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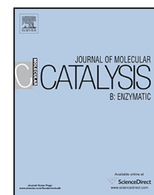

New metabolites of drospirenone obtained in Mucorales fungi culture

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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.

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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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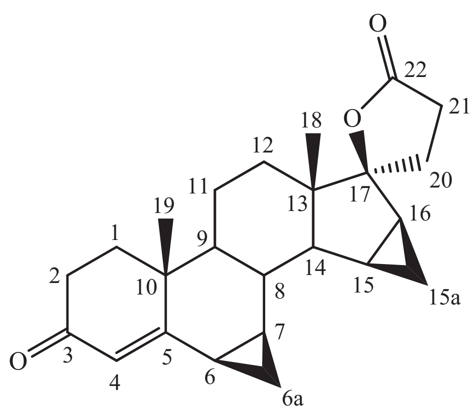


Fig. 1. Drospirenone (**1**).

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 ^{1}H 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.

2.5.2. Growing cells

A spore suspension (1 mL) of each fungal strain (10^4 spores/mL) was inoculated into 25 mL Erlenmeyer flasks containing 19 mL of sterile medium (MEB, pH 5) in an orbital shaker (170 rpm) at 30 °C. After four days, 10 mg of DRSP was added and incubated at 30 °C for 4 days (170 rpm). Biotransformation progress was monitored everyday by TLC and HPLC analysis. Blank assays without DRSP and without fungi were carried out in parallel. Experiments were performed in triplicate.

2.5.3. Preparative scale biotransformation of drospirenone by A. corymbifera BAFC 1072

DRSP (2× 100 mg) was evenly distributed among two Erlenmeyer flasks containing each 190 mL of stage I culture medium and incubated as in Section 2.5.2 (optimal conditions for *A. corymbifera* BAFC 1072).

2.5.4. Extraction, purification and identification of biotransformation products

After 4 days of fermentation, the cultures were filtered and the filtrates were extracted with dichloromethane (3× 10 mL), dried (anhydrous Na_2SO_4) and evaporated to dryness under reduced pressure. The crude extract (234 mg) was analyzed by HPLC and purified by column chromatography on silica gel with a stepwise elution with hexane-ethyl acetate from 70:30 to 20:80. The fraction containing compounds **3** and **4** was further purified by preparative plates (PTLC) using toluene-ethyl acetate 60:40 as eluant. Four products were obtained: **2** (60.1 mg), **3** (27.2 mg), **4** (9.5 mg) and **5** (2.0 mg). All the products were identified on the basis of their spectroscopic data (^{1}H and ^{13}C NMR (HMBC, HSQC, NOESY) FT-IR and HR-ESI-MS).

2.6. Biotransformation products

*2.6.1. 6β,7β,15β,16β-Dimethylene-11α-hydroxy-3-oxo-l7α-pregn-4-en-21,17-carbolactone (**2**)*

Yield: 28.6%. White solid; m.p. 239–240 °C. (Reported: m.p.: 238.8 °C [35]; $[\alpha]_D$ (c = 1.02, $CHCl_3$): −15.6°; FT-IR (film): ν_{max} cm^{-1} 3423, 1767, 1652, 1593; ^{1}H NMR ($CDCl_3$) see Table 3; ^{13}C NMR ($CDCl_3$) see Table 4; HR-ESI-MS: $[M+H]^+$ = 383.22086 (calcd. for $C_{20}H_{30}O_4H$: 383,22093); $[M+Na]^+$ = 405.20437 (calcd. for $C_{20}H_{30}O_4Na$: 405.20399).

*2.6.2. 6β,7β,15β,16β-Dimethylene-11α-hydroxy-3-oxo-l7β-pregn-4-en-21,17-carbolactone (**3**)*

Yield: 13.0%. White solid; m.p. 225–226 °C; $[\alpha]_D$: −42.4° (c = 0.01; $CHCl_3$); FT-IR (film): ν_{max} cm^{-1} 3424, 1767, 1653, 1593; RMN ^{1}H ($CDCl_3$) see Table 3; ^{13}C NMR ($CDCl_3$) see Table 4; HR-ESI-MS: $[M+H]^+$ = 383.22100; $[M+Na]^+$ = 405.20363.

