizing of substrate progesterone and/or product 11β-hydroxyprogesterone in the active site of 11β-hydroxylase is not permitted. The amount of 11β-hydroxyprogesterone, produced in the first 2 h of incubation, was reduced thereafter mainly on account of secondary hydroxylations on the reverse side of the steroid ring (Fig. 2) producing both the 11β,14α- and 7α,14α-dihydroxy derivatives. Progesterone itself, or the newly synthesized 11β-hydroxyprogesterone, could serve as inducer and/or substrate for at least one other steroid hydroxylase which is activated in the late phase of progesterone transformation. This question remains open and will be elucidated in future experiments.

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