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Development of a high-yielding bioprocess for 11-α hydroxylation of canrenone under conditions of oxygen-enriched air supply

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ABSTRACT

A high yielding bioprocess for 11-α hydroxylation of canrenone (**1a**) using *Aspergillus ochraceus* ATCC 18500 was developed. The optimization of the biotransformation involved both fermentation (for achieving highly active mycelium of *A. ochraceus*) and biotransformation with the aim to obtain 11-α hydroxylation with high selectivity and yield. A medium based on sucrose as C-source resulted particularly suitable for conversion of canrenone into the corresponding 11-hydroxy derivative, whereas the use of O_2-enriched air and dimethyl sulfoxide (DMSO) as a co-solvent for increasing substrate solubility played a crucial role for obtaining high yields (>95%) of the desired product in high chemical purity starting from 30 mM (10.2 g/L) of substrate. The structure of the hydroxylated product was confirmed by a combination of two-dimensional NMR proton-proton correlation techniques.

1. Introduction

The regio- and stereoselective hydroxylation of steroids by fungal strains is a well-known method for the preparation of corticosteroids and progestogens having an oxygen substituent at C-11 position [1,2]. To overcome synthetic laborious steps, different species of eumycetes (i.e., *Rhizopus* and *Aspergillus*) are employed to perform the 11-hydroxylation of steroids because of the high regio- and stereoselective specificity of their oxygenating enzymes [3]. Although microbial oxidation is a good method for the simplification of the steroid drugs production, large-scale biotransformations are sometimes limited by low yields because of the poor solubility of substrates in the reaction medium and formation of different oxygenated by-products, which sometimes are very-difficult to separate [4,5]. Another problem is that many fungal species are very specific in their nutritional needs, so the use of cultural growing media is drastically limited. Microbial 11-hydroxylation of steroids using *Aspergillus ochraceus* is a well-known process and applicable on different substrates; the stereoselectivity of the hydroxylation mostly depends on the substrate employed. α-Hydroxylation is preferred with progesterone [6–10], 2-oxatestosterone [11], 13β-ethyl-gona-4-en-3,17-dione [12], canrenone [13], estr-4-en-3,17-dione [13], 16α, 17-epoxyprogesterone [14], 5α-androstan-3-one [15], whereas β-hydroxylation was found with cortexolone [16]. Generally, the bioconversion yield and selectivity is affected by different parameters, such as substrate concentration, aeration, and agitation; the chosen strain can be also important for regio- and enantioselectivity [2], as observed for other steroid biotransformation as well [17]. High substrate concentrations are needed to achieve industrially relevant biotransformations, but often dramatically limit conversion and selectivity. In the case of 11α-hydroxylation of canrenone, good yields and selectivity were obtained only when pure substrate concentration was kept below 5–6 g/L (14.7–17.6 mM) [13,18]; higher substrate concentrations not only limited overall conversions, but also yielded undesired by-products due to hydroxylation of different positions [19].

In this study we have investigated an efficient, economical and industrially scalable method for the conversion of canrenone into the corresponding 11α-canrenone using *Aspergillus ochraceus* ATCC 18500 as the biocatalyst. This microorganism is able to grow on

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different inexpensive nutrient media and to be stored, after lyophilisation, without losing its oxygenating skills [6,7]. *Aspergillus ochraceus* ATCC 18500 was employed for the preparation of 11α-hydroxy-canrenone, a key intermediate for the preparation of eplerenone (an important potassium sparing diuretic useful in the heart failure treatment) [20].

2. Materials and methods

2.1. Materials

All the solvents and reagents were purchased from Sigma-Aldrich, whereas canrenone and reference products were kindly furnished by Industriale Chimica (Saronno, Varese, Italy).

2.2. Microorganism and cultivation

Aspergillus ochraceus ATCC 18500 was routinely maintained on M5YE agar (malt broth, yeast extract 0.5%, agar 1.5% pH 5.6).

Growth in shake flasks: Erlenmeyer flasks (1 L) containing 100 mL of liquid medium (malt extract 3%, sucrose 2%, yeast extract 0.3%, pH 5.6) were inoculated from slants prepared with M5YE-agar medium. Each flask was inoculated with one slant, suspending the culture with 5 mL of sterile water. The flasks were incubated on a rotatory shaker at 28 °C, 150 rpm, for 48 h. Culture media were prepared using different combinations of the following products:

C-sources: glucose (1–5%), sucrose (1–5%), lactose (1–5%), glycerol (1–5%), maltose (1–5%), soluble starch (1–5%).