*2.6.3. 6β,7β,15β,16β-Dimethylene-11β-hydroxy-3-oxo-l7α-pregn-4-en-21,17-carbolactone (**4**)*

Yield: 4.5%. White solid; m.p. 170–171 °C; $[\alpha]_D$: −56,2° (c = 0,001; $CHCl_3$); FT-IR (film): ν_{max} cm^{-1} 3420, 1760, 1656, 1595; RMN ^{1}H ($CDCl_3$) see Table 3; ^{13}C NMR ($CDCl_3$) see Table 4; HR-ESI-MS: $[M+H]^+$ = 383.22133; $[M+Na^+]$ = 405.20515.

*2.6.4. 6β,7β,15β,16β-Dimethylene-2β-hydroxy-3-oxo-l7α-pregn-4-en-21,17-carbolactone (**5**)*

Yield: 1.0%. White solid; m.p. 185–187 °C; $[\alpha]_D$: −83.0° (c = 0,001; $CHCl_3$); FT-IR (film): ν_{max} cm^{-1} 3422, 1765, 1656, 1590; RMN ^{1}H ($CDCl_3$) see Table 3; ^{13}C NMR ($CDCl_3$) see Table 4; HR-ESI-MS: $[M+H^+]$ = 383.22118; $[M+Na^+]$ = 405.20500.

3. Results and discussion

3.1. Biotransformation

Six fungal strains from different genus and species were evaluated in the biotransformation of drospirenone: *A. coerulea*, *A. corymbifera* (BAFC 1080), *A. corymbifera* (BAFC 1072), *M. plumbeus* (BAFC 2314) and *R. oryzae* belonging to the Fam. Mucoraceae, and *S. racemosum* of the Fam. Syncephalastraceae. The biotransformation was studied applying two methodologies: growing cells and resting cells.

3.1.1. Biotransformation with resting cells

The methodology of resting cells is broadly used in microbial transformations. This process is generally cleaner and the work-up is much easier than with growing cells. Unfortunately, when DRSP was used as a substrate we obtained poor results with all the fungi screened.

After harvesting, fungal cells were suspended in acetate buffer or organic solvent (hexane, toluene, diisopropyl ether and dioxane) and DRSP was added. These experiments have shown that the best results were achieved with *A. coerulea* and *A. corymbifera* BAFC 1072 suspended in acetate buffer. This transformation gave a 16% conversion whereas the cells suspended in each of the solvents were completely inactive.

Usually the steroid hydroxylase in fungi is a monooxygenase enzyme system [36,37]. These multienzyme complexes, situated on the microsomal membrane of the endoplasmic reticulum in cells, contain a P450 as substrate binding terminal oxidase and a flavoprotein for transferring electrons from reduced coenzyme to P450 [38,39]. The manipulation of cells in the resting cells methodology could inhibit the hydroxylation reaction through a decrease in activity of enzymes responsible for the oxidation or those involved in the regeneration of the redox cofactors.

3.1.2. Biotransformation with growing cells

When the six strains were screened using the growing cells procedure, DRSP was only transformed by *A. coerulea*, *A. corymbifera* BAFC 1072 and *S. racemosum*.

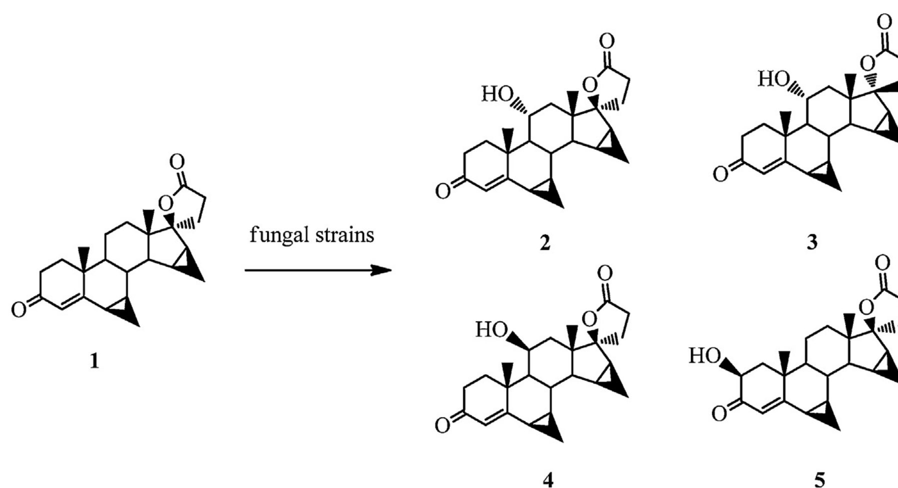
The four hydroxylation products obtained (**2**–**5**) are shown in Scheme 1.

