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Predominant allylic hydroxylation at carbons 6 and 7 of 4 and 5-ene functionalized steroids by the thermophilic fungus *Rhizomucor tauricus* IMI23312

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Abstract

This paper demonstrates for the first time transformation of a series of steroids (progesterone, androst-4-en-3,17-dione, testosterone, pregnenolone and dehydroepiandrosterone) by the thermophilic fungus *Rhizomucor tauricus.* All transformations were found to be oxidative (monohydroxylation and dihydroxylation) with allylic hydroxylation the predominant route of attack functionalizing the steroidal skeleta. Timed experiments demonstrated that dihydroxylation of progesterone, androst-4-en-3,17-dione and pregnenolone all initiated with hydroxylation on ring-B followed by attack on ring-C. Similar patterns of steroidal transformation to those observed with *R. tauricus* have been observed with some species of thermophilic *Bacilli* and mesophilic fungi. All metabolites were isolated by column chromatography and were identified by ^{1}H, ^{13}C NMR, DEPT analysis and other spectroscopic data. The application of thermophilic fungi to steroid transformation may represent a potentially rich source for the generation of new steroidal compounds as well as for uncovering inter and intraspecies similarities and differences in steroid metabolism.

Keywords: *Rhizomucor tauricus*; Allylic oxidation; Steroid transformation; Thermophilic fungus; Hydroxylation

1. Introduction

Thermophilic fungi have a remarkable range of secretory (e.g. proteases, lipases, amylases, cellulases, xylanases) and cell associated enzymes (e.g. trehalase, invertase, β-glycosidase) [1–3]. The majority of these enzymes have high temperature stability and tolerance to a wide range of pH [1]. These distinctive properties have resulted in significant application of thermostable enzymes in both the sugar and paper industries [4]. To date of all the species of thermophiles only thermophilic *Bacilli* have been applied to steroid transformation and have achieved a range of oxidative reactions and generation of novel metabolites [5–8]. The application of thermophilic fungi to steroid transformation has not been investigated and represents a potentially rich source for the generation of new steroidal compounds and determination of metabolic information [9,10]. We have focussed in this study on *Rhizomucor tauricus* which is highly a unique strain of thermophilic fungus that was originally isolated from forest soil in the Ukraine [11] and although closely related to *Rhizomucor pusillus* it retains significant morphological and metabolic differences [12]. In order to determine the pattern of metabolic handling of steroids by this organism we have incubated a range of structurally diverse compounds namely progesterone (**1**), androst-4-en-3,17-dione (**2**), testosterone (**3**), pregnenolone (**13**), dehydroepiandrosterone (**14**) and determined the transformation products and sequence of hydroxylation.

2. Materials and methods

2.1. Chemicals and Reagents

All steroids were purchased from Steraloids Ltd. (UK) and were used as supplied. Solvents were of analytical grade; petroleum ether refers to the fraction b.p. 60–80 °C. Silica

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for column chromatography was Merck 9385 and TLC was performed with Macherey-Nagel Alugram® SIL G/UV_{254}.

2.2. Microorganism

R. tauricus (IMI23312) was obtained from the collection at CABI Bioscience (UK). Stock cultures were grown on potato dextrose agar slopes (14 days) and maintained at 4 °C until use. Steroid transformation studies were carried out in 3% malt extract medium (Oxoid, UK) containing 20% sucrose.

2.3. Conditions of cultivation and transformation

Spores were transferred aseptically in a laminar flow hood into 500 ml Erlenmeyer flasks containing 300 ml of sterile media and were incubated for 72 h at 40 °C. The cultures were shaken at 180 rpm on an orbital shaker. Five millilitres aliquots from the seed flask were transferred aseptically to 10 flasks and grown for a further 72 h as above at the end of which the fungus is in log phase growth. After this time period steroid dissolved in dimethylformamide (DMF) was evenly distributed between the flasks (1 mg/ml) under sterile conditions and incubated for a further 5 days after which the metabolites were extracted from the broth.

