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Enantioselective production of 3-hydroxy metabolites of tibolone by yeast reduction

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7α-Methyl-19-norpregn-5(10)-en-20-in-3β,17β-diol

ABSTRACT

The enantioselective reduction of tibolone into the corresponding 3α-hydroxy or 3β-hydroxy metabolite can be controlled by choosing suited strains of yeasts and biotransformation conditions. A restricted screening performed among 52 yeasts showed that the 3α-epimer was preferentially obtained with high epimeric purity with various strains (i.e. with *Kluyveromyces lactis* CBS 2359), while only *Saccharomyces cerevisiae* CBS 3093 gave the 3β-epimer as major product. The reduction of tibolone with *K. lactis* CBS 2359 and *S. cerevisiae* CBS 3093 was optimised. *S. cerevisiae* CBS 3093 furnished a 96:4 ratio of 3β/3α with complete molar conversion within 72 h when the initial concentration of substrate was below 2.5 g/L. *K. lactis* CBS 2359 gave a 99:1 ratio of 3α/3β with complete conversion in 64 h.

1. Introduction

Tibolone (**1**, 17α-hydroxy-7α-methyl-19-norpregn-5(10)-en-20-in-3-one) is a synthetic pro-hormone with estrogenic, progestagenic and androgenic actions [1]. Results of a randomised controlled trial of tibolone versus continuous combined estrogen-only HRT (Hormone Replacement Therapy) suggest both options are equally effective at relieving vasomotor symptoms of the menopause [2]. Moreover, all existing studies have shown a positive action of tibolone on bone mineral density [3]. Tibolone is quickly metabolised into its 3α-hydroxy metabolite (**2**, 7α-methyl-19-norpregn-5(10)-en-20-in-3α,17β-diol) and 3β-hydroxy metabolite (**3**, 7α-methyl-19-norpregn-5(10)-en-20-in-3β,17β-diol) by 3α/3β hydroxysteroid dehydrogenases (HSDH) (Fig. 1) in the intestine and the liver; the 3-OH-epimers can be interconverted, but only through their previous conversion into mono- or disulphated compounds [4]. Only 3β-OH-tibolone can be converted into the Δ4 isomer (**4**, Fig. 1) which shows a clear binding to the progesterone receptor, which is comparable to that of natural progesterone;

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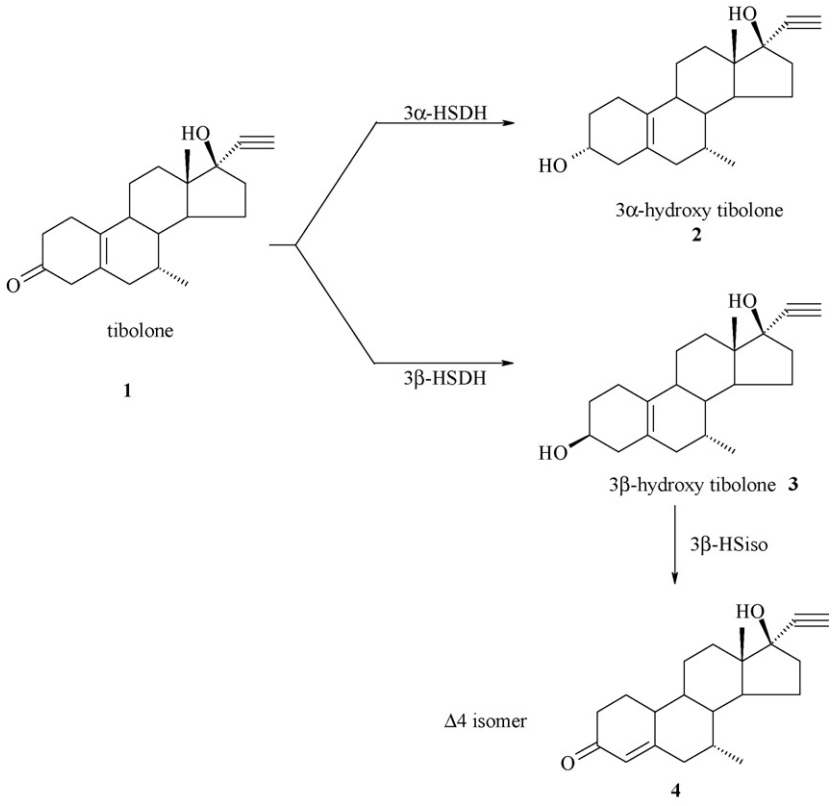

Fig. 1 – Metabolism of tibolone and its active metabolites.

the Δ4 isomer activates both the progesterone receptor and the androgen receptor but not the estrogen receptor [5].

