Microbial Metabolism of Danazol: A Contribution to Doping Analysis

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Microbial metabolism of danazol (17α-pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17β-ol) by *Beauveria bassiana* ATCC 7159 and *Glyocladium viride* ATCC 10097 afforded four metabolites. The isolated metabolites were identified by different spectroscopic techniques as 6β-hydroxy danazol, which is a not yet reported danazol metabolite, 17β-hydroxy-17α-pregn-4-en-20-yn-3-one (ethisterone) and 17β-hydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (2α-hydroxymethyl ethisterone), which represent the major danazol metabolites detected in human urine. The last metabolite, 6β,17β-dihydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one, is also a minor human metabolite, for which the NMR data are described here for the first time. The metabolites were isolated in quantities that allowed their use for direct comparison in routine doping analysis.

Key words: Microbial Transformation, Mammalian Metabolism, Danazol, Doping Analysis

Introduction

Danazol (17α-pregna-2,4-dien-20-yno[2,3-*d*]isoxazol-17β-ol) is a synthetic derivative of ethisterone that is used in the treatment of a wide variety of medical conditions, mainly endometriosis and benign fibrocystic mastitis (Barbieri and Ryan, 1981; De Oca Porto *et al*., 2006). It has also been reported that danazol, as an attenuated androgen, may have some efficacy in increasing the platelet count of patients with myelodysplastic syndrome (Chan *et al*., 2002). Beyond its medical uses, danazol is classified as class S1 anabolic agent in the list of forbidden substances in sports by the World Antidoping Agency (WADA) because danazol is structurally related to the anabolic steroid stanozolol (De Boer *et al*., 1992). In fact, some anabolic effect attributed to danazol itself has been reported (Choi and Chung, 1998). Like most steroids, danazol is extensively biotransformed, and several metabolites have been reported in human urine and serum (Potts, 1977; Rosi *et al*., 1977; Haning *et al*., 1982). Identification of anabolic steroids in sports laboratories depends mostly on gas chromatography/mass spectrometry (GC-MS) and liquid chromatography/mass spectrometry (LC-MS) analyses. Nevertheless, many of these metabolites have remained unknown despite the availability of a large body of data. Thus, the solution for more confident detection would be the isolation, in large quantities, and proper identification of the metabolites through full structure elucidation by NMR spectroscopy for their use as reference standards in forensic analysis by GC-MS or LC-MS. Determining factors in the applicability of this approach to human biological samples are the low magnitude and the short life-time of many of these metabolites in human urine and serum, as well as the complexity of analysis due to high concentrations of endogenous compounds. In this work, we used microbial transformation by whole cells as a model to mimic the human metabolism of danazol for large-scale isolation and full structure elucidation of its metabolites. Microbial transformation provides many advantages over other systems adopted for metabolic studies such as chemical derivatization (Kieslich, 1976; Clark *et al*., 1989), *in vitro* incubation with cellular or subcellular (microsomal) systems (reviewed in Levesque *et al*., 2002), or the use of chimeric mice with humanized liver (Lootens *et al*.,

2009). These advantages include high catalytic activity and high regio- and stereospecificity, easier handling not requiring specific culture conditions, as well as higher economy and versatility. Steroids are readily transformed by microbes, as documented by many studies (Hanson *et al*., 1996; Wilson *et al*., 1999; Choudhary *et al*., 2005). In this study, four major microbial metabolites of danazol were obtained and identified by different spectroscopic means, among other several minor ones.

Results and Discussion

Screening for danazol biotransformation using 23 microorganisms revealed that *Beauveria bassiana* ATCC 7159 and *Glyocladium viride* ATCC 10097 gave best results.