Reactions performed in triplicate, blank culture control and substrate control were monitored by HPLC. It was observed that DRSP was stable in the blank culture control. Table 1 shows the conversion of DRSP and the composition of the mixture obtained under the conditions in which the three active strains produced the four products.

It was noticed that every strain showed similar activity toward DRSP biotransformation affording the products between 44.3 and 52.6% conversion. The two strains that belong to the *Absidia* genus led to a mixture of the same four products (**2**–**5**) but with differences in their quantitative composition.

These strains afforded hydroxylation products at C-11 (**2**) and at C-2 (**5**) positions. The C-11α position of DRSP was preferentially hydroxylated with a high diasteromeric excess ranging from 85.5 to 87.4%. This outcome was predictable since the hydroxylation of steroids at C-11 by a broad variety of microorganisms is well known [40].

On the other hand we would like to point out that the hydroxylation at C-2 is also a very interesting result. It is difficult to carry out in only one step and selectively by chemical procedures. There



Scheme 1. Biotransformation of drospirenone (**1**) with Mucoral fungi.

are few reports of hydroxyl derivatives at C-2 obtained by biotransformation [41,42].

The two active strains were also able to catalyze the epimerization at C-17 to obtain product **3**. Concerning microbial epimerization of steroids there are reports on epimerization at C-3, -7 and -12 in bile acids by different microorganisms, including bacterial and fungal strains [43] and 3-OH in bufadienolides by *Mucor polymorphosporus* [35]. Regarding steroids containing a C-17 spironolactone side chain, no epimerization was detected by biotransformation with fungi such as *Beauveria bassiana* and *Mortierella isabellina* [44]. The only epimerization at C-17 in steroids was described in the *in vivo* biotransformation of 17α-methyltestosterone in the horse [45]. To the best of our knowledge this is the first work reporting the epimerization at C-17 of steroids carried out by microorganisms and particularly by Mucorales fungi.

As it can be observed in Table 1, *A. corymbifera* BAFC 1072 showed higher stereoselectivity than *A. coerulea*. It afforded **2** with almost 70% of diasteromeric excess.

When DRSP was biotransformed with *S. racemosum* as a bioagent, **2** was almost the only product (92.8%) in high diasteromeric excess (96.2% of the 11α-hydroxy derivative, Table 1). *S. racemosum* showed the best regio- and stereoselective behavior in the biotransformation of DRSP.

In every case both cyclopropane rings in DRSP remained unaltered by microbial transformation. In comparison, these rings showed to be more stable than the cyclopropane ring of the 3α,5-cyclosteroids. These being sensitive to a variety of reagents, were transformed by growing cells of *Candida aphidicola* [46]. Additionally, the cyclopropane ring of 3β-hydroxy-5,6β-cyclopropano-5β-cholestane afforded elimination products by fermentation with *Mycobacterium* sp. [47].

3.1.3. Optimization of biotransformation conditions

Considering that *A. corymbifera* BAFC 1072 gave the best results in terms of DRSP conversion and variety of products, we decided to choose this strain to optimize the biotransformation conditions with growing cells. During submerged cultivation of filamentous fungi in shake culture, mycelium can be either in a dispersed form or it can form mycelial pellets by aggregation of growing hyphae. Fungal pellets provide a natural and convenient way of immobilizing biomass and directing metabolism toward specific metabolites, unavailable under conditions of free mycelial growth [48]. The formation of spherical and stable fungal pellets is greatly influenced by the rate of agitation, media composition and strain and inoculum concentration.

To obtain a better performance of the biotransformation, we performed several experiments by changing reaction parameters such as: inoculum size, agitation speed, pH, temperature, substrate concentration, solvent and reaction time (Table 2).

3.1.3.1. Inoculum size and agitation speed. To evaluate the optimum inoculum size in the biotransformation, we performed experiments with three spore concentrations: 10^4, 10^5 and 10^6 spores/mL. Previous experiments showed that concentrations lower than 10^4 produced poor growth. Under these conditions the fungus was not able to perform the biotransformation.