The pharmacological importance of these metabolites has stimulated the search for synthetic methods able to provide both the 3-OH-epimers of tibolone. Reduction of tibolone with lithium tri-*tert*-butoxyaluminum hydride (LTTBA) predominantly furnishes the 3α-OH-tibolone in a ratio α/β of 96:4; inversion of the configuration can be achieved by a Mitsunobu reaction [6]. Stereoselective preparation of the 3-hydroxy metabolites of tibolone can be also accomplished by a chemoenzymatic approach where tibolone is reduced with diisobutylaluminum hydride (DIBAL) giving a 7/3 mixture of 3α/3β-OH-tibolone; *Candida antarctica* lipase B (CALB) catalysed only the acetylation of the 3α-OH functionality, therefore allowing for the recovery of 3β-OH-tibolone [7]. These methods are laborious and characterised by low overall yields, especially for the production of the 3β-OH-epimer. Microbial reduction of the 3-keto group of tibolone is a more straightforward method for obtaining both 3α- and 3β-OH-tibolone. In this paper, the enantioselective microbial reduction of tibolone has been developed using yeasts belonging to different genera.

2. Experimental

Solvents and reagents were purchased from Sigma–Aldrich. TLC was performed on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany). NMR spectra were recorded in $CDCl_3$ solutions with a Bruker AMX-600 (Bruker Spectrospin, Rheinstetten, Germany), operating at 600.1 MHz frequency for the proton nucleus. Chemical shifts were measured in δ (ppm), using the acetone residual signal as a reference and setting the methyl proton resonance at 2.1. The $[\alpha]_D$ were measured in methanol (*ca.* 1.0) using a Perkin-Elmer Polarimeter (Model 343 Plus). Melting points were measured by differential scanning calorimetry analysis (Perkin-Elmer DSC 6).

2.1. *Microorganisms and microbial growth*

The microorganisms were from CBS (Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands). They were cultured in 750 mL Erlenmeyer flasks with 100 mL of malt broth pH 6.0 for 48 h, at 27 °C on a rotary shaker set at 200 rpm. Cells from 48 h submerged cultures were centrifuged and washed with 0.1 M phosphate buffer, pH 7.0. *Saccharomyces cerevisiae* CBS 3093 and *Kluyveromyces lactis* CBS 2359 were employed in optimization studies accomplished using cells from a 8 L fermenter containing 4.0 L of liquid medium for 48 h, pH 6.0, at 27 °C and agitation speed 100 rpm. The dry weights were determined after centrifugation of 100 mL of cultures, cells were washed with distilled water and dried at 110 °C for 24 h.

2.2. *Biotransformations*

The substrate was kindly furnished by Industriale Chimica (Saronno, Italy). Washed cells were suspended in aqueous buffers (200 mL) to reach the same biomass concentration

obtained at the end of the growth. Reactions were started by addition of the substrate (either as micronized powder or dissolved in co-solvents) and/or co-substrates; the incubation was performed under reciprocal shaking (120 spm) and the reaction was followed by HPLC.

The biotransformation with *S. cerevisiae* CBS 3093 under optimised conditions was carried out following this procedure: tibolone (2 g) was added to 63 mL of ethanol (95%) and the solution heated at 65 °C until complete dissolution of tibolone; the solution at 65 °C was added to 1 L of phosphate buffer 0.1 M at pH 6.0 and 28 °C, containing 20 g (dry weight) of yeasts. The biotransformation mixture was maintained under agitation (100 rpm).

When the reaction was over, the biotransformation medium was paper-filtered and the aqueous phase extracted with ethyl acetate. The organic extracts were dried over Na_2SO_4 and the solvent removed; the crude product was purified by flash chromatography (hexane/ethyl acetate, 7/3, vanillin stained). Products were assigned on the basis of their chemico-physical data which were in agreement with those reported in Ref. [6]: 7α-methyl-19-norpregn-5(10)-en-20-in-3α,17β-diol (**2**): $[\alpha]_D$ +68 (*ca.* 1, ethyl acetate); 1H NMR d 0.76 (d, J = 12 Hz, 3H); 0.84 (s, 3H); 2.56 (s, 1H); 3.80 (m, 1H). MS (EI): *m/z* 314 (M^+); 7α-methyl-19-norpregn-5(10)-en-20-in-3β,17β-diol (**2**): $[\alpha]_D$ +16 (*ca.* 1, ethyl acetate); 1H NMR d 0.76 (d, J = 12 Hz, 3H); 0.84 (s, 3H); 2.56 (s, 1H); 4.04 (m, 1H). MS (EI): *m/z* 314 (M^+)