The ethyl acetate extracts from the culture medium and the whole cells of the two organisms after danazol biotransformation gave several spots on thin-layer chromatography (TLC) plates. Scaling up the reaction resulted in the isolation and identification of four major metabolites, **2** – **5** (Fig. 1). Two of these metabolites, identified as ethisterone (**3**) and 2α-hydroxymethyl ethisterone (**4**), are known major human metabolites of danazol (Davison *et al*., 1976; Potts, 1977; Rosi *et al*., 1977; Rose *et al*., 1988; Murakami *et al*., 1993). Metabolite **2**, 6β-hydroxy danazol, is a new, hitherto unidentified metabolite. 6β-Hydroxy derivatives are important metabolites in the detection of positive cases of steroid abuse that made their synthesis necessary (reviewed in Schänzer *et al*., 1995). Moreover, a previous metabolic study on human cytochrome P450 2J2 reported the detection of hydroxylated derivatives of danazol based on molecular ion peaks in LC-MS/MS spectra, but exact structures could not be assigned to these ions (Lee *et al*., 2010). Metabolite **2** could possibly be another minor human metabolite of danazol that has not yet been identified. Metabolite **5**, 6β,17β-dihydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one, is also a minor danazol human metabolite that was tentatively identified in human urine by Rosi *et al*. (1977) and De Oca Porto *et al*. (2006). Its structure was also hypothesized by Kim *et al*. (2001), based on GC-MS data for danazol metabolites in equine urine. The full NMR data of this metabolite are presented here for the first time. Danazol metabolites in this study were isolated by column chromatography (CC) in quantities sufficient for their identification through NMR spectroscopy and mass spectrometry which allowed proper structure elucidation. They were obtained in a simple single-step bioconversion process, compared to the elaborative and multi-step procedure of Rosi *et al*. (1977), which comprised chemical and microbiological conversions of several substrates and utilized a number of different organisms for the preparation of each metabolite of danazol. This illustrates the importance of the selection of suitable microorganisms in a screening step (see Experimental).

The isolated metabolites were identified as follows:

Metabolite **2** gave a pseudomolecular ion peak at m/z 354 ([$M^+ + H$]) in the positive FAB mass spectrum. An increase of 16 mass units over the substrate suggested hydroxylation. HRFAB-MS (positive ion mode) gave a molecular ion peak at m/z 353.1952 ([M^+]) (calcd. 353.1991), indicating a molecular formula of $C_{22}H_{27}NO_3$. Hydroxylation was confirmed by the appearance of a new methine carbinol proton at δ_H 4.24 ppm (1H, br s) in its ^{1}H NMR spectrum (Table I). This proton was correlated by HMQC to a carbon resonance at δ_C 70.5 ppm which was assigned to position 6 by an HMBC experiment (Fig. 2). H-6 showed ^3J correlations to C-4 and C-10 (δ_C 112.4 and 40.0 ppm,

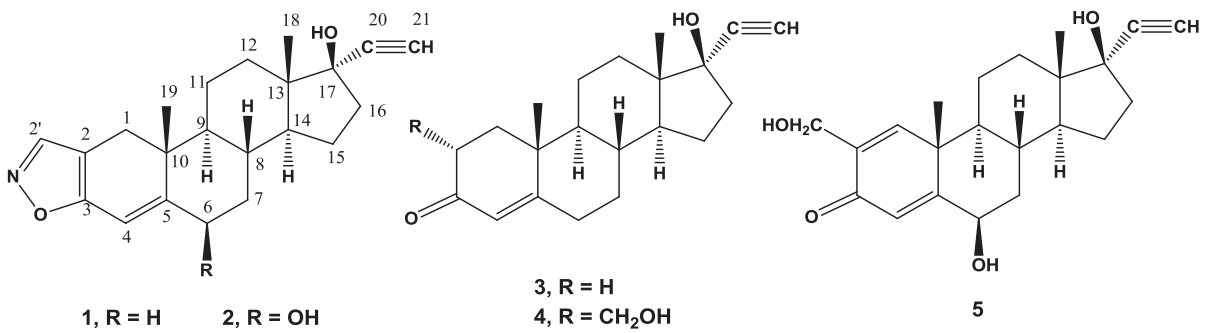

Fig. 1. Chemical structure of danazol (**1**) and its metabolites **2** – **5**.

Table I. ^{1}H NMR (DMSO-d$_6$, 500 MHz) data (δ in ppm, J values in parentheses in Hz) of danazol (**1**) and its metabolites **2**–**5**.

