Transformations of Steroids by *Beauveria bassiana*

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The course of transformations of testosterone and its derivatives, including compounds with an additional C1,C2 double bond and/or a 17α-methyl group, a 17β-acetyl group or without a 19-methyl group, by a *Beauveria bassiana* culture was investigated. The fungi promoted hydroxylation of these compounds at position 11α, oxidation of the 17β-hydroxyl group, reduction of the C1,C2 or C4,C5 double bonds and degradation of the progesterone side-chain, leading to testosterone. The structure of 4-ene-3-oxo-steroids had no influence on regio- and stereochemistry of hydroxylation. In a similar manner, dehydroepiandrosterone was hydroxylated by *Beauveria bassiana* at position 11α, however, a small amount of 7α-hydroxylation product was also formed.

Key words: Beauveria bassiana, Biotransformation, Steroids

Introduction

Beauveria, belonging to the Moniliaceae family of fungi imperfecti, is a naturally occurring soil fungus (Bidochka *et al.*, 1998). It is a recognized pathogen of more than 100 insect species (Hajek and St. Leger, 1994), which has found an application in agricultural biocontrol programs (Bing and Lewis, 1991, 1992; Krueger and Roberts, 1997; Mulock and Chandler, 2000).

The fungus *Beauveria bassiana* ATCC 7159 (also known as *B. sulfurescens* or *Sporotrichum sulfurescens*) is one of the most frequently used biocatalysts capable of performing reactions of a different type, *e.g.* hydroxylation of saturated and aromatic carbon atoms, keto-alcohol redox reaction, alkene redox reaction, sulfide oxidation, Baeyer-Villiger oxidation, glucosidation, epoxide and ester hydrolysis and heteroatom dealkylation. These results have been summarized in the review article of Grogan and Holland (2000). The most significant is the use of *B. bassiana* for selective hydroxylation of a wide range of organic compounds.

In contrast to many other fungi currently used for biocatalysis, *Beauveria* has not been extensively used for transformations of steroids. Previous research showed that *B. bassiana* promotes hydroxylation of 4-ene-3-oxo-steroids mainly at position 11α (Griffiths *et al.*, 1993; Bayunova *et al.*, 1989; Čapek and Fassatiova, 1977; Čapek *et al.*, 1966; Schubert *et al.*, 1962) and, rarely, at positions 6β and 11α (Griffiths *et al.*, 1993; Čapek and Fassatiova, 1977; Čapek *et al.*, 1966). Hydroxylation at positions 6α, 11α, 11β and 15α was observed in the B-norsteroid 17α,21-dihydroxy-B-nor-pregn-4-en-3,20-dione (Sanada *et al.*, 1977). Also the ability of *B. bassiana* to reduce the 17-ketone to a 17β-alcohol (Bayunova *et al.*, 1989), and to degrade the progesterone side-chain leading to testosterone (Schubert *et al.*, 1962) was reported.

Therefore, it was of interest to us performing comparative studies on various 4-ene-3-oxo-steroids. We wanted to check whether the additional C17 methyl group, the lack of the C19 methyl group, and the additional C1,C2 double bond do not alter the localisation of the hydroxylation process.

Because the knowledge of biotransformations of 5-ene-steroids is much less documented compared to 4-ene-3-oxo-steroids, we have chosen dehydroepiandrosterone (DHEA) as an additional substrate for the tests with *Beauveria bassiana.* There are no previous reports on 19-nortestosterone and dehydroepiandrosterone transformation in a *Beauveria bassiana* culture.

Materials and Methods

Microorganism

Beauveria bassiana AM446 was obtained from the Institute of Biology and Botany of the Medical University of Wrocław. It was isolated from the

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insect *Pyrrhocoris apterus* (Pyrrhocoridae) (imago).

Conditions of cultivation and transformation

The fungi were incubated in 3% glucose and 1% peptone, pH 5.9, and shaken at 27 °C in 2 l Erlenmeyer flasks with 300 ml of medium. After 3 d of growth, 120 mg of a substrate, dissolved in 5 ml of acetone or ethanol, were added, and the flasks returned to the shaker. The products were extracted with chloroform after 3–10 d of transformation (until the substrate was metabolized).