The tests were performed in triplicate, with DRSP concentration of 0.5 mg/mL, at 30 °C and 170 rpm. After four days the culture was filtrated and the products extracted with dichloromethane. Conversion percentage in the organic extract was determined by HPLC and the results are depicted in Table 2 (entries 1–12). It can be observed that conversion decreased with the increase in inoculum size.

Regarding agitation speed (Table 2, entries 13–20), the best results were obtained working at 170 rpm. At lower speed of 90 rpm DRSP conversion decreased and the product **5** was not detected. An experiment carried out without agitation showed no biotransformation products. In this case the mycelium was in a dispersed form without the formation of spherical pellets. The static culture is not favorable to the diffusion of the substrate toward the enzymatic

Table 1
Biotansformation of drospirenone by Mucorales fungal strains.

Strain	Conversion (%)[a]					Composition (%)				Diasteromeric excess (%)	
	2	3	4	5	DRSP	2	3	4	5	C-17β	C-11α
A. corymbifera BAFC 1072	31.9 ± 0.5	13.7 ± 1.0	5.7 ± 0.5	1.3 ± 0.1	52.6	60.6	26.0	10.8	2.4	69.9	85.8
A. coerulea	22.2 ± 6.6	15.6 ± 2.8	3.2 ± 0.7	2.9 ± 0.1	43.9	50.5	35.5	7.3	6.6	59.7	87.4
S. racemosum	47.6 ± 2.1	–	1.9 ± 0.1	1.8 ± 0.1	51.3	92.8	–	3.7	3.5	––	96.2

Biotransformation conditions: inoculum size: 10^4 spores/mL, [DRSP]: 0.5 mg/mL, temperature: 30°C, agitation speed: 170 rpm, pH: 5, time: 4 days.
[a] Determined by HPLC.

Table 2
Optimization of reaction parameters for drospirenone biotransformation.

Entry	[Spore] (spores/mL)	[DRSP] (mg/mL)	pH	DRSP solvent	Temperature (°C)	Agitation speed (rpm)	Product conversion (%)
Inoculum size							
1	10^4	0.5	5	–	30	170	2 (29.9)
2	10^4	0.5	5	–	30	170	3 (13.2)
3	10^4	0.5	5	–	30	170	4 (5.5)
4	10^4	0.5	5	–	30	170	5 (1.3)
5	10^5	0.5	5	–	30	170	2 (27.6)
6	10^5	0.5	5	–	30	170	3 (8.5)
7	10^5	0.5	5	–	30	170	4 (3.5)
8	10^5	0.5	5	–	30	170	5 (0.8)
9	10^6	0.5	5	–	30	170	2 (22.0)
10	10^6	0.5	5	–	30	170	3 (7.8)
11	10^6	0.5	5	–	30	170	4 (3.2)
12	10^6	0.5	5	–	30	170	5 (0.6)
Agitation speed[a]							
13	10^4	0.5	5	–	30	90	2 (23.9)
14	10^4	0.5	5	–	30	90	3 (8.5)
15	10^4	0.5	5	–	30	90	4 (1.5)
16	10^4	0.5	5	–	30	90	5 (n.d.)
17	10^4	0.5	5	–	30	170	2 (29.9)
18	10^4	0.5	5	–	30	170	3 (13.2)
19	10^4	0.5	5	–	30	170	4 (5.5)
20	10^4	0.5	5	–	30	170	5 (1.3)
DRSP concentration							
21	10^4	0.1	5	–	30	170	2 (31.9)
22	10^4	0.1	5	–	30	170	3 (13.7)
23	10^4	0.1	5	–	30	170	4 (5.7)
24	10^4	0.1	5	–	30	170	5 (1.0)
25	10^4	0.2	5	–	30	170	2 (31.1)
26	10^4	0.2	5	–	30	170	3 (12.7)
27	10^4	0.2	5	–	30	170	4 (5.3)
28	10^4	0.2	5	–	30	170	5 (1.3)
29	10^4	0.3	5	–	30	170	2 (31.4)
30	10^4	0.3	5	–	30	170	3 (15.8)
31	10^4	0.3	5	–	30	170	4 (6.2)
32	10^4	0.3	5	–	30	170	5 (1.8)
33	10^4	0.4	5	–	30	170	2 (28.1)
34	10^4	0.4	5	–	30	170	3 (10.2)
35	10^4	0.4	5	–	30	170	4 (4.0)
36	10^4	0.4	5	–	30	170	5 (1.4)
37	10^4	0.5	5	–	30	170	2 (29.9)
38	10^4	0.5	5	–	30	170	3 (13.2)
39	10^4	0.5	5	–	30	170	4 (5.5)
40	10^4	0.5	5	–	30	170	5 (1.1)
DRSP solvent[b]							
41	10^4	0.5	5	AcOEt	30	170	2 (28.3)
42	10^4	0.5	5	AcOEt	30	170	3 (8.5)
43	10^4	0.5	5	AcOEt	30	170	4 (1.5)
44	10^4	0.5	5	AcOEt	30	170	5 (1.1)
45	10^4	0.5	5	MeOH	30	170	2 (4.1)
46	10^4	0.5	5	MeOH	30	170	3 (1.9)
47	10^4	0.5	5	MeOH	30	170	4 (0.7)
48	10^4	0.5	5	MeOH	30	170	5 (n.d.)
pH[c]							
49	10^4	0.5	5	–	30	170	2 (31.9)
50	10^4	0.5	5	–	30	170	3 (13.7)
51	10^4	0.5	5	–	30	170	4 (5.7)
52	10^4	0.5	5	–	30	170	5 (1.3)
53	10^4	0.5	7	–	30	170	2 (27.8)
54	10^4	0.5	7	–	30	170	3 (6.5)
55	10^4	0.5	7	–	30	170	4 (4.1)
56	10^4	0.5	7	–	30	170	5 (0.9)
Temperature[d]							
57	10^4	0.5	5	–	25	170	2 (33.6)
58	10^4	0.5	5	–	25	170	3 (10.0)
59	10^4	0.5	5	–	25	170	4 (2.1)
60	10^4	0.5	5	–	25	170	5 (n.d.)
61	10^4	0.5	5	–	30	170	2 (31.9)
62	10^4	0.5	5	–	30	170	3 (13.7)
63	10^4	0.5	5	–	30	170	4 (5.7)
64	10^4	0.5	5	–	30	170	5 (1.3)