H	**1**	**2**	**3**	**4**	**5**
1	2.44, d (16.0)	2.32, d (16.0)	1.43, m	1.42, m	6.80, s
	2.67, d (16.0)	2.66, d (16.0)	2.00, m	1.81, m	
2	–	–	2.37, m	2.37, m	–
4	6.22, s	6.35, s	5.89, s	5.57, s	5.94, s
6	2.31, m	4.24, br s	2.27, m	2.20, m	4.32, br s
	2.34, m		2.34, m	2.32, m	
7	1.02, m	1.12, m	1.04, m	0.88, m	0.97, m
	1.68, dd (10.7, 4.0)	1.73, m	1.90, m	1.74, m	1.70, m
8	1.69, m	1.71, m	1.59, m	1.49, m	1.48, m
9	0.88, m	0.92, m	0.71, m	0.80, m	0.93, m
11	1.34, m	1.35, m	1.22, m	1.31, m	1.33, m
	1.53, m	1.53, m	1.56	1.56, m	1.55, m
12	1.44, m	1.30, m	1.44, m	1.42, m	1.40, m
	1.52, m	1.52, m	1.75, m	1.55, m	1.53, m
14	1.38, m	1.33, m	1.42, m	1.34, m	1.34, m
15	1.05, m	1.21, m	1.17, m	1.21, m	1.18, m
	1.74	1.74, m	1.52, m	1.52, m	1.50, m
16	1.81, ddd (16.0, 9.3, 4.6)	1.78, m	2.04, m	2.02, ddd (13.4, 10.7, 4.6)	2.04, m
	2.03, ddd (14.5, 9.6, 5.8)	2.00, m	2.24, m	2.02, m	2.07, m
18	0.73, s	0.74, s	0.65, s	0.74, s	0.71, s
19	0.91, s	1.04, s	1.09, s	1.13, s	1.05, s
21	3.27, s	3.26, s	2.88, s	3.27, s	3.31, s
2′	8.28, s	8.25, s	–	3.47, ddd (16.9, 11.5, 5.3)	3.22, d (10.8)
				3.66, ddd (15.3, 9.9, 5.3)	3.37, d (10.8)
OH 2′	–	–	–	4.42, t (5.3)	4.34, t (5.3)
OH 17	5.30, br s	5.16, s	–	5.30, s	5.25, s
OH 6	–	5.54, br s	–	–	5.48, br s

respectively). Mutually, H-4 (δ_H 6.35 ppm, s) showed cross-peaks with each of C-6 and C-10. Hydroxylation at position 6 was further confirmed by a downfield shift of H-19 by 0.14 ppm (Table I) and by β- and γ-effects on carbon atoms in the vicinity of C-6. The β-effect was evidenced by deshielding of C-7 (δ_C 37.9 ppm) by 7.0 ppm, while the γ-effect was evidenced by shielding of C-8 (δ_C 30.7 ppm) by 6.1 ppm compared to danazol (**1**) (Table II) (Hanson *et al.*, 1996; Swizdor and Kolek, 2005). β-Orientation of the 6-hydroxy group was assigned based on the width at half height of H-6 ($W_{1/2} = 7.4$ Hz) (Choudhary *et al.*, 2005). Other signals in the ^{1}H and ^{13}C NMR spectra of metabolite **2** confirmed that the isoxazole ring is still intact. Proton H-2′ appeared at the same position in the substrate (δ_H 8.25 ppm, s and 8.28 ppm, s, respectively, Table I) and showed ^2J and ^3J correlations to carbon signals at δ_C 109.9 and 164.3 ppm (C-2 and C-3, respectively). Similarly, the carbon signals of the corresponding positions in the isoxazole ring of danazol (**1**) and its metabolite **2** exhibited comparable shifts, *viz.*, positions 2, 3, and 2′ (Table II). These data collectively confirmed the structure of **2** as 6β-hydroxy danazol (Fig. 1), which is a new danazol metabolite.

All other danazol metabolites obtained by scaling up the reaction with *G. viride* ATCC 10097 showed cleavage of the isoxazole ring as follows:

Metabolite **3** showed a pseudomolecular ion peak at m/z 313 ($[M^+ + H]$) in the ESI mass spectrum (positive ion mode) indicating a molecular formula of $C_{21}H_{28}O_2$. NMR spectral data revealed disappearance of the hallmarks of the isoxazole ring. The characteristic signal due to H-2′ (singlet at δ_H 8.28 ppm) was absent from the ^{1}H NMR spectrum, also both H-1 protons appeared as multiplets at δ_H 1.43 and 2.00 ppm instead of the characteristic doublets with large coupling constant ($J = 16.0$ Hz) of the corresponding position in danazol (δ_H 2.44 and 2.67 ppm) (Table I). The three olefinic resonances characteristic of positions 2, 3, and 2′ of the isoxazole ring in danazol (**1**) were also absent from the ^{13}C NMR spectrum of **2** (Table II). Instead, new olefinic resonances appeared at δ_C 122.4 and 171.4 ppm together with a ketonic group at δ_C 207.5 ppm. These resonances could be assigned to carbon atoms 4, 5, and 3, respectively. These data char-

