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Transformations of testosterone and related steroids by *Botrytis cinerea*

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Abstract

Botrytis cinerea (strain AM235) was used to investigate the transformations of testosterone and related steroids. It was found that the position and stereochemistry of the introduced hydroxyl group, as well as the yield of products, depended on the structure of the substrate. *Botrytis cinerea* converts the examined substrates mainly to 7α-hydroxy derivatives. 1-Dehydrotestosterone was also significantly hydroxylated at a 14α-position.

Keywords: *Botrytis cinerea*; Testosterone; Testosterone relatives; Biotransformation

1. Introduction

Botrytis species are widespread fungi able to infect aerial parts of a number of commercial plants, in particular grapes, lettuces, carrots, strawberries and tobacco (Coley-Smith et al., 1980). Concerning the pathogenicity of *Botrytis cinerea* some reports have been made on biotransformation of potential antifungal agents (Poole and Whitaker, 1997; Aleu et al., 1999) and metabolites playing a role in the pathogenicity of this fungus (Collado et al., 1998; Duran et al., 1999; Cichewicz et al., 2000; Duran-Patron et al., 2000). These results have been summarized (Aleu and Collado, 2001).

Microbial hydroxylation of steroids is a route both detoxication and oxidative degradation of xenobiotics. Our previous research on metabolism of α-camphelenone derivatives by *B. cinerea* (AM235) revealed that the fungus is able to introduce an oxygen function by hydroxylation in α position with respect to the double bond, or by epoxidation of the double bond itself (Dmochowska-Gładysz et al., 1986). However, Farooq and Tahara (2000) exploring the epoxidation possibility of testosterone and pregnenolone double bonds by *B. cinerea* (AHU9424), proved that the constitutive epoxidase enzymes of this fungus are unable to epoxidize double bonds of steroidal rings.

B. cinerea is known as a hydroxylator of 4-ene-3-oxo-steroids mainly at C-11α, C-11β and rarely at C-2β, C-6β in C-21 steroids (Charney and Herzog, 1967), at C-7β in testosterone and C-11α, C-16β in pregnenolone (Farooq and Tahara, 2000). Because of the observation that our strain of *B. cinerea* hydroxylated testosterone uncommonly (at C-7α) in a good yield, we initiated this study in order to investigate more thoroughly the transformation of testosterone relatives. We intend to determine the hydroxylation preference of this fungus.

It is noteworthy that 7α-hydroxy derivatives of steroids are difficult to synthesize by chemical methods and can be used, for example, for manufacturing of diuretics.

2. Results and discussion

In order to examine the effect of an additional C_1-C_2 double bond and/or additional C-17α methyl group or the lack of C-19 methyl group, the transformation of testosterone (**1**), 17α-methyltestosterone (**3**), 19-nortestosterone (**5**), 1-dehydrotestosterone (**7**) and 1-dehydro-17α-methyltestosterone (**10**) by *B. cinerea* were compared.

The fungus *B. cinerea* was incubated with the substrates until they were metabolized (3–11 days). The mixtures of many compounds were obtained, but only the main products were isolated and identified. The

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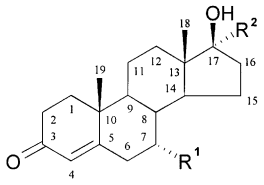

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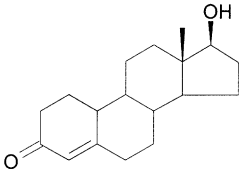

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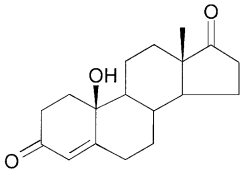

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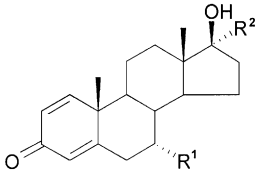

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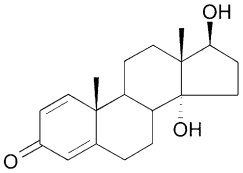

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yield of the products was determined using GC analysis of the crude product (see Experimental).

The structure of biotransformation products was determined by IR, ^{1}H NMR and ^{13}C NMR spectra. The position and configuration of the introduced hydroxy group were recognized mainly with respect to changes in NMR spectra compared with the ones of the starting material and data in literature (Jones, 1973; Kirk et al., 1990).