Experiments were performed in triplicate using *Absidia corymbifera* BAFC 1072 as biotransformation agent.
[a] Agitation speed: 0, no pellet formation.
[b] DRSP solubility: AcOEt: 35 mg/mL, MeOH: 50 mg/mL.
[c] pH 10: no growth.
[d] Temperature: 35 °C, no pellet formation.
n.d.: non detected.

active site, which is a necessary condition for the biotransformation to happen. These results are in accordance with a previous work [48] about the pelleted growth form of *R. nigricans* as a biocatalyst for progesterone 11α-hydroxylation.

The authors reported the requirements to maintain the biotransformation capacity for pellets obtained from the growth phase of cultivation at high agitation speed and with low inoculum size.

3.1.3.2. Substrate concentration and solvent effect. To improve the substrate/biocatalyst ratio, the effect of increasing substrate concentration on the biotransformation was evaluated. Working conditions were similar to those already described, varying only the substrate concentration between 0.1 and 0.5 mg/mL. Samples were withdrawn at the fourth day of biotransformation and the results are depicted in Table 2 (entries 21–40). It was observed that, in this range of concentrations, there was no significant variation in DRSP conversion. No product was detected at DRSP concentrations lower than 0.1 mg/mL or higher than 0.5 mg/mL. As DRSP was not soluble in the aqueous culture medium, the conversion decreased (data not shown).

With the aim of increasing DRSP solubility in the culture medium, we performed experiments with DRSP dissolved in ethyl acetate and methanol at 35 mg/mL and 50 mg/mL respectively. According to the results showed in Table 2 (entries 41–48) the addition of both solvents was not useful to improve the biotransformation. Methanol caused a remarkable decrease in the conversion DRSP. This fact could be attributed to a toxic effect caused by this alcohol on the activity of the biomass.

3.1.3.3. Influence of pH and temperature. To evaluate pH effect, the biotransformation of DRSP was studied at different pH values: 5, 7 and 10 in the culture medium. The medium was inoculated to obtain spore concentration of 10^4 spores/mL, and the biotransformation was performed at the above mentioned conditions. The results, shown in Table 2 (entries 49–56), indicate similar conversion at pH 5 and 7 with the exception of compound **3** which was obtained in double yield at pH 5. It seems that epimerization at C-17 is highly favored at a slight acidic pH.