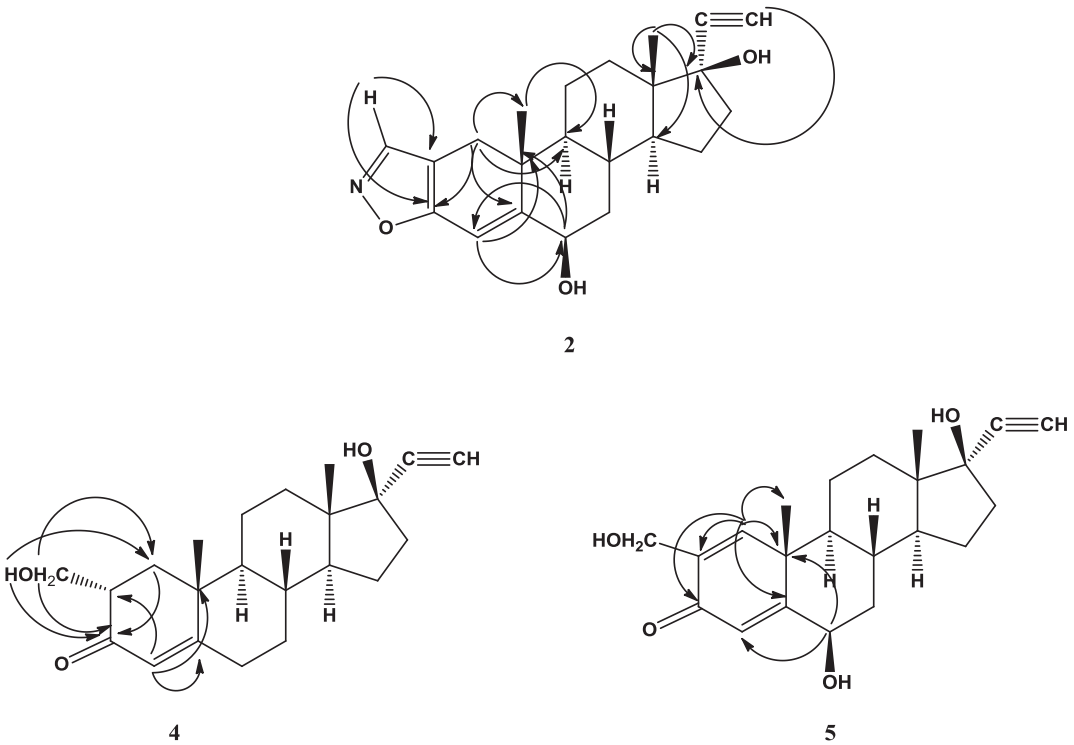

Fig. 2. Important HMBC correlations of metabolites **2**, **4**, and **5**.

acterized metabolite **3** as a $\Delta 4$ C_{21} steroid with a keto group at position 3. Since the substituents at position 17 remained unchanged in this metabolite as well as in the other metabolites, as can be seen from the spectral data in Tables I and II, the structure of metabolite **3** could be deduced as ethisterone or 17β-hydroxy-17α-pregn-4-en-20-yn-3-one (Fig. 1). The ^{13}C NMR data were found comparable to those published for ethisterone (Rodriguez-Molina *et al*., 2010). Ethisterone is known to be the major human metabolite of danazol (Davison *et al*., 1976; Potts, 1977; Rosi *et al*., 1977; Haning *et al*., 1982).

Metabolite **4** showed a pseudomolecular ion peak in the ESI mass spectrum (positive ion mode) at m/z 343 ($[M^+ + H]$) indicating a molecular formula of $C_{22}H_{30}O_3$. Its ^{13}C NMR spectrum (Table II) showed the appearance of a ketonic carbon signal at δ_C 199.2 ppm. This carbon resonance was assigned to position 3 by HMBC (Fig. 2), where H-1_a and H-1_b (δ_H 1.24 ppm, m and 1.81 ppm, m) showed ^3J correlation to this carbon atom. Its ^{1}H NMR spectrum (Table I) showed also the absence of the isoxazole proton (H-2′), a singlet at δ_H 8.28 ppm in **1**, and the appear-

Table II. ^{13}C NMR (DMSO-d_6, 125 MHz) data (δ in ppm) of danazol (**1**) and its metabolites **2**–**5**.