Product analysis

The composition of crude biotransformation mixtures was analysed by TLC and GC. TLC was carried out using silica-gel 60 plates (Merck) with hexane/acetone (2:1 or 1:1 v/v) as eluent. Steroids were detected by spraying the plates with H_2SO_4/EtOH (1:1 v/v) followed by heating. Analytical GC analysis was performed on a Hewlett Packard 5890A Series II GC instrument, using a HP-5 capillary column (cross-linked 5% Ph-Me-Silicone, 30 m × 0.53 mm × 0.88 μm film thickness; temperature program: 240 °C – 1 min, gradient 5 °C/min to 300 °C – 5 min). Biotransformation products were separated by column chromatography using silica gel 0.05–0.2 mesh (Merck) with a hexane/acetone mixture (2:1 v/v) as eluent. Structures of biotransformation products were determined on the basis of ^{1}H NMR spectra, which were recorded on a DRX 300 Bruker 300 MHz spectrometer in $CDCl_3$, CD measurements, which were done on a JASCO-715 spectropolarimeter in chloroform, and optical rotation measurements, which were performed on an AUTOPOL IV polarimeter in acetone at 25 °C.

Results and Discussion

In order to examine different structural factors of a steroid on the biotransformation course, including the presence of the C1,C2 double bond and/or the additional 17α-methyl group and the absence of either C19-methyl or C17-acetyl groups, the following substrates have been chosen for transformations by *Beauveria bassiana* AM446: testosterone (**1**), 17α-methyltestosterone (**2**), 19-nortestosterone (**3**), 1-dehydrotestosterone (**4**), 1-dehydro-17α-methyltestosterone (**5**) and progesterone (**6**). As there is much less information about hydroxylation of 5-androstenes compared to 4-ene-3-oxo-steroids, we also decided to explore the bioconversion of dehydroepiandrosterone (**7**).

The fungus *Beauveria bassiana* was incubated with the substrates until they were metabolised (3–10 d). The results of the biotransformations are presented by Fig. 1. The yield of products was determined by GC analysis of the chloroform extract (Table I).

The structures of biotransformation products were assigned mainly based on ^{1}H NMR spectra. Both location and configuration of the newly introduced hydroxyl group were determined by analysing differences between NMR spectra of the

Table I. Biotransformation of steroids by *Beauveria bassiana*.

Substrate	Products	Yield[a] (%)
Testosterone (**1**)	11α-hydroxytestosterone (**8**)	55.4
	5α-androstan-11α,17β-diol-3-one (**9**)	14.8
	11α-hydroxyandrost-4-ene-3,17-dione (**10**)	10.5
	5α-androstan-11α-ol-3,17-dione (**11**)	8.4
17α-Methyltestosterone (**2**)	11α-hydroxy-17α-methyltestosterone (**12**)	95.3
19-Nortestosterone (**3**)	11α-hydroxy-19-nortestoterone (**13**)	40.5
1-Dehydrotestosterone (**4**)	11α-hydroxy-1-dehydrotestosterone (**14**)	56.4
	11α-hydroxyandrost-1,4-diene-3,17-dione (**15**)	13.2
	11α-hydroxytestosterone (**8**)	11.4
	11α-hydroxyandrost-4-ene-3,17-dione (**10**)	9.3
1-Dehydro-17α-methyltestosterone (**5**)	11α-hydroxy-1-dehydro-17α-methyltestosterone (**16**)	86.7
Progesterone (**6**)	11α-hydroxytestosterone (**8**)	94.1
Dehydroepiandrosterone (**7**)	5-androsten-3β,11α,17β-triol (**17**)	59.6
	7α-hydroxydehydroepiandrosterone (**18**)	13.1
	androstenediol (**19**)	8.3

[a] Yield determined by GC.

