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New metabolites of drospirenone obtained in Mucorales fungi culture

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ABSTRACT

Microbial transformation of 6,7,15,16-dimethylene-3-oxo-17-pregn-4-ene-2,17-carbo-lactone, the well-known contraceptive drospirenone (**1**), using fungal cells was carried out. Six fungal strains of different species of Order Mucorales were evaluated in this study, namely *Absidia corymbifera* BAFC 1072, *Absidia corymbifera* BAFC 1080, *A. coerulea*, *Mucor plumbeus* BAFC 2314, *Rhizopus oryzae* and *Syncephalastrum racemosum*. Four products were obtained by hydroxylation at C-11 and C-2 and epimerization at C-17 of drospirenone by *A. corymbifera* BAFC 1072, *A. coerulea* and *S. racemosum*. The structures were elucidated as 6β,7β,15β,16β-dimethylene-11α-hydroxy-3-oxo-l7α-pregn-4-en-21,17-carbolactone (**2**), 6β,7β,15β,16β-dimethylene-11α-hydroxy-3-oxo-l7β-pregn-4-en-21,17-carbolactone (**3**), 6β,7β,15β,16β-dimethylene-11β-hydroxy-3-oxo-l7α-pregn-4-en-21,17-carbolactone (**4**) and 6β,7β,15β,16β-dimethylene-2β-hydroxy-3-oxo-l7α-pregn-4-en-21,17-carbolactone (**5**), on the basis of extensive spectral data including 2D NMR spectroscopy and MS. Products **3**, **4** and **5** were found to be new compounds. Several biotransformation parameters such as the employment of growing or resting cells, inoculum size, agitation speed, drospirenone concentration, temperature, pH and presence of co-solvent were seen to be important to the optimization of the process.

1. Introduction

Drospirenone (DRSP), generic name of 6,7,15,16-dimethylen-3-oxo-17-pregn-4-ene-2,17-carbo-lactone, is an anti-mineralocorticoid progestin derived from spironolactone [1]. Its structure consists of a 19-carbon chemical structure with two methylene groups, one of which is attached to C-6 and C-7 and the other to C-15 and C-16 (Fig. 1).

Because of its progesterone-like action DRSP is used in contraceptives and hormone therapy [2]. When DRSP and ethinyl estradiol (EE) are combined in a contraceptive tablet, the anti-mineralocorticoid action of DRSP controls the increase of angiotensinogen related to the action of EE [3]. Moreover, the combination DRSP-EE is also useful in the treatment of premenstrual syndrome, premenstrual dysphoric disorder and acne [4–6].

In the field of contraception the objective is to create the "ideal" progestin, which could produce the benefits of progesterone without any of the interaction of the androgenic, estrogenic or glucocorticoid receptors. Despite the advances achieved by the application of DRSP-EE, it was not possible to eliminate some unwanted side-effects such as many somatic symptoms of premenstrual dysphoric disorder and an increased cardiovascular risk [7–9].

Considering the relevant pharmacological interest in DRSP, the production of analogous products leading to the ideal progestin shows very interesting aspects.

In the field of steroids, it has been reported that hydroxyl derivatives show much higher biological activity than the less polar substrate. For example, as far as immunoprotective and immunoregulatory properties are concerned, the 7-hydroxy derivative of dehydroepiandrosterone (DHEA) is several times more active than DHEA itself [10].

Microbial biotransformation is an important tool for the modification of organic compounds, especially natural products with complicated structures [11–13].

Filamentous fungi are particularly used in a number of bioconversions, including ketoreduction, hydroxylation, ester hydrolysis and hydrogenation of double bonds in steroids, and conversions of alkaloids and xenobiotics. Fungi belonging to the Order Mucorales, especially some species of *Mucor* and *Rhizopus*, are an important biochemical resource to mediate steps in organic synthesis, due to their fast growth and high enzymatic activity [14,15]. Another

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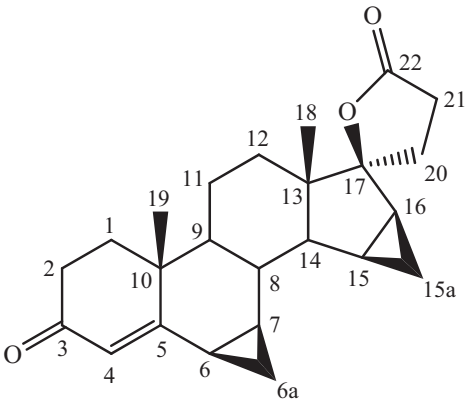

Drospirenone (1)

Fig. 1. Drospirenone (**1**).

advantage of this group of fungi is that they rarely produce mycotoxins, making them good candidates to be investigated in the bioconversion of products for clinical use.