C	**1**	**2**	**3**	**4**	**5**
1	33.0	34.0	33.8	39.4	151.0
2	108.7	109.9	32.7	44.4	136.5
3	164.5	164.3	207.5	199.2	185.8
4	108.1	112.4	122.4	123.9	128.0
5	155.3	154.9	171.4	170.9	171.1
6	31.6	70.5	32.0	32.1	70.1
7	30.9	37.9	29.4	31.8	35.8
8	36.8	30.7	35.8	36.0	32.0
9	54.1	53.8	54.1	54.3	57.9
10	40.3	40.0	43.9	39.0	43.5
11	21.3	21.2	23.3	20.8	23.3
12	32.9	32.8	29.4	32.8	32.7
13	46.9	46.9	47.1	46.7	48.1
14	49.9	49.8	49.7	50.0	49.8
15	23.4	23.3	25.1	23.3	23.3
16	39.6	39.9	41.3	39.2	43.3
17	78.6	78.7	78.4	78.5	78.4
18	13.2	13.2	13.3	13.3	13.4
19	18.9	20.9	20.2	17.8	19.0
20	89.4	89.2	89.0	89.3	88.9
21	75.6	75.5	75.7	75.6	75.8
2′	149.5	149.7	–	60.5	63.2

ance of downfield methylene proton signals at δ_H 3.47 (1H, ddd, $J = 16.9$, 11.5, and 5.3 Hz) and 3.66 ppm (1H, ddd, $J = 15.3$, 9.9, and 5.3 Hz) which were correlated by HMQC to a carbon resonance at δ_C 60.5 ppm. This indicated the presence of a hydroxymethyl functionality. The hydroxymethyl group was assigned by an HMBC experiment (Fig. 2) to position 2, where the methylene protons showed ^3J correlation to C-3 (δ_C 199.2 ppm) and C-1 (δ_C 39.4 ppm). Running the spectra in DMSO-d_6 allowed observation of signals due to protons of OH groups at C-2′ (δ_H 4.42 ppm, t, $J = 5.3$ Hz) and C-17 (δ_H 5.30 ppm, s), respectively. In the HMBC experiment (Fig. 2), the proton of the C-2′ OH group showed also cross-peaks with signals due to C-1 (δ_C 39.4 ppm) and C-3 (δ_C 199.2 ppm). Other correlations were observed between H-4 (δ_H 5.57 ppm, s) and each of C-2 (δ_C 44.4 ppm), C-5 (δ_C 170.9 ppm), and C-10 (δ_C 39.0 ppm). The NMR data of metabolite **4** were found comparable to those published by Choudhary *et al*. (2002), which confirmed the structure of this metabolite as 2-hydroxymethyl ethisterone or 17β-hydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (Fig. 1). This metabolite was identified previously in human urine as a major danazol metabolite (Davison *et al*., 1976; Potts, 1977; Rosi *et al*., 1977). It was also obtained as a microbial metabolite of danazol upon biotransformation by *Aspergillus niger*, *Bacillus cereus*, *Cephalosporium aphidicola* and *Fusarium lini* (Choudhary *et al*., 2002), and biotransformation by *Fusarium solani* and *Gibberella fujikuorii* (Azizuddin and Choudhary 2010).