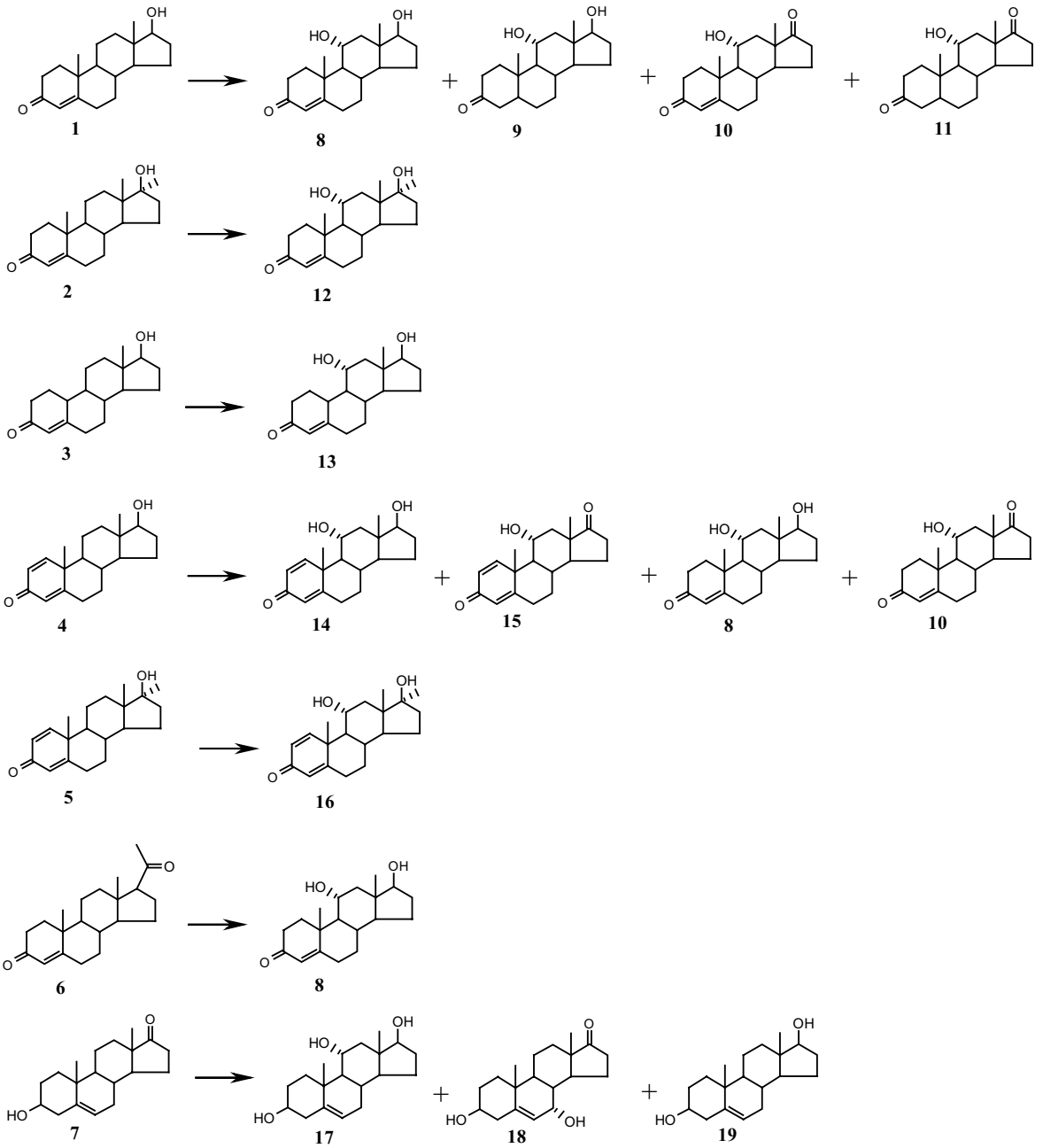

Fig. 1. Metabolism of testosterone (**1**), 17α-methyltestosterone (**2**), 19-nortestosterone (**3**), 1-dehydrotestosterone (**4**), 1-dehydro-17α-methyltestosterone (**5**), progesterone (**6**) and dehydroepiandrosterone (**7**) by *Beauveria bassiana*.

starting material and products (Table II, III), supported by literature data (Jones, 1973; Kirk *et al.*, 1990).

All the products obtained from 4-ene-3-oxo-steroids transformations contained a 11α-hydroxyl group, which was proved by a large downfield shift of the 19-H_3 signal (but not 18-H_3) and by the broad multiplet profile for the 11β-H signal in the region of δ 3.86 ppm to 4.11 ppm as was reported by Jones (1973) and Kirk *et al.* (1990). The spectral

Table II. ^{1}H NMR data for *B. bassiana* 4-ene-3-oxo-steroids transformation products and some substrates[a].