All the products (except **6** and **8**) contained a 7α-hydroxy group. The presence of such orientation of hydroxy group was confirmed by the presence of a characteristic narrow peak of 7β-H ($W_{1/2}$ = 8 Hz) in a region δ 3.95 ppm to 4.03 ppm as was described by Jones (1973) and Kirk et al. (1990). Additionally 7α-hydroxylation indicate coupling constants of the 6-H protons (*dd* ca. 2.4 ppm for 6α-H and a distinctive *ddd* at δ ca. 2.8 ppm for 6β-H) (Smith et al., 1989b; Kirk et al., 1990). Data of these compounds corresponded very closely to those already described for 7α-hydroxytestosterone (Kirk et al., 1990), 7α-hydroxy-17-methyltestosterone, 7α-hydroxy-1-dehydro-17α-methyltestosterone (Brzezowska et al., 1996) and other related 7α-hydroxysteroids (Smith et al., 1989a,b, 1990). Only one substrate, 1-dehydrotestosterone (**7**), underwent hydroxylation at a 14α-position. ^{13}C NMR spectrum of this metabolite suggested that the androstene skeleton was intact, though with the addition of one tertiary hydroxyl group (δ = 83.2 ppm). Compared with 1-dehydrotestosterone (**7**) the ^{1}H NMR spectrum of 14α-hydroxy-1-dehydrotestosterone (**8**) shows a downfield shift (0.12 ppm) of the 18-methyl group with no significant change of the 19-methyl group position. Another substrate with a C_1–C_2 double bond, 1-dehydro-17α-methyltestosterone (**10**), was hydroxylated only at a 7α-position. It was in agreement with earlier observations for *Mucorales* that a bulky substituent at C-17α would prevent hydroxylation at C-14α (Singh et al., 1967; Brzezowska et al., 1996) whereas steroids with a 17β-hydroxy group, as in testosterone, were hydroxylated at a C-14α position (Krishnan et al., 1991).

The bioconversion of 19-nortestosterone (**5**) afforded a single metabolite. It was concluded that the fungus inserted a hydroxy group at C-10β and oxidized the 17β hydroxy to keto group. The IR spectrum of **6** showed hydroxy absorption at 3413 cm^{-1} and two carbonyl bands at 1724 and 1671 cm^{-1}. The H-17α triplet (at 3.64 ppm), which is characteristic of testosterones, had disappeared from the ^{1}H NMR spectrum, whilst the ^{13}C NMR spectrum showed a new carbon signal at δ 220.5 ppm of carbonyl. A peak at 70.3 ppm in DEPT spectrum showed the presence of tertiary 10β-hydroxy group in compound **6**. 19-Nor-4-ene-3-oxosteroids are commonly hydroxylated at the allylic C-10 position (Holland and Diakow, 1978; Hanson et al., 1996). The comparison of transformation routes of testosterone (**1**) and 17α-methyltestosterone (**3**) showed that an additional 17α-methyl group did not influence the position of hydroxylation, however a partial reduction of C_1-C_2 double bond occurred only in the presence of this function, when 1-dehydro-17α-methyltestosterone (**10**) was used as a substrate.

3. Concluding remarks

To sum up, as far as we know *B. cinerea* transformation of steroids to 7α-hydroxy derivatives has never been reported before. We have found *B. cinerea* to be an efficient 7α-hydroxylator of steroidal 4-ene-3-ketones. Compared with many other microorganisms, *B. cinerea* metabolizes these compounds efficiently. The amount of 7α-hydroxy derivatives comprised about 26–82% of total metabolites. Besides, we have identified 14α-hydroxy-1-dehydrotestosterone (**8**) as the main product of 1-dehydrotestosterone (**7**) transformation.

4. Experimental

4.1. General experimental procedures

The composition of crude mixtures was analyzed by TLC and GC. TLC was carried out using silica gel 60, 0.2 mm thick plates with hexane–acetone (2:1 or 1:1 v/v) as eluent. The spots were sprayed with H_2SO_4–EtOH (1:1 v/v). The products were separated chromatographically on silica gel 0.05–0.2 (Merck) with hexane–acetone mixture (2:1 v/v) as eluent. GC analysis was performed using a Hewlett Packard 5890A Series II gas chromatograph; fitted with a flame ionization detector, and HP-5 (cross-linked 5% Ph–Me–Silicone) 30 m×0.53 mm×0.88 μm film thickness column (240 °C−1 min, 5 °C min^{-1}−300 °C−10 min). H_2 at flow rate of 2 ml min^{-1} was used as a carrier gas. The structure of biotransformation products was determined based on IR, ^1H NMR and ^{13}C NMR spectra. IR spectra were recorded in KBr on a Mattson IR 300 spectrometer. NMR spectra were obtained using a DRX 300 Bruker, 300 MHz spectrometer in $CDCl_3$ with TMS as internal standard.

4.2. Microorganism

B. cinerea AM235 was obtained from the collection of the Institute of Biology and Botany, Medical University of Wrocław. It was isolated from straw protecting roses.

4.3. Conditions of cultivation and transformation

The fungi were maintained on potato agar slants at 4 °C and incubated in potato medium containing 3% glucose, potatoes extract obtained from 800 g of potatoes (washed, peeled and cut up, gently boiled in 1000 ml of distilled water), water (to 1000 ml). Cultures were shaken at 27 °C in 2 l Erlenmeyer flasks with 300 ml of medium. After three days of growth, 120 mg of the substrate, dissolved in 5 ml of acetone were added to 300 ml of culture. The products were extracted with chloroform after 3–11 days of transformation (until the substrate was metabolized).

