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Biotransformation of dehydro-*epi*-androsterone by *Aspergillus parasiticus*: Metabolic evidences of BVMO activity

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ABSTRACT

The research on the synthesis of steroids and its derivatives is of high interest due to their clinical applications. A particular focus is given to molecules bearing a D-ring lactone like testolactone because of its bioactivity. The *Aspergillus* genus has been used to perform steroid biotransformations since it offers a toolbox of redox enzymes. In this work, the use of growing cells of *Aspergillus parasiticus* to study the bioconversion of dehydro-epi-androsterone (DHEA) is described, emphasizing the metabolic steps leading to D-ring lactonization products. It was observed that *A. parasiticus* is not only capable of transforming bicyclo[3.2.0]hept-2-en-6-one, the standard Baeyer-Villiger monooxygenase (BVMO) substrate, but also yielded testololactone and the homo-lactone 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one from DHEA. Moreover, the biocatalyst degraded the lateral chain of cortisone by an oxidative route suggesting the action of a BVMO, thus providing enough metabolic evidences denoting the presence of BVMO activity in *A. parasiticus*. Furthermore, since excellent biotransformation rates were observed, *A. parasiticus* is a promising candidate for the production of bioactive lactone-based compounds of steroidal origin in larger scales.

1. Introduction

Steroids are a large family of molecules widely distributed in Nature. They are structurally diverse solid alcohols sharing a common four-ring nucleus [1]. These molecules exhibit vital roles in animal, fungal and plant cells. Likewise, they are used to treat severe human health disorders such as cancer, diabetes and obesity [2,3], among others. In particular, the discovery of the antineoplasic properties of testolactone in 1962 [4] triggered the search for bioactive D-ring lactone-based compounds [5].

The generation of oxyfunctionalized steroidal products is a multistep, complex task to achieve by chemical means [6–8]. Alternatively, the use of microbial catalysts represents a simpler and greener strategy to perform regio- and stereoselective hydroxylations, regioselective C=C reductions and isomerizations and, to a lesser extent, C=O reductions and Baeyer-Villiger oxidation reactions on the D-ring and the lateral chain of these molecules [9–11]. In particular, the use of filamentous fungi to biotransform androstan- and pregnan-based steroids has been comprehensively documented [12,13]. Among them, *Fusarium* [14] species have been shown to display excellent dehydrogenation activities. On the other hand, strains from *Beauveria* [15], *Cunninghamella* [16], *Mucor* [17–19] as well as *Fusarium* [20] were reported to perform regio- and stereoselective hydroxylations. Recently, Wu and coworkers described the hydroxylation of dehydro-*epi*-androsterone (DHEA) by *Colletotrichum lini* [21]. Furthermore, the genus *Penicillium* [22,23] has been employed to carry out D-ring lactonizations. Nevertheless, *Aspergillus* strains might be the biocatalysts of choice to study the transformation profile of a particular steroidal molecule, since this genus is capable of performing all the aforementioned reactions, as extensively referred by several authors [24–28].

It is demonstrated that the *Aspergillus* genus provides a toolbox of redox enzymes [29,30]. Nowadays, the Baeyer-Villiger monooxygenase (BVMO) activity is one of the most studied from biomolecular as well as biotechnological perspectives [31–33]. In this context, the purpose of this work was to find *Aspergillus* strains capable of yielding steroidal D-ring lactonization products using DHEA as an androstan-derived model compound.

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2. Experimental

2.1. Chemicals and microorganisms

All the chemicals were purchased in analytical grade from Sigma Aldrich or Merck and were used without further purification. Culture media components were obtained from Britania. The *Aspergillus* strains used comprised *A. flavus, A. fumigatus* and *A. japonicus*, which were acquired from the collections of Universidad de Buenos Aires (Argentina), Universidad Nacional de Rio Cuarto (Argentina) and Colección de Cultivos Fúngicos IIB-INTECH (Argentina), respectively, as well as *A. candidus* and *A. parasiticus*, which were both obtained from Universidad Nacional del Litoral (Argentina). Microorganisms stored at 4 °C in Czapek Yeast (CY) agar slopes were inoculated to erlenmeyers containing CY liquid medium, which composition *per* 1 L distilled H_2O is 5 g yeast extract, 30 g sucrose, 1 g K_2HPO_3, 0.3 g $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.005 g $CuSO_4{\cdot}5H_2O$ and 0.01 g $ZnSO_4{\cdot}7H_2O$. Cultures were incubated at 28 °C and 150 rpm.

2.2. Microbial screening

To test the capability of the *Aspergillus* strains to transform the model BVMO substrate *rac*-bicyclo[3.2.0]hept-2-en-6-one (**1**), biocatalysts were prepared according to previous reports [31,34]. Then, **1** (2.5 mM final concentration) was added to 50 mL erlenmeyer flasks containing 500 mg wet cells (2.5% (*p*/*v*)) of an individual strain each in 20 mL of CY medium. Biotransformations were run by duplicate –mean values are given- and incubated at 28 °C and 150 rpm during 72 h. Controls without **1** and without biocatalyst were also run. Samples were taken as described in Section 2.3.1 and analyzed by GC-FID using a Clarus 500 instrument (Perkin Elmer) and pure analytic standards. Conversion was determined employing a Rt-βDEXse (Restek) chiral capillary column and calculated using relative areas of the substrate and the lactone-based products only. The enantiomeric excesses were calculated as done by Chen et al. [35]. See Supplementary Information for further details.