C- and *N*-sources: malt extract (0–1%), yeast extract (0–1%), corn steep liquor (0–1%).

Growth in stirred tank reactor: inoculum was prepared using two 1 L baffled Erlenmeyer flasks containing 100 mL of liquid medium (malt extract 3%, sucrose 2%, yeast extract 0.3%, pH 5.6) pre-inoculated from slants prepared with M5YE-agar medium. Each flask was inoculated with one slant, suspending the culture with 5 mL of sterile water and incubated on a rotatory shaker at 28 °C, 150 rpm, for 48 h. A 20 L stirred-tank bioreactor Applikon Biobench 20 L (Applikon Biotechnology B.V.) equipped with an on-line data acquisition, control system, gas mixer, and pH and O_2 electrodes was employed as stirred tank reactor (STR). The STR was filled with 5 L of liquid medium (malt extract 3%, sucrose 2%, yeast extract 0.3%, pH 5.6). The bioreactor was inoculated with 200 mL of inoculum and incubated at 28 °C for 48 h with air inlet 300 vvm, stirring 300 rpm, pH maintained at 5.6. Dry weight was determined after filtration of the mycelium with a Buchner funnel and dried for 24 h at 110 °C. Results were an average of five replicates.

2.3. Biotransformations

After 48 h of growth, the substrate (neat or solubilized in solvents) was added to the whole culture to start the biotransformation. Pure air and different air/O_2 ratios (5/1, 3/1, and 1/1) were used during the biotransformation. The reaction was followed by HPLC. When the bioconversion was over, mycelium was filtered and washed with dichloromethane; the supernatant was extracted 3 times with dichloromethane and the organic extracts collected, dried over Na_2SO_4 and the solvent was removed; the crude product was purified by flash chromatography (*n*-hexane/EtOAc 1:1).

2.4. Analyticals

Molar conversions were evaluated by HPLC (Purospher Star RP18e 250 * 4.6 mm, 5 μm column (Merck, Darmstadt, Germany), mobile phase: CH_3CN/water 60:40, flow rate 0.5 mL/min, detection at 299 nm for **1a,** and at 240 nm for **2a**. The mobility of substrate and product was: **1a** = 16.3 min, **2a** = 7.9 min.

NMR spectroscopy. ^1H NMR and ^{13}C NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer using the residual signal of the deuterated solvent as internal standard. ^1H and ^{13}C chemical shifts (δ) are expressed in ppm, and coupling constants (J) in hertz (Hz).

Two-dimensional proton-proton NOESY experiments were performed using standard pulse sequences, present in the spectrometer library.

2a ^1H NMR (300 MHz,$CDCl_3$): δ = 1.05 (s, 3H), 1.25 (s, 3H), 1.29–1.59 (m, 4H), 1.62 (s, br, OH), 1.86–2.06 (m, 5H), 2.28–2.46 (m, 4H), 2.5–2.65 (m, 4H), 4.12 (sest, J = 4.8 Hz, 1H), 5.7 (s, 1H), 6.03 (dd, J = 9.7, 1.8 Hz, 1H), 6.03 (dd, J = 9.7, 2.2 Hz, 1H) ppm; ^{13}C NMR (300 MHz, $CDCl_3$): δ = 15.6, 17.2, 22.5, 29.1, 31.1, 34.2, 35.5, 35.5, 35.8, 36.3, 37.7, 43.8, 46.6, 55.7, 67.9, 94.8, 124.7, 128.8, 138.0, 162.9, 176.4, 199.9 ppm.

3. Results

3.1. Optimization of the growth conditions in shake flasks

Firstly, an optimization of the growth conditions of *A. ochraceus* in shake flasks was performed for obtaining high and selective activity towards canrenone **1a.** The substrate (15 mM, 5.1 g/L) was added after 48 h of growth. Yields of 11α-canrenone (**2a**) (expressed as g/L $g_{mycelium}$ of **2a** produced after 24 h) and chemical purity of **2a** produced (evaluated by HPLC) were used as response parameters. It is known that hydroxylation of different position of **1a** may decrease the selectivity of 11-α hydroxylation with *A. ochraceus* [19]. Simultaneous evaluation of different combinations and concentrations of C-sources and *N*-sources (glucose, sucrose, lactose, glycerol, maltose, hydrolysed starch, malt extract, yeast extract, corn steep) were evaluated using the Multisimplex experimental design, already used for optimizing fermentations and biotransformations [21,22]. The best hydroxylation conditions were found when *A. ochraceus* was grown for 48 h at 28 °C, 300 rpm in a medium composed with 2% sucrose, 3% malt extract, 0.3% yeast extract at pH 5.6, giving the highest molar conversion (68–70%, analytical yield) of **1a** into **2a** (chemical purity 99.5%), starting from 15 mM substrate concentration.