The ^{1}H NMR spectrum of metabolite **5** (Table I) also showed the absence of a signal due to H-2′ (δ_H 8.28 ppm, s) in danazol. Similarly to metabolite **4**, it showed the presence of hydroxymethyl protons at C-2′, but in this case they appeared as a pair of doublets with a J value of 10.8 Hz (signals at δ_H 3.22 and 3.37 ppm), instead of the pair of ddd at δ_H 3.47 and 3.66 ppm in metabolite **4**. This indicated absence of the C-2 proton, probably due to reductive cleavage of the isoxazole ring. Formation of a new double bond at C-1 was confirmed by the appearance of a new signal due to an olefinic proton singlet in the ^{1}H NMR spectrum (Table I) at δ_H 6.80 ppm, assigned to H-1, as well as the presence of two new olefinic resonances in the ^{13}C NMR spectrum of **5** at δ_C 151.0 and 136.5 ppm compared to **4** (Table II). These resonances were assigned by HMBC (Fig. 2) to positions 1 and 2, respectively, where ^2J correlations were observed between H-1 (δ_H 6.80 ppm, s) and each of C-2 and C-10 (δ_C 136.5 and 43.5 ppm), while it showed ^3J correlations with C-3, C-5, and C-19 (δ_C 185.8, 171.1, and 19.0 ppm, respectively). These data suggested an 1,2-dehydro derivative of **4**. A new methine carbinol proton signal in the ^{1}H NMR spectrum of **5** at δ_H 4.32 ppm (br s) suggested hydroxylation of this derivative. This proton was correlated by HMQC to the carbon resonance at δ_C 70.1 ppm. The assignment of this carbon resonance to position 6 was confirmed by the observation of β- and γ-effects on chemical shifts of carbon atoms in its vicinity as well as by HMBC. The β-effect was evident by deshielding of C-7 (δ_C 35.8 ppm) by 4.9 ppm, while the γ-effect caused shielding of C-8 (δ_C 32.0 ppm) by 4.8 ppm, compared to danazol (Table II). In the HMBC experiment (Fig. 2), H-6 (δ_H 4.32 ppm, br s) showed cross-peaks with each of C-4 and C-10 (δ_C 128.0 and 43.5 ppm). Correspondingly, H-4 (δ_H 5.94 ppm, s) showed ^3J correlation to C-6 (δ_C 70.1 ppm) and C-10 (δ_C 43.5 ppm). H-6 was assigned an α (equatorial)-orientation based on width at half height ($W_{1/2} = 7.4$ Hz) (Choudhary *et al*., 2005). The C-6 hydroxy group must therefore have a β (axial)-orientation. These data collectively identified **5** as 6β,17β-dihydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (Fig. 1). The structure of this compound was hypothesized previously based only on GC-MS data obtained for danazol metabolites in equine and human urine samples, respectively (Kim *et al*., 2001; De Oca Porto *et al*., 2006). The NMR data are presented here for the first time.

In conclusion, this work demonstrates the capacity of microorganisms to mimic mammalian drug metabolism. In case of danazol, four microbial metabolites could be isolated and their structures fully elucidated. Three of the isolated metabolites are also known human metabolites of danazol. Thus, the purified and identified compounds can be further used as standards for direct comparison in routine analyses of anabolic steroids by GC-MS and LC-MS in urine and blood samples, in which these metabolites are usually present in trace amounts.

Experimental

General experimental procedures

^{1}H, ^{13}C NMR, HMBC, and HMQC spectra were obtained with a JEOL JNM-ECA500 spectrometer (JEOL Instruments, Tokyo, Japan), operating at 500 MHz (^{1}H) and 125 MHz (^{13}C); δ values are expressed in ppm. Mass spectrometry electrospray ionization (ESI-MS) spectra were carried out on a VG-

ZAB-HF reversed-geometry mass spectrometer (VG Analytical, Waltham, MA, USA). FAB mass spectra were taken on a JEOL JMS600 mass spectrometer (JEOL Instruments). Column chromatography (CC) was performed on silica gel (70 – 230 mesh; Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) was carried out on precoated silica gel 60 GF_{254} plates (Fluka, Darmstadt, Germany). Developed chromatograms were visualized by spraying with 0.01 % vanillin/H_2SO_4, followed by heating until maximum development of spot colour, or spraying with modified Dragendorff's reagent. Preparative-scale TLC was carried out on 1 mm thick silica gel plates. Solvents were of reagent grade.

Substrate

Danazol (**1**) was extracted from Danol capsules (Winthorp, Sheffield, England) using CH_2Cl_2, and crystallized from the same solvent. Its identity was confirmed by comparison of its spectroscopic data with those in the literature (Choudhary *et al*., 2002).

Fermentation method

Fermentation in liquid cultures was carried out according to the standard two-stage fermentation protocol of Betts *et al*. (1974). For screening experiments, solid cultures, kept on either potato dextrose agar (PDA) or Sabouraud maltose agar (SMA), of the following organisms were used: *Absidia pseudocylinderospora* ATCC 24169, *Amycolata autotrophica* ATCC 35203, *Aspergillus alliaceus* UI 315, *Aspergillus niger* ATCC 9142, *Aspergillus ochraceous* ATCC 1008, *Bacillus cereus* NRRL 14591b, *Bacillus megaterium* ATCC 14581, *Beauveria bassiana* ATCC 7159, *Botrytis allii* NRRL 2502, *Candida tropicalis* UI 2312, *Comamonas testosteroni* ATCC 11996, *Cunninghamella echinulata* ATCC 8688a, *Cunninghamella elegans* ATCC 9245, *Curvularia lunata* NRRL 2178, *Glyocladium viride* ATCC 10097, *Mortierella isabellina* ATCC 38063, *Mucor mucedo* UI 5513, *Nocardia species* NRRL 5646, *Rhizopus stolonifer* NRRL 1478, *Rhodotorula rubra* ATCC 20129, *Sepedonium chrysanthosporum* ATCC 13378, *Streptomyces griseus* ATCC 13273, and *Thamnidium elegans* ATCC 18191. Each culture was used separately to inoculate 100-ml flasks containing 20 ml of the following media: 2 % glucose, 0.5 % soybean meal, 0.5 % yeast extract, 0.5 % NaCl, 0.5 % K_2HPO_4. The pH value was adjusted to 7.0 before autoclaving for 20 min at 121 °C. The inoculated flasks were incubated at 27 °C and 250 rpm for 72 h (stage I cultures) before being used to inoculate stage II culture flasks, in which 10 % inoculum volumes of stage I were used to inoculate another batch of sterile medium, and were incubated under the same conditions for 24 h. Danazol (30 mg) dissolved in 1.5 ml of CH_2Cl_2/DMSO (1:1, v/v) was added to the 24-h-old stage II cultures in 50-μl aliquots to give a final content of 1 mg substrate per flask. The flasks were incubated again and sampled periodically for analysis.