With the aim of proving that the epimerization product **3** was not formed by acid catalysis, we carried out experiments at pH 5 but without *A. corymbifera*. In this case no product was detected, confirming the microorganism role in the epimerization of DRSP at carbon 17.

The influence of temperature on the microorganism's growing and the activity of enzymatic complex involved in the biotransformation were also studied. The reaction was carried out under standard conditions at 25, 30 and 35 °C. The results (Table 2, entries 57–64) show that increasing the temperature from 25 to 30 °C decreases the biotransformation regioselectivity. Epimerization at carbon 17 shows a slight increase. It was not observed growing the cells at 35 °C although this is the optimum growing temperature for *A. corymbifera* according to previous reports [49].

3.2. Identification of products

The structure of products **2**–**5** was determined by FT-IR, HRMS, ^{1}H NMR and ^{13}C NMR spectra. FT-IR analysis confirmed the presence of hydroxyl groups in the steroid skeleton of the four products through the presence of strong bands at 3420–4 cm^{-1} due to the O–H stretching.

The molecular formula was determined as $C_{24}H_{30}O_4$, on the basis of the HR-ESI-MS spectrum [M+H: $[M+H]^+$: 383.22086–383.22133; $[M+Na]^+$ = 405.20205–405.20515]. It was identical for the four products (**2**–**5**) and the molecular ion was16 mass units higher than that of DRSP, which indicated the monohydroxylation of the substrate in every case.

Compound **2** was obtained as a white solid. Its melting point (238–239 °C) was in accordance with a previous work reporting the chemical and bio-transformation of DRSP [50]. Compared to its parent compound, the ^{1}H NMR spectrum of **2** exhibited a new proton signal at δ 3.89 (*dt*, *J* = 9.8, 5.6 Hz) corresponding to a methine proton due to the secondary alcohol at C-11 (Table 3).

There was also a significant difference in the chemical shift of the methyl-19 protons relative to DRSP, which moved from 1.10 to 1.21 ppm and the down-field shifts of the signals of H-9 (δ 1.13–1.27) and H-12 (δ 1.46 and 1.42 to 1.86 and 1.44). In the ^{13}C NMR spectrum of **2**, it can be observed a new signal at δ 67.6 assigned to C-11 and the disappearance of the signal at δ 37.0 corresponding to the same carbon in DRSP (Table 4).

In the COSY spectrum, correlations between H-11, H-9 and H-12 were observed. H-9 and H-12 signals were determined from ^{1}H NMR spectrum and carbon signals were assigned taking into account two-dimensional spectra HSQC and HMBC, showing short and long-range H–C correlation signals. H-9 signal at δ 1.27 correlated with C-8 (δ 34.2), C-11 (δ67.6) and C-19 (δ 18.5) and H-12 at δ 1.86 and 1.44, correlated with C-11 (δ67.6), C-14 (δ 51.8) and C-19 (δ 18.5) (Supplementary data). The stereochemistry of the hydroxyl group at C-11 was established using correlation signals in the NOESY spectrum. NOE enhancement between H-11 (δ 3.89), methyl-18 (δ 0.98) and methyl-19 (δ 1.21) indicated that the hydroxyl group is α configuration. Moreover, the multiplet at the signal at δ 3.89 in the ^{1}H NMR spectrum confirmed equatorial configuration which is consistent with a 11α-hydroxyl group. From these results, it seems that DRSP was easily hydroxylated at C-11 α by *A. corymbifera* BAFC 1072. The chemical shifts assignments, made on the basis of the information obtained from ^{1}H NMR, ^{13}C NMR, HSQC, HMBC and COSY, made possible the identification of **2** as 6β,7β,15β,16β-dimethylene-11α-hydroxy-3-oxo-l7α-pregn-4-en-21,17-carbolactone.

Compound **3** was obtained as a white solid of melting point 225–226 °C. In the ^{1}H NMR spectrum, we could also see a signal at δ 3.82 indicating the hydroxylation at C-11α. Chemical shifts of the signal at δ 67.7 assigned to C-11 and the lack of the signal corresponding to the same carbon in DRSP confirmed the C-11α hydroxylation.