4.4. Biotranformations of steroids with B. cinerea

4.4.1. Testosterone (1)

After 5 days of incubation the main metabolite was 7α-hydroxytestosterone (**2**) (76% yield): IR ν_{max} cm^{-1}: 3365, 1654, 1608, ^1H NMR: δ 0.78 (3H, *s*, H-18), 1.19 (3H, *s*, H-19), 2.43 (1H, *dd*, $J=14.8$ Hz, $J=3$ Hz, H-6α), 2.66 (1H, *ddd*, $J=14.2$ Hz, $J=3$ Hz, $J=2$ Hz, 6β-H), 3.69 (1H, *t*, $J=8.4$ Hz, H-17α), 3.95 (1H, *m*, $W_{1/2}=8$ Hz, H-7β), 5.79 (1H, *s*, H-4).

4.4.2. 17α-Methyltestosterone (3)

After 6 days of incubation the main metabolite was 7α-hydroxy-17α-methyltestosterone (**4**) (82% yield): IR ν_{max} cm^{-1}: 3416, 1657, ^1H NMR: δ 0.90 (3H, *s*, H-18), 1.21 (3H, *s*, H-19), 1.24 (3H, *s*, H-17), 2.43 (1H, *dd*, $J=14.5$ Hz, $J=3$ Hz, H-6α), 2.66 (1H, *ddd*, $J=14.4$ Hz, $J=3$ Hz, $J=2$ Hz, 6β-H), 3.96 (1H, *m*, $W_{1/2}=8$ Hz, H-7β), 5.78 (1H, *s*, H-4).

4.4.3. 19-Nortestosterone (5)

After 6 days (as well as after 10 days) of incubation the main metabolite was 10β-hydroxy-19-norandrost-4-ene-3,17-dione (**6**) (78% yield): IR ν_{max} cm^{-1}: 3413, 1724, 1671, 1624, ^1H NMR: δ 0.93 (3H, *s*, H-18), 5.78 (1H, *s*, H-4), ^{13}C NMR: δ 13.7 (C-18), 19.7 (C-11), 21.8 (C-15), 30.52 (C-7), 31.0 (C-12), 31.7 (C-6), 33.6 (C-2), 33.7 (C-1), 34.8 (C-8), 35.7 (C-16), 47.6 (C-13), 50.4 (C-14), 52.6 (C-9), 70.3 (C-10), 124.9 (C-4), 163.9 (C-5), 198.9 (C-3), 220.5 (C-17).

4.4.4. 1-Dehydrotestosterone (7)

After 10 days of incubation the main product of transformation was 14α-hydroxy-1-dehydrotestosterone (**8**) (68% yield): IR ν_{max} cm^{-1}: 3417, 1650, 1609, ^1H NMR: δ 0.94 (3H, *s*, H-18), 1.27 (3H, *s*, H-19), 4.30 (1H, *t*, $J=8$ Hz, H-17α), 6.06 (1H, *s*, H-4), 6.22 (1H, *dd*, $J=10$ Hz, $J=2$ Hz, H-2), 7.05 (1H, *d*, $J=10$ Hz, H-1), ^{13}C NMR: δ 15.0 (C-18), 18.4 (C-19), 21.6 (C-11), 27.8 (C-16), 28.7 (C-7), 29.6 (C-15), 32.4 (C-6), 33.0 (C-12), 38.9 (C-8), 43.4 (C-10), 45.9 (C-9), 47.2 (C-13), 78.5 (C-17), 83.2 (C-14), 124.0 (C-4), 127.5 (C-2), 155.7 (C-1), 168.3 (C-5), 186.3 (C-3).

7α-Hydroxy-1-dehydrotestosterone (**9**) (27% yield) was the minor product: IR ν_{max} cm^{-1}: 3343, 1656, 1614, ^1H NMR: δ 0.92 (3H, *s*, H-18), 1.24 (3H, *s*, H-19), 2.47 (1H, *dd*, $J=14$ Hz, $J=3$ Hz, H-6α), 2.78 (1H, *ddd*, $J=14.1$ Hz, $J=3$ Hz, $J=2$ Hz, 6β-H), 4.03 (1H, *m*, $W_{1/2}=8$ Hz, H-7β), 6.13 (1H, *s*, H-4), 6.23 (1H, *dd*, $J=10$ Hz, $J=2$ Hz, H-2), 7.08 (1H, *d*, $J=10$ Hz, H-1).

4.4.5. 1-Dehydro-17α-methyltestosterone (10)

After 11 days of incubation the main product of biotransformation was 7α-hydroxy-1-dehydro-17α-methyltestosterone (**11**) (52% yield): IR ν_{max} cm^{-1}: 3451, 1655, 1615, ^1H NMR: δ 0.92 (3H, *s*, H-18), 1.20 (3H, *s*, H-19), 1.24 (3H, *s*, H-17), 2.47 (1H, *dd*, $J=14.2$ Hz, $J=3$ Hz, H-6α), 2.75 (1H, *ddd*, $J=14.5$ Hz, $J=3$ Hz, $J=2$ Hz, 6β-H), 4.03 (1H, *m*, $W_{1/2}=8$ Hz, H-7β), 6.13 (1H, *s*, H-4), 6.23 (1H, *dd*, $J=10$ Hz, $J=2$ Hz, H-2), 7.08 (1H, *d*, $J=10$ Hz, H-1).

The minor product was 7α-hydroxy-17α-methyltestosterone (**4**) (26% yield).

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