Sampling

Samples of 1 ml were taken after 12, 24, 36, and 48 h and then every other day for 2 weeks following substrate addition. Each sample was extracted by shaking three times with 0.5 ml of EtOAc and spun at $3000 \times g$ for 1 min in a desktop centrifuge. The upper layer was pipetted off, and the extracts were concentrated and spotted on silica gel 60 GF_{254} TLC plates and developed with CH_2Cl_2/MeOH (95:5). The developed chromatograms were visualized by spraying with vanillin/H_2SO_4, followed by heating on a hot plate until maximum development of the spot colour.

Preparative-scale conversion

Danazol (1 g each) was added to stage II cultures of microorganisms which had given the best results in the screening, *i.e. Beauveria bassiana* ATCC 7159 and *Glyocladium viride* ATCC 10097, at a concentration of 1 mg per ml culture medium in 1-l flasks. After incubation for 2 weeks under the same conditions, the broth and the fungal mycelia were combined, homogenized in a blender, and exhaustively extracted with 3×1 l EtOAc. The combined extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield 900 and 1250 mg of residue, respectively.

Isolation and purification of the metabolites

Isolation of danazol metabolites of *B. bassiana* ATCC 7159

The EtOAc extract (850 mg) was fractionated on a silica gel column (70 cm $\times$ 1.5 cm) eluted with mixtures of CH_2Cl_2 and MeOH. Fractions eluted with 0.5 % MeOH in CH_2Cl_2 (413 mg) afforded untransformed substrate (R_f 0.52, CH_2Cl_2/MeOH, 95:5; and R_f 0.66, petroleum ether/EtOAc, 60:40; positive with

Dragendorff's reagent; orange pink on heating with vanillin/H_2SO_4 spray reagent). Further fractions, also eluted with 0.5% MeOH in CH_2Cl_2 (157 mg), contained mainly one major spot, which was purified by rechromatography on a silica gel column (50 cm × 1.0 cm) using mixtures of EtOAc in petroleum ether. Fractions eluted with 30% EtOAc yielded metabolite **2** as yellowish needles (55 mg; R_f 0.30, CH_2Cl_2/MeOH, 95:5; and R_f 0.28, petroleum ether/EtOAc, 60:40; positive with Dragendorff's reagent; blue violet on heating with vanillin/H_2SO_4 spray reagent).

Isolation of danazol metabolites of *G. viride* ATCC 10097

The EtOAc extract (1200 mg) was fractionated on a silica gel column (80 cm × 1.5 cm) and eluted with mixtures of petroleum ether and EtOAc. Fractions eluted with 20% EtOAc in petroleum ether (570 mg) contained unchanged substrate (see above). Fractions eluted with 30% EtOAc in petroleum ether (45 mg) contained one major spot along with several minor spots. Purification by rechromatography using the same solvent system yielded metabolite **3** as amorphous solid (3 mg; R_f 0.51, CH_2Cl_2/MeOH, 95:5; and R_f 0.30, petroleum ether/EtOAc, 65:35; negative with Dragendorff's reagent; pinkish-violet on heating with vanillin/H_2SO_4 spray reagent). Fractions eluted with 35% EtOAc in petroleum ether (71 mg) contained mainly one spot. Crystallization from CH_2Cl_2 afforded metabolite **4** as colourless needles (55 mg; R_f 0.40, CH_2Cl_2/MeOH, 95:5; and R_f 0.21, petroleum ether/EtOAc, 65:35; negative with Dragendorff's reagent; blue violet on heating with vanillin/H_2SO_4 spray reagent). Fractions eluted with 40% EtOAc in petroleum ether (31 mg) contained a mixture of three spots. Purification on preparative TLC plates using petroleum ether/EtOAc (70:30) afforded metabolite **5** as amorphous solid (5 mg; R_f 0.37, CH_2Cl_2/MeOH, 95:5; and R_f 0.20, petroleum ether/EtOAc, 60:40; negative with Dragendorff's reagent; blue violet on heating with vanillin/H_2SO_4 spray reagent).