3.2. Optimization of 11-α hydroxylation of canrenone in stirred tank (bio)reactor

Once found the best conditions of growth in shake flask, the fermentation and biotransformation were carried out in a conventional stirred tank reactor (STR). *A. ochraceus* was grown in the STR employing the medium optimized in shake flasks (2% sucrose, 3% malt extract, 0.3% yeast extract at pH 5.6 rpm); the first experiment was carried out with an agitation of 400 rpm and aeration of 2.0 vvm. Biotransformation was started by addition of 15 mM of **1a** after 48 h of growth, furnishing a molar conversion of 82% into **2a** (chemical purity 99.5%). The growth and biotransformation in STR allowed for a remarkable increase of the yield of **2a** with respect to what obtained in shake flasks (maximum yield 70% from 15 mM substrate concentration). This first positive result in STR led us to study different conditions of aeration and agitation during the process of growth and biotransformation. O_2 is poorly soluble in water (7.5 mg/L at 1 atm and 30 °C [23]) and, therefore, transfer of O_2 from the gas to the liquid phase is often the limiting step in aerobic bioprocesses, including enzymatic hydroxylations; different conditions of aeration and agitation may have a strong impact on dissolved oxygen. Neat substrate (15 mM) was added after 48 h and pH maintained at 5.6 (Table 1).

Table 1
Effect of aeration and agitation on 11-α hydroxylation of canrenone (**1a**) with *Aspergillus ochraceus* ATCC 18500 in stirred tank reactor. Biotransformation was started by adding 15 mM of neat substrate. *DOT = Dissolved oxygen tension.

Entry	Agitation (rpm)	Aeration (vvm)	Molar conversion (%)
1	200	1.0	75
2	200	1.5	78
3	200	2.0	78
4	400	1.0	76
5	400	1.5	80
6	400	2.0	82
7	600	1.0	82
8	600	1.5	84
9	600	2.0	84

Table 2
Effect of substrate concentration on 11-α hydroxylation of canrenone (**1a**) dissolved in hot DMSO (4% v/v).

Entry	Substrate (mM)	Molar conversion (%)	Chemical purity of **2a** (%)	Time (h)
1	7.5	>97	>99.5	36
2	15.0	95	>99.5	48
3	22.5	85	>99.5	48
4	30.0	72	99.4	72
5	37.5	30	98.7	72

Table 3
Effect of substrate concentration on 11-α hydroxylation of canrenone (**1a**) dissolved in hot DMSO (4% v/v).

Entry	O_2 fraction (%)	Substrate (mM)	Molar conversion (%)	Chemical purity of **2a** (%)
1	15	15.0	>97	>99.5
2	15	30.0	93	>99.5
3	15	37.5	76	99.4
4	25	15.0	>97	>99.5
5	25	30.0	>97	>99.5
6	25	37.5	84	99.3
7	35	15.0	>97	>99.5
8	35	30.0	>97	>99.5
9	35	37.5	85	99.1

The application of different levels of agitation and aeration had no dramatic effect on the monooxygenase activity of the mycelium of *A. ochraceus*; in fact, the dissolved oxygen tension (DOT) measured during these experiments resulted to be low and in a narrow range (3–5%), indicating that O_2 was largely consumed and only low concentrations of O_2 were available as substrate for the steroid oxygenation.

One of the main problem in steroids biotransformation is the low solubility of substrates in aqueous media. Different methodologies for improving their solubility were investigated such as the use of different amounts of co-solvents (acetone, ethanol, DMSO, DMF), the use of micronized powders, and sonicated suspensions (1/10 in deionized water, sonication: 3 cycle for 7 min).