Compound	4-H	17α-H	17α-CH_3	18-H	19-H	11β-H	Other significant signals
8	5.72	3.69 t, *J* = 8.3 Hz	–	0.82	1.32	4.03 m	–
9	–	3.69 t, *J* = 8.5 Hz	–	0.76	1.13	3.98 m	–
10	5.73	–	–	0.93	1.32	4.05 m	–
11	–	–	–	0.89	1.15	4.02 m	–
2	5.71	–	1.20	0.90	1.19	–	–
12	5.72	–	1.23	0.92	1.32	4.05 m	–
13	5.80	3.64 t, *J* = 8.5 Hz	–	0.78	–	–	–
13	5.81	3.68 t, *J* = 8.3 Hz	–	0.80	–	3.86 m	–
4	6.04	3.61 t, *J* = 8.4 Hz	–	0.79	1.21	–	7.03 d, *J* = 10.2 Hz (1-H) 6.19 d, *J* = 10.1 Hz (2H)
14	6.06	3.63 t, *J* = 8.4 Hz	–	0.81	1.30	4.03 m	7.79 d, *J* = 10.3 Hz (1-H) 6.13 dd, *J* = 10.2 Hz, 2 Hz (2-H)
15	6.10	–	–	0.93	1.32	4.11 m	7.74 d, *J* = 10.3 Hz (1-H) 6.14 dd, *J* = 10.5 Hz, 2 Hz (2-H)
5	6.05	–	1.18	0.92	1.24	–	7.04 d, *J* = 10.2 Hz (1-H) 6.21 dd, *J* = 10.2 Hz, 2 Hz (2-H)
16	6.08	–	1.20	0.93	1.32	4.09 m	7.79 d, *J* = 10.3 Hz (1-H) 6.13 dd, *J* = 10.2 Hz, 2 Hz (2-H)

[a] Chemical shifts in ppm relative to Me_4Si; solvent, $CDCl_3$; *J*, coupling constant.

Table III. ^{1}H NMR data for dehydroepiandrosterone and its transformation products[a].

Compound	6-H	3α-H	C*H*OR	17α-H	18-H	19-H
7	5.34 d, *J* = 5.1 Hz	3.51 m, Wh = 23 Hz	–	–	0.86	1.02
17	5.41 d, *J* = 5.8 Hz	3.53 m, Wh = 22 Hz	4.08 m (11β-H)	3.69 t, *J* = 8.5 Hz	0.78	1.16
18	5.62 d, *J* = 5.3 Hz	3.56 m, Wh = 22 Hz	3.95 m, Wh = 11 Hz (7β-H)	–	0.87	1.00
19	5.33 d, *J* = 5.2 Hz	3.50 m, Wh = 22 Hz	–	3.63 t, *J* = 8.5 Hz	0.75	1.01

[a] Chemical shifts in ppm relative to Me_4Si; solvent, $CDCl_3$; *J*, coupling constant.

data of these compounds (Table II) correspond very closely to those described in the literature for 11α-hydroxytestosterone (**8**) (Smith *et al.*, 1990; Kirk *et al.*, 1990), 11α-hydroxyandrost-4-ene-3,17-dione (**10**) (Kirk *et al.*, 1990), 11α-hydroxy-17α-methyltestosterone (**12**) (Huszcza and Dmochowska-Gładysz, 2003), 11α-hydroxy-1-dehydrotestosterone (**14**) and 11α-hydroxyandrost-1,4-diene-3,17-dione (**15**) (Ahmed *et al.*, 1996).

Apart from the hydroxylation, two other redox reactions took place in the transformations promoted by *B. bassiana.* The metabolites with saturated C4,C5 or C1,C4 bonds were identified in testosterone (**1**) and 1-dehydrotestosterone (**4**) transformations, respectively. The configuration of 5β for compound **9** was determined from the negative Cotton effect observed in the CD spectrum ($[\Theta]_{304}$ = – 1450). The presence of C3 and C17 carbonyl groups in the metabolite **11** resulted a total positive Cotton effect, therefore the 5β configuration was confirmed by optical rotation measurement ($[\alpha]_{589}^{25}$ = + 72.9°), which was in a good agreement with the literature data (Allard, 1965). Similar reduction of the C4,C5 double bound to the 5β configuration was observed for *Beauveria globulifera* (Protiva *et al.*, 1968).