2.3. Biotransformation of steroids

Biocatalysts were prepared as referred above. In the analytical scale, 50 mL erlenmeyer flasks containing 500 mg wet cells (2.5% (*p*/*v*)) of *A. parasiticus* in 20 mL CY medium were used. To start the biotransformations, each substrate was dissolved in DMSO or DMF (1% (*v*/*v*) final concentration) and added to the erlenmeyer flasks. Biotransformations were incubated at 28 °C and 150 rpm and were run by duplicate, mean values are shown. Controls without biocatalyst and without substrate were also included. To perform time-course experiments, multiples batches were prepared and samples were taken by withdrawing batches and controls in duplicate at a time. In the semipreparative scale, the same procedure described for the analytical scale was followed, but using a biotransformation volume of 200 mL in a 500 mL erlenmeyer flask. A total of five batches were run and combined after 96 h.

2.3.1. Work-up and analysis

Each batch was filtered *in vacuo* to remove cells and then reaction mixtures were extracted three times with one volume of ethyl acetate. Organic layers were combined, dried with sodium sulfate, concentrated *in vacuo* and spotted on silica gel 60 F254 TLC plates (Merck) to qualitatively assess the substrate consumption and the appearance of biotransformation products. The chromatographic analysis was done using the mixture *n*-hexane: ethyl acetate as mobile phase and plates were revealed under UV light and by spraying the *p*-anisaldehyde reagent, as described elsewhere. To assess the biotransformation profile of each substrate, samples were analyzed by GC-FID using a Clarus 500 instrument (Perkin Elmer) using pure analytic standards. Conversion was determined using a 007 (methyl 5% phenyl silicone) capillary column (Quadrex) and calculated using relative areas. Product purifications were done by column chromatography on silica gel 60 (230-400 mesh, Merck). Mixtures of Hx: EtOAc were initially used as mobile phases, changing the composition from 100:0 to 30:70 by a 10% *per* column volume (V_c). Finally, two V_c of pure EtOAc followed by two V_c of EtOAc: MeOH (90:10) were employed. The identity of the isolated compounds was determined by NMR, HRMS-ESI and GC/LRMS-EI. The ^1H and ^{13}C NMR spectra were recorded on a AC-200 or AMX-400 spectrometer (at 200.13 or 400.16 MHz, and at 50.23 or 100.62 MHz, respectively) (Bruker) using $CDCl_3$ as solvent with TMS as internal standard. HRMS-ESI measurements were done on a microTOF-Q II (Bruker) coupled to a UPLC-DAD 1200 (Agilent) at a resolution of 5000 (5% valley definition), by 70 eV electron ionization, at an accelerating voltage of 8 kV. GC/LRMS-EI were performed at 70 eV using an ion trap (GCQ Plus) with MSn (Finnigan, Thermo-Quest), operated at a fundamental *rf*-drive of 1.03 MHz. Helium was used as the damping gas at an uncorrected gauge reading of $6{\cdot}10^{-5}$ Torr. An OV-5 (5% diphenyl 95% dimethylpolysiloxane) capillary column (OVS) was used. Compounds were analyzed by comparing their mass spectra with the NIST spectral library (Wiley). Structural data is given in the Supplementary Information.

3. Results and discussion

3.1. Screening of BVMO activity in Aspergillus strains

Considering previous reports in literature describing the potential of the *Aspergillus* genus as a biocatalyst to perform Baeyer-Villiger oxidations of the D-ring in several steroids [36–39], we selected a small collection of strains from this genus to screen for this enzymatic activity by using *rac*-bicyclo[3.2.0]hept-2-en-6-one (**1**) as model substrate [40]. Since redox cofactors are needed, we employed growing cell cultures in order to display self-sufficient transformations. Although all the strains assayed were capable to oxidize the model substrate **1** to the corresponding normal and/or abnormal lactones (Table 1, Supplementary Information), *A. parasiticus* exhibited the highest regio- and enantioselectivity by yielding mainly the normal (1*S*, 5*R*) and the abnormal (1*R*, 5*S*) lactones from the enantiomers (1*R*, 5*S*) and (1*S*, 5*R*) of substrate **1**, respectively (Fig. 1). Since dehydrogenase activity is ubiquitous and constitutive in these fungi, carbonyl reduction products were observed as well (data not shown). Because of these results, *A. parasiticus* was selected as the biocatalyst to perform the transformation of DHEA (**2**).

3.2. Biotransformation of dehydro-epi-androsterone (DHEA)

When the biotransformation of **2** was assayed in analytical scale (100 mg), five products were detected (Fig. 2) by GC/ LRMS-EI and NMR analyses (see Supplementary Information). Among them, three compounds are supposed to directly derive from the substrate, being androstenediol (**3**) –a product of the reduction of the carbonyl in the D-ring-, androstenedione (**4**) –obtained by the oxidation of hydroxyl in position 3 and the subsequent isomerization of the C=C from C5 to C4- and the homo-lactone 3β-hydroxy-17a-oxa-D-homoandrost-5-en-17-one (**5**), produced after a D-ring lactonization reaction. Supported in literature reports, it is possible to propose that these molecules were obtained by the action of a dehydrogenase, a 3β-HSD/isomerase (E_2) and a BVMO