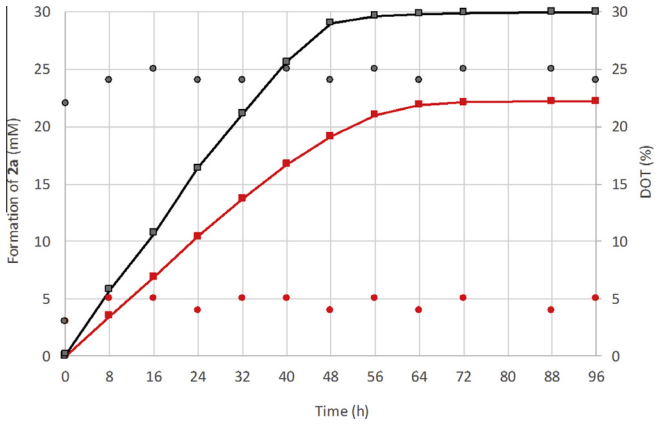

Fig. 2. 11-α Hydroxylation of canrenone (**1a**, 30 mM dissolved in hot DMSO) with *Aspergillus ochraceus* ATCC 18500 performed in STR: aeration with pure air (red) or 25% O_2-enriched air (black). Circles refer to dissolved oxygen tension (DOT) registered during the biotransformation.

The use of hot DMSO (4% v/v) gave the highest yield (95%) starting from 15 mM **1a** (under the same conditions, the addition of neat substrate allowed for 82% yield). These conditions were hence applied for the biotransformation of different concentrations of **1a** in STR (Table 2).

Yields and hydroxylation rates tended to decrease by increasing substrate concentrations (entries 2–5), and, for substrate concentrations above 22.5 mM (entries 4 and 5), the chemical purity of the product decreased as well.

Under the hypothesis that the diffusion of O_2 was limiting the overall yield of the biotransformation, we evaluated the effect of enriching the inlet air with pure O_2. The dissolved oxygen tension (DOT) can be increased in bioprocesses with filamentous fungi by applying different oxygen concentrations in the inlet gas [24]. We investigated the effect of different oxygen fraction in the inlet air, ranging from 15 to 35%, on the biotransformation of **1a** with *A. ochraceus* (Table 3). It was observed that an O_2 fraction of 25% allowed for accumulation of **2a** up to yield >97% starting from 30 mM of **1a**, with chemical purity of the recovered **2a** >99.5%. At higher substrate concentration, yields in the range of 76–85% were obtained with less chemical purity, no matter the concentration of O_2 applied. No significant increase in the biotransformation yield could be observed by increasing the O_2 fraction up to 35%. (See Fig. 1).

These experiments showed that the use of air enriched with O_2 was crucial for obtaining higher yields in the hydroxylation catalysed by mycelium-bound monooxygenase of *Aspergillus ochraceus* ATCC 18500 with the possibility to totally convert 30 mM **1a** into chemically pure **2a**, whereas only 72% yield was obtained from 30 mM **1a** in the absence of added O_2 (see Table 2). Fig. 2 shows the different profile of the biotransformation of **1a** (30 mM, 10.2 g/L) and the correspondent registered dissolved oxygen tension (DOT) using pure air and O_2-enriched air.

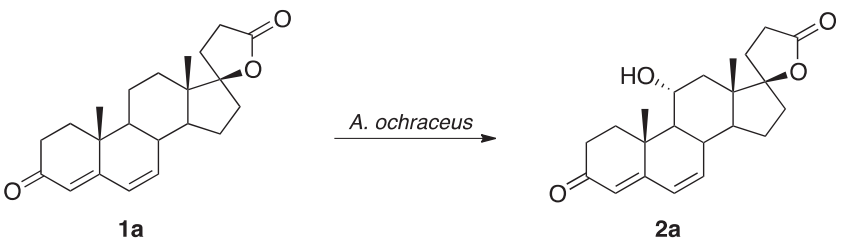

Fig. 1. 11-α Hydroxylation of canrenone (**1a**) with *Aspergillus ochraceus* ATCC 18500.

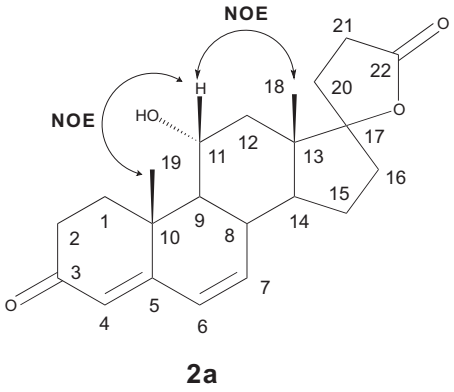

Fig. 3. Assignment of 11-hydroxyl group configuration using NOESY experiments.

3.3. Determination of the hydroxylation positions and relative configurations by NMR

The structure of the hydroxylation products of steroids can be determined by combination of NMR techniques [25]. In the case of the derivative **2a**, the observation of NOESY correlations from H-19 to H-11, and from H-11 to H-18, indicated that the H-11 and both the methyl groups are in *syn* configuration (Fig. 3).