Conversion of the alcohol at C17 into the ketones **1** and **4** also occurred. The presence of C17α methyl group inhibited reduction of both C4,C5 double bond in 17α-methyltestosterone (**2**) and C1,C2 double bond in 1-dehydro-17α-methyltestosterone (**5**).

It is noteworthy that the products of C4,C5 double bond reduction and/or C17 oxidation were not found by transformation of 19-nortestosterone (**3**). This substrate was relatively poorly metabolized, which is in agreement with the results obtained by Shibahara *et al.* (1970). They observed that in spite of the induction of *Aspergillus ochraceus* hydrolase only a low level of 11α-hydroxylation of 19-nortestosterone could be achieved.

We have found that the main profile of biotransformation of progesterone (**6**) by *B. bassiana* is the

side chain cleavage. Thus, the metabolite of progesterone was found to be the derivative of testosterone **8**. Interestingly, 11α-hydroxytestosterone (**8**), which was formed as the single product in high yield, was not observed for other *B. bassiana* strains.

The main result of our study is the fact that structural differences in the 4-ene-3-oxo-steroids substrates not effect the regio- and stereoselectivity of the hydroxylation process. The steroid skeleton was always attacked only at α-face of C11. Although the 6β and 11α positions are expected to be equivalent in enzyme-substrate complexes, 6β-hydroxy and 6β,11α-dihydroxy products were not found in any our experiments.

The correlation between the structure of the substituent at C17 and the site specifity of hydroxylation of different steroid compounds by known 11α-hydroxylators *e.g. Rhizopus nigricans* (Žakelj-Narvič and Belič, 1987), *Aspergillus ochraceus* (Tan and Smith, 1968) and *Cephalosporium aphidicola* (Boynton *et al.*, 1997) was previously investigated. Unlike in our study, it was shown that the side chain at C17 had a strong influence on the position and yield of hydroxylation by these fungi.

Introduction of the C5,C6 double bond to the steroid skeleton slightly alters the transformation course by the *B. bassiana* culture. Apart from the major metabolite, 5-androstene-3β,11α,17β-triol (**17**), incubation of dehydroepiandrosterone (**7**) with *B. bassiana* gave a small quantity of 7α-hydroxydehydroepiandrosterone (**18**). 7α-Hydroxylation was confirmed by a down-field shift of the 6-H signal (0.28 ppm as compared to substrate) and the existence of a narrow signal of 7β-H at 3.95 ppm. The presence of the other minor product, androstenediol (**19**), suggests that the 11α-hydroxylation was followed by the reduction of the 17-ketone to the 17β-alcohol. The hydroxylation of dehydroepiandrosterone (**7**) at position 11α without further oxidation at C3 is a typical feature of 11α-hydroxylating fungi such as *Rhizopus nigricans* (Raspe and Richler, 1960), *Rhizopus arrhizus* (Holland and Diakow, 1979) and *Aspergillus niger* (Bell *et al.*, 1972).

7α-Hydroxydehydroepiandrosterone (**18**) and androstenediol (**19**) are metabolites of dehydroepiandrosterone (**7**), which were detected in several mice tissues. Compound **18** was described as a more potent activator of immune processes in mice than **7** (Morfin and Courchay, 1994).

To sum up, we have found *Beauveria bassiana* to be an efficient 11α-hydroxylator of dehydroepiandrosterone (**7**) and 4-ene-3-ones, especially of 17α-methyltestosterone (**2**) and 1-dehydro-17α-methyltestosterone (**5**). Compared to many other microorganisms, *Beauveria bassiana* showed particularly high regioselectivity and very low substrate specifity in steroid hydroxylation. This phenomenon was observed also for *B. bassiana* catalysed hydroxylations of a variety of substrates *e.g.* amides, lactams, carbamates, azides and sulfonamides (Grogan and Holland, 2000). Additionally, we have identified a new dehydroepiandrosterone (**7**) transformation product: 5-androstene-3β,11α,17β-triol (**17**).

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