Some reactions, such as hydroxylation at specific positions, which are difficult for chemical synthesis, could be readily accomplished with fungi [16–19]. Among all sites of steroid structure accessible for fungi hydroxylation, the 11α-, 11β-, 15α- and 16α-hydroxylations are the most valuable in the manufacturing of anti-inflammatory drugs [20]. The use of *Rhizopus stolonifer* and *Rhizopus nigricans* is well known in the 11α-hydroxylation of progesterone, a decisive step in the production of cortisone [21,22]. Other Mucorales, such as *Cunninghamella blakesleeana* and *Absidia orchidis*, were used for this hydroxylation reaction at position C-11 [23]. Moreover, the application of *Absidia cuneospora* was reported in the biotransformation of compounds with cholesterol lowering activity [24].

Therefore, in conformity with our previous studies on fungi biotransformation and biocatalytic steroid modification [14,15,25–34], in the present work various strains of different species of Mucorales have been screened for their capabilities to biotransform DRSP.

The optimal biotransformation conditions were determined for the most efficient strain. Under these conditions, the biotransformation afforded four products, three of them new compounds, which were completely identified by spectroscopic methods.

2. Experimental

2.1. Materials

Drospirenone was a generous gift from Laboratorio Gador S.A., Argentina. All solvents and reagents were of analytical grade and were purchased from Merck Argentina. TLC and PTLC silica gel 60F-254 aluminum sheets (0.2 mm thickness) and column silica gel 60 (230-400 mesh) were purchased from Sigma Aldrich.

2.2. Analytical methods

Biotrasformation reactions were carried out on Innova 4000 digital incubator shaker, New Brunswick Scientific Co. The course of biotransformation was controlled by means of TLC and HPLC. TLC analyses were performed on silica gel 60 F254. Compounds were detected by spraying the plates with H_2SO_4/CH_3OH mixture (1:1, v/v). HPLC analyses were performed on a Waters instrument equipped with a reversed phase Waters Symmetry C-18 column, 4.6 mm × 150 mm (5 μm) and UV detector at 254 nm; mobile phase: acetonitrile:water (55:45); flow rate: 0.3 mL/min, isocratic. Retention time (min): **1**: 9.20, **2**: 4.31, **3**: 4.63, **4**: 5.07 and **5**: 5.53. Melting points were determined on a Fisher Johns apparatus and are uncorrected. Optical purities of isolated products were determined by specific rotation with PerkinElmer 343 and Jasco P-1010 polarimeters. Solvents are indicated. FT-IR measurements were performed on a Shimadzu FTIR-8300 spectrophotometer in film with KBr windows. Proton and carbon NMR spectra were acquired on a Bruker AM-500 (500 MHz for ^1H and 125.1 for ^{13}C) in $CDCl_3$. Chemical shifts (δ) are reported in ppm downfield from TMS as the internal standard. Coupling constant (J) values are given in Hz. Solvents are indicated. The assignment of the proton signals is based on the homonuclear-shift-correlation spectroscopy (COSY) while the carbon nuclei were assigned from the heteronuclear correlation experiments *via* one-bond (HSQC) coupling constants and long-range (HMBC) coupling constants. Stereochemistry was determined from nuclear Overhauser enhancement spectroscopy (NOESY). HR-ESI-MS were measured in a Bruker microTOF-Q II mass spectrometer.

2.3. Microorganisms and culture medium

Strains of *Absidia corymbifera* BAFC 1072, *A. corymbifera* BAFC 1080 and *Mucor plumbeus* BAFC 2314 were obtained from the BAFC (Buenos Aires Facultad de Ciencias, Culture Collection of the University of Buenos Aires). *Absidia coerulea, Rhizopus oryzae* and *Syncephalastrum racemosum* were provided from the Food Microbiology Laboratory Collection (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires). Each fungal strain was inoculated in Malt Extract Agar (which contains 30 g of malt extract and 3 g of peptone per liter) slants and incubated at 25 °C for 5–7 days to obtain heavily sporulating culture.

Inocula were prepared by collecting spores adding 10 mL of Malt Extract Broth (MEB) to the slant. After shaking 30 s, the suspensions were removed, counted using a Neubauer chamber and adjusted to different concentration.

2.4. General procedures for biotransformation

Cultures were grown according to the standard two-stage fermentation protocol: stage I of fermentation was used for the growth of microorganisms, while the stage II was used for the biotransformation of DRSP (**1**).

2.5. Screening procedures

2.5.1. Resting cells

A spore suspension (1 mL) of each fungal strain (2×10^6 spores/mL) was inoculated into Erlenmeyer flasks (25 mL) containing 19 mL of Malt Extract Broth (MEB, pH 5) and incubated at 30 °C for 4 days in an orbital shaker (170 rpm). After incubation, fungal cells were harvested by filtration and washed with acetate buffer (pH 5, 0.1 M). Cells (1 g per flask) were suspended in acetate buffer (20 mL) or in 5 mL of organic solvent (diisopropyl ether, dioxane, toluene and hexane) in 25 mL and 10 mL Erlenmeyer flask respectively, stoppered and sealed. Drospirenone (**1**) (0.5 mg/mL) was added and bio-reaction was incubated in orbital shaker at 30 °C for 7 days (170 rpm). Biotransformation progress was monitored everyday by TLC and/or HPLC analysis. Blank assays without DRSP and without fungi were carried out in parallel. Experiments were performed in triplicate.

DRSP was also added to filtration supernatant under the same conditions.