Danazol (17α-pregna-2,4-dien-20-yno[2,3-d]isoxazol-17β-ol) (**1**): Crystalline solid. – M. p. 225–226 °C (dec.) (lit. 225 °C). – Yield: 413 mg. – R_f 0.52 [CH_2Cl_2/MeOH (95:5)] and 0.66 [petroleum ether/EtOAc (60:40)]. – UV (MeOH): $\lambda_{max}(\log \in) =$ 284 nm (5.6). – ^{1}H and ^{13}C NMR (500, 125 MHz, DMSO-d_6): see Tables I and II. – FAB-MS (positive ion mode): m/z (rel. int.) = 338 (9) [$M^+ + H$], 355 (38) [$M^+ + H_2O$].

6β-Hydroxy danazol (**2**): Crystalline solid. – M. p. 243–244 °C (dec.). – Yield: 55 mg. – R_f 0.30 [CH_2Cl_2/MeOH (95:5)] and 0.28 [petroleum ether/EtOAc, (60:40)]. – UV (MeOH): $\lambda_{max}(\log \in)$ = 283 nm (5.6). – ^{1}H and ^{13}C NMR (500, 125 MHz, DMSO-d_6): see Tables I and II. – HRFAB-MS (positive ion mode): m/z = 353.1952 [M^+] (calcd. 353.1991 for $C_{22}H_{27}NO_3$). – FAB-MS (positive ion mode): m/z (rel. int.) = 354 (19) [$M^+ + H$], 371 (22) [$M^+ + H_2O$].

17β-Hydroxy-17α-pregn-4-en-20-yn-3-one (ethisterone) (**3**): Amorphous solid. – M. p. 268–269 °C (lit. 266–273 °C). – Yield: 3 mg. – R_f 0.51 [CH_2Cl_2/MeOH, (95:5)] and 0.30 [petroleum ether/EtOAc (65:35)]. – UV (MeOH): $\lambda_{max}(\log \in) =$ 238 nm (6.2). – ^{1}H and ^{13}C NMR (500, 125 MHz, DMSO-d_6): see Tables I and II. – ESI-MS (positive ion mode): m/z (rel. int.) = 313 (11) [$M^+ + H$], 330 (8) [$M^+ + NH_4$].

17β-Hydroxy-2α-(hydroxymethyl)-17α-pregn-4-en-20-yn-3-one (2α-hydroxymethylethisterone) (**4**): Crystalline solid. – M. p. 162–163 °C (lit. 164–165 °C). – Yield: 55 mg. – R_f 0.40 [CH_2Cl_2/MeOH (95:5)] and 0.21 [petroleum ether/EtOAc (65:35)]. – UV (MeOH): $\lambda_{max}(\log \in)$ = 241 nm (5.5). – ^{1}H and ^{13}C NMR (500, 125 MHz, DMSO-d_6): see Tables I and II. – ESI-MS (positive ion mode): m/z (rel. int.) = 343 (15) [$M^+ + H$], 360 (22) [$M^+ + NH_4$], 365 (15) [$M^+ + Na$].

6β,17β-Dihydroxy-2-(hydroxymethyl)-17α-pregna-1,4-dien-20-yn-3-one (**5**): Amorphous solid. – M. p. 227–228 °C (lit. 226–227 °C). – Yield: 5 mg. – R_f 0.37 [CH_2Cl_2/MeOH (95:5)] and 0.20 [petroleum ether/EtOAc (60:40)]. – UV (MeOH): $\lambda_{max}(\log \in)$ = 248 nm (4.3). – ^{1}H and ^{13}C NMR (500, 125 MHz, DMSO-d_6): see Tables I and II. – ESI-MS (positive ion mode): m/z (rel. int.) = 357 (18) [$M^+ + H$], (19) [$M^+ + NH_4$].

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