4. Conclusions

The hydroxylation of canrenone (**1a**) by *Aspergillus ochraceus* ATCC 18500 has been optimized in terms of yields and selectivity. The optimization was carried out in a stirred tank (bio)reactor, showing that the most crucial parameters for obtaining high-yielding conversion of the substrates were the use of a suited co-solvent (hot DMSO 4% vol/vol) and in particular the employment of O_2-enriched air for providing high dissolved oxygen tension. Specifically, the use of an inlet air containing 25% of O_2 allowed for high yields of the desired 11α-derivative (starting from 30 mM in of **1a**) in high chemical purity (>99.5% by HPLC). The structure and relative configuration of the hydroxylated product were confirmed by a two-dimensional NMR proton-proton correlation techniques (NOESY).

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References

[1] H.L. Holland, Steroids 64 (1999) 178–186.
[2] P. Fernandes, A. Cruz, B. Angelova, H.M. Pinheiro, J.M.S. Cabral, Enzyme Microb. Technol. 32 (2003) 688–705.
[3] S. Petric, T. Hakki, R. Bernhardt, D. Zigon, B. Cresnar, J. Biotechnol. 150 (2010) 428–437.
[4] S.B. Mahato, S. Garai, Steroids 62 (1997) 332–345.
[5] H.N. Bhatti, R.A. Khera, Steroids 77 (2012) 1267–1290.
[6] C. Vezina, S.N. Sehgal, K. Singh, Appl. Environ. Microbiol. 11 (1963) 50–57.
[7] S.N. Sehgal, K. Singh, C. Vezina, Can. J. Microbiol. 14 (1968) 529–532.
[8] M. Shibahara, J.A. Moody, L.L. Smith, Biochim. Biophys. Acta 202 (1970) 172–179.
[9] J.Y. Houng, W.P. Chiang, K.C. Chen, C. Tiu, Enzyme Microb. Technol. 16 (1994) 485–491.
[10] K.C. Chen, W.S. Yin, C. Tiu, J.Y. Houng, Enzyme Microb. Technol. 16 (1994) 551–555.
[11] H.L. Holland, G. Lakshmaiah, J. Mol. Catal. B: Enzym. 6 (1999) 83–88.
[12] P. Grisenti, F. Pecora, E. Verza, M. Leoni, L. Bossi, U.S. Patent 7667056 B2, 2010.
[13] M. Wiersma, P. van der Meijden, WO Patent 9721830, 1997.
[14] S. Mao, B. Hua, N. Wang, X. Hu, Z. Ge, Y. Li, S. Liu, F. Lu, J. Chem. Technol. Biotechnol. 88 (2013) 287–292.
[15] A.M. Bell, J.W. Browne, W.A. Denny, E.R.H. Jones, A. Kasal, G.D. Meakins, J. Chem. Soc., Perkin Trans. 1 (1972) 2930–2936.
[16] F. Naghibi, P. Ataie, M. Mosaddegh, Int. J. Pharm. Pharm. Sci. 4 (2012) 402–403.
[17] D. Romano, V. Ferrario, D. Mora, R. Lenna, F. Molinari, Steroids 73 (2008) 112–115.
[18] X. Zhang, S. Rong, B.M. Ding, Chin. J. Pharm. 43 (2012) 17–20.
[19] S.F. Rong, X.L. Zhang, B.M. Ding, Y.F. Wang, X.H. Pan, J. Zhang, Y. Shen, Chin. Chem. Lett. 23 (2012) 313–316.
[20] A.J. Reyes, W.P. Leary, G. Crippa, M.F.C. Maranhao, R. Hernandez-Hernandez, Eur. J. Intern. Med. 16 (2005) 3–11.
[21] M.L. Contente, F. Molinari, I. Serra, A. Pinto, D. Romano, Eur. J. Org. Chem. 2016 (2016) 1260–1263.
[22] D. Romano, R. Gandolfi, S. Guglielmetti, F. Molinari, Food Chem. 124 (2011) 1096–1098.
[23] P.E. Liley, G.H. Thomson, D.G. Friend, T.E. Daubert, E. Buck, Physical and chemical data, in: R.H. Perry, D.W. Green (Eds.), Perry's Chemical Engineers' Handbook, McGraw-Hill, New York, 1999.
[24] Y.Q. Cui, R.G.J.M. van der Lans, K.C.A.M. Luyben, Biotechnol. Bioeng. 57 (1998) 409–419.
[25] A. Romano, D. Romano, E. Ragg, F. Costantino, R. Lenna, R. Gandolfi, F. Molinari, Steroids 71 (2006) 429–434.