The shifts of the signals of lactone protons compared to DRSP: H-15 (δ 1.60–1.67), H-15′ 1.33 and 0.54 to 0.97 and 0.56) and H-16 (δ 1.37–1.52) and NOESY correlations between H-11 and H-18 and H-19 and H-20 with H-18 indicated the epimerization in C-17 of the lactone ring. The assignments were also made on the basis of the information obtained from COSY, HSQC and HMBC. The structure of **3** was identified as 6β,7β,15β,16β-dimethylene-11α-hydroxy-3-oxo-l7β-pregn-4-en-21,17-carbolactone, epimer of **2** at C-17.

Compound **4** was isolated as a white solid (m.p.: 170–171 °C). The ^{1}H and ^{13}C NMR spectra of **4** were similar to those of **2** and **3**, showing a multiplet signal at δ 3.73 assigned to H-11 and at δ 67.7 assigned to C-11, indicating hydroxylation at C-11 of DRSP. Correlation signals in the NOESY spectrum between H-11 and H-1 and H-12 and the lack of NOE signals between H-11 and protons from methyl-18 and methyl-19 indicated that, in this case, the hydroxyl group is β configuration. The structure of **4** was identified as 6β,7β,15β,16β-dimethylene-2β-hydroxy-3-oxo-l7α-pregn-4-en-21,17-carbolactone, epimer of **2** at C-11.

Compound **5** was obtained as a white solid of melting point 185–187 °C. While comparing the ^{1}H NMR spectrum of **5** to DRSP (**1**), we may notice the presence of a new hydroxyl-bearing methine proton at δ 4.38 (dd, *J* = 4.5, 10.5 Hz), with corresponding carbon at δ 68.5 in the HSQC spectrum (Supplementary data). The signal resonating at δ 68.5 was assigned to C-2 on the basis of long-range correlation between H-9 (δ 1.13), H-1α (δ 2.40) and H-1β (δ 2.64) in the HMBC spectrum. A down-field shift of the signal of methyl-19 protons relative to DRSP from δ 1.10 to 1.35 was also

Table 3
^{1}H NMR data of compounds **1–5**.

H N°	1	2	3	4	5
H-1α	2.10 m	2.11 m	2.10 m	2.08 m	2.40 m
H-1β	2.43 m	2.28 ddd (2.3, 4.9, 13.5)	2.35 m	2.32 m	2.64 m
H-2α	2.37 m	2.41 m	2.43 m	2.41 m	4.38 dd (3.2, 6.6)
H-2β	2.55 m	2.60 m	2.58 m	2.59 m	–
H-3	–	–	–	–	–
H-4	6.05 s	6.05 s	6.05 s	6.05 s	5.96 s
H-5	–	–	–	–	–
H-6α	1.50 m	1.51 m	1.57 m	1.55 m	1.59 m
H-6aα	0.88 dd (5, 10)	0.81 dd (5, 10)	0.79 dd (5, 10)	0.79 dd (5, 10)	1.06 dd (5, 10)
H-6aβ	1.23 m	1.25 m	1.24 m	1.24 m	1.20 m
H-7α	1.60 m	1.66 m	1.68 m	1.68 m	1.64 m
H-8	1.79 m	1.78 m	1.74 m	1.74 m	2.26 m
H-9	1.13 dd (4.5, 11)	1.27 m	1.26 m	1.25 m	1.13 dd (3.9, 12.5)
H-10	–	–	–	–	–
H-11α	1.65 m	–	–	3.82 td (5.7, 10.4)	1.82
H-11β	1.86 m	3.89 td (5.6, 9.8)	3.82 td (5.6, 10.3)	–	2.17
H-12α	1.46 m	1.44 m	1.66 m	1.67 m	1.68 m
H-12β	1.42 m	1.86 dd (5.5, 11.0)	1.73 m	1.75 m	1.68 m
H-13	–	–	–	–	–
H-14	1.95 dd (3.9, 12)	2.08 m	2.45 m	2.45 m	1.98 m
H-15α	1.60 m	1.61 m	1.67 m	1.67 m	1.64
H-15aα	0.54 dd (8, 14.5)	0.55 dd (8.0, 15.0)	0.56 dd (8.2, 15.2)	0.56 dd (8.2, 15.2)	0.57dd (8.2, 15.2)
H-15aβ	1.33 m	1.29 m	0.97 m	1.29 m	1.49 m
H-16α	1.37 m	1.41 m	1.52 m	1.51 m	1.36 m
H-17	–	–	–	–	–
H-18	1.00	0.98 s	0.84 s	0.84 s	1.25 s
H-19	1.10	1.21 s	1.20 s	1.20 s	1.35 s
H-20α	2.45 m	2.44 m	2.20 m	2.21 m	2.11 m
H-20β	2.12 m	2.15 m	2.29 m	2.15 m	2.37 m
H-21α	2.64 m	2.66 m	2.73 m	2.74 m	2.53 m
H-21β	2.57 m	2.57 m	2.59 m	2.29 m	2.65 m