2.4. Extraction and identification of metabolites

The fungal mycelium was separated from the broth by filtration under vacuum. Following completion the mycelium was rinsed with ethyl acetate (0.5 l) to ensure the entire available steroid was removed. The mycelial broth was then extracted thrice with ethyl acetate (1.5 l). The organic extract was dried over sodium sulfate and the solvent evaporated *in vacuo* to give a gum. This gum was adsorbed onto silica and chromatographed on a column of silica; the steroidal metabolites were eluted with increasing concentrations of ethyl acetate in petroleum ether. The solvent was collected in aliquots (10 ml) and analysed by thin layer chromatography (TLC) to identify the separated metabolite fractions. The solvent systems used for running the TLC plates were 50:50 petroleum ether in ethyl acetate or pure ethyl acetate. A 50:50 sulfuric acid in methanol spray was used to develop the TLC plates.

2.5. Analysis and identification of metabolites

Characteristic shift values [13,14] and splitting patterns [15] in the ^{1}H and ^{13}C NMR spectra from the starting compounds were used to determine metabolite structure in combination with DEPT analysis to identify the nature of the carbon (Tables 1–4). Spectra were recorded on a Bruker WM 360 Spectrometer, all samples were analysed in deuteriochloroform or deuteriopyridine using tetramethylsilane as the internal standard. High-resolution mass measurement (HRMS) was determined in electrospray ionization (ESI) mode using a Bruker Daltonics Microtof spectrometer. Infra-red spectra were recorded directly on a Nicolet avatar 320 FT-IR fitted with a Smart Golden Gate®. Infra-red absorption signals and product yields are listed in Tables 5 and 6.

2.6. Time course experiment to determine order of dihydroxylation

Experimental conditions were identical to those in Section 2.3 except that steroids (**1**, **2**, **13**) were placed into 200 ml of media in six individual flasks at a concentration of 1 mg/ml. These flasks were harvested at 6 h then every 24 h and extracted as in Section 2.4. Following 4 h under high vacuum, the product ^{1}H NMR spectra were determined to confirm the nature of the extracts.

3. Results

3.1. Products of metabolism and structural identification

Incubation of progesterone (**1**) (Fig. 1) resulted in two transformation products. 6β-Hydroxyprogesterone (**4**) was identified by a new triplet signal in the ^{1}H NMR spectrum of the metabolite at 4.36 ppm ($J = 3$ Hz) indicating monohydroxylation. The ^{13}C NMR spectrum, on comparison to (**1**), demonstrated downfield shifts for C6 (39.13 ppm), C7 (5.18 ppm) and a gamma carbon upfield shift for C8 (6.73 ppm), which are consistent with literature values [13] for substitution at C-6. This metabolite crystallized from acetone/hexane as needles m.p. 176 °C, which agreed with literature values, 174–177 °C [16] (HRMS ESI: calc. for $M + Na^{+}$: 353.208 obsd. 353.207).

The ^{1}H NMR spectrum of the major dihydroxylated metabolite, isolated as a gum, contained signals at 4.33 ppm (t) and 4.56 ppm (td) in the ^{1}H NMR spectrum consistent with equatorial substitution at carbons 6 and 11 [15] supporting the structure 6β,11α-dihydroxyprogesterone (**5**) as did methyne signals in the ^{13}C NMR spectrum at 73.20 ppm and 68.91 ppm for carbons 6 and 11, respectively. Accurate mass measurement also fully supported the proposed structure (HRMS ESI: calc. for $M + Na^{+}$ 369.203 obsd. 369.201) as did infra-red absorption signals. Direct comparison between the spectra of (**4**) and (**5**) due to NMR determination in different deuterated solvents was not possible. However, further supporting structural evidence for (**5**) was achieved through acetylation that afforded 6β,11α-diacetoxyprogesterone (**6**) which contained two new acetate (–O–CO–CH_3) signals at 2.05 and 2.06 ppm and (*cf.* **4**) containing identical splitting signals for the downfield shifted axial protons of C-6 (5.43 ppm, s) and C-11 (5.29 ppm, td) with required accurate mass measurement (HRMS ESI: calc. for $M + Na^{+}$ 453.224 obsd. 453.224).

Following incubation of androst-4-en-3,17-dione (**2**) (Fig. 1) three monohydroxylated and one dihydroxylated