Solvent: $CDCl_3$. Assignments are based on homonuclear COSY and NOESY experiments (Supplementary data).

Table 4
^{13}C NMR data of compounds **1–5**.

Carbon atom	Compounds				
	1	**2**	**3**	**4**	**5**
1	30.6	38.4	38.5	38.4	33.8
2	34.1	34.2	34.2	34.3	68.5
3	197.8	198.3	198.3	198.3	198.1
4	125.7	126.8	126.8	126.9	124.1
5	171.2	171.0	171.0	171.0	172.5
6a	19.7	20.1	20.5	20.5	19.6
6b	18.8	19.2	19.2	19.2	17.8
7	18.7	18.3	18.3	18.3	16.3
8	33.8	34.2	33.8	33.8	30.4
9	51.6	56.5	57.4	57.3	56.7
10	37.3	39.1	39.0	39.0	36.7
11	37.0	67.6	67.7	67.8	36.5
12	37.3	48.9	46.5	46.6	45.9
13	41.6	41.8	42.3	42.6	41.9
14	51.7	51.0	52.4	52.5	52.7
15a	16.6	16.8	17.3	17.3	16.9
15b	9.9	9.6	9.5	9.6	10.5
16	24.2	24.6	24.2	24.4	24.3
17	96.1	95.5	96.9	96.9	96.2
18	19.6	20.1	20.3	20.3	21.8
19	17.5	18.5	18.6	18.6	21.5
20	30.6	30.6	27.0	27.0	30.9
21	29.3	29.2	29.4	29.4	29.3
22	176.6	176.5	176.4	176.4	176.5

Solvent: $CDCl_3$. Assignments are based on heteronuclear HSQC and HMBC experiments (Supplementary data).

observed. Regarding hydroxyl configuration, NOE enhancement between H-2 (δ 4.38) and H-1β (δ 2.64) and H-1α (δ 2.40) indicated that the hydroxyl group at C-2 is in β configuration. On the basis of the information provided by NMR experiments, compound 5 was identified as 6β,7β,15β,16β-dimethylene-2β-hydroxy-3-oxo-l7α-pregn-4-en-21,17-carbolactone.

While compound **2** has been previously reported in literature [41], the other three biotransformation products of DRSP reported in this work (**3**–**5**) are new compounds.

4. Conclusions

The ability of various species of Mucorales to perform the biotransformation of the progestin drospirenone (**1**) was screened. As a result, the microbial transformation of DRSP with the fungal strains *A. corymbifera* BAFC 1072, *A. corymbifera* BAFC 1080, *A. coerulea, M. plumbeus* BAFC 2314, *R. oryzae* and *S. racemosum* was studied.

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 processes.

Looking at the results of this study, growing cells of *A. corymbifera* BAFC 1072, *A. coerulea* and *S. racemosum* afforded four hydroxylation products, three of them new compounds. The structures of all products were completely determined by spectroscopic methods. The compounds **2–4** have a hydroxyl group at C-11 and the compound **5** at C-2. The latter being rarely obtained by biotransformation. The microbial epimerization at C-17 is also remarkable. This is the first report on this reaction performed by Mucorales fungi.

Finally, remarkable regio- and stereoselectivity is demonstrated by *S. racemosum* in affording compound **2** in excellent yield and diasteromeric excess.

In summary, we are reporting here an efficient method for the transformation of the oral contraceptive steroid drospirenone using fungal cultures. This strategy can be used effectively for the synthesis of new products with potential oral contraceptive activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.08.005.

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