2.3. *Analytical methods*

Samples (0.5 mL) were taken at intervals and extracted with an equal volume of ethyl acetate; substrate and product concentrations were determined by HPLC using a Hypersil ODS (250 mm × 4.6 mm) column (Supelco Inc., Bellefonte, PA, USA), UV detection at 210 nm with a Merck-Hitachi 655-22 detector; a mixture of acetonitrile/water (1/1) was used as eluent with a flow rate of 0.8 mL min^{-1}. The mobility of substrate and products was: **1** = 12.5 min; **2** = 9.0 min; **3** = 9.9 min, allowing for the independent measurement of 3α- and 3β-HSDH activity.

3. Results

A preliminary screening was performed among 52 yeasts belonging to different genera grown for 48 h on medium containing malt extract as carbon source. Neat substrate (1 g/L) and glucose (25 g/L) were added to whole cultures and the reaction followed by TLC and HPLC. Table 1 shows the strains able to reduce tibolone into its 3-hydroxy derivatives with molar conversions above 10% after 48 h.

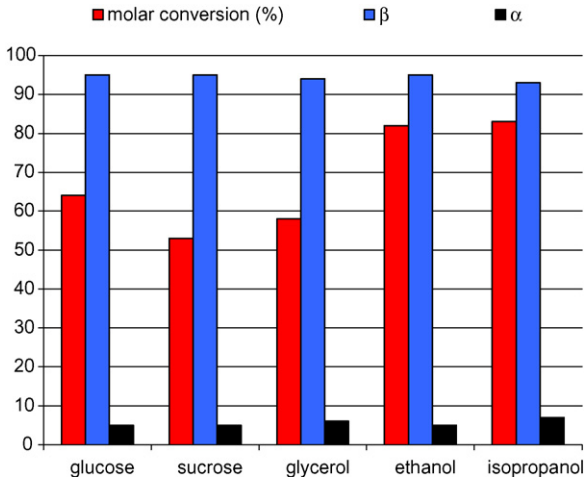

Fig. 2 – Influence of co-substrate on the reduction of tibolone with *S. cerevisiae* CBS 3093.

The biotransformation with *Kluyveromyces* and *Pichia* strains furnished 3α-OH-tibolone **2** with high purity, while only *S. cerevisiae* CBS 3093 gave predominantly 3β-OH-tibolone **3**, although with a slow reaction rate.

Since the synthetic difficulty in obtaining the epimer **3**, further experiments were aimed at the optimization of the biotransformation with *S. cerevisiae* CBS 3093. The optimization was performed by simultaneously evaluating parameters affecting both dehydrogenase expression during the growth (carbon source supplied, pH, aeration) and the biotransformation (temperature, type of buffer and cell concentration) using the Multisimplex experimental design [8]. A medium based on malt extract added with 0.5% of yeast extract and kept at pH 5.8 with an aeration rate of 1.2 vvm gave 9.3 g/L of dry *S. cerevisiae* CBS 3093 with the highest 3β-HSDH activity. These cells were employed under optimised conditions of biotransformation (28 °C, concentration of the cells = 20 g L^{-1} in phosphate buffer 0.1 M at pH 6.0) for testing the influence of different co-substrates (25 g/L) on the transformation. Fig. 2 shows the molar conversion and the epimeric ratio obtained after 24 h.

Ethanol and isopropanol proved to be the best co-substrate and it was assumed that they might contribute to the solubilization of the steroid substrate as well. Biotransformations

Table 1 – Reduction of tibolone with different yeasts

	3α–OH (**2**)/3β-OH (**3**)	Molar conversion (%)	Time (h)
Kluyveromyces lactis CBS 2359	>99/1	62	48
Kluyveromyces marxianus CBS 397	>99/1	33	48
Kluyveromyces marxianus CBS 607	98/2	26	48
Kluyveromyces marxianus CBS 1553	98/2	12	48
Kluyveromyces marxianus CBS 2231	99/1	35	120
Pichia anomala CBS 110	89/11	26	48
Pichia pastoris CBS 2612	95/5	64	168
Saccharomyces cerevisiae CBS 3093	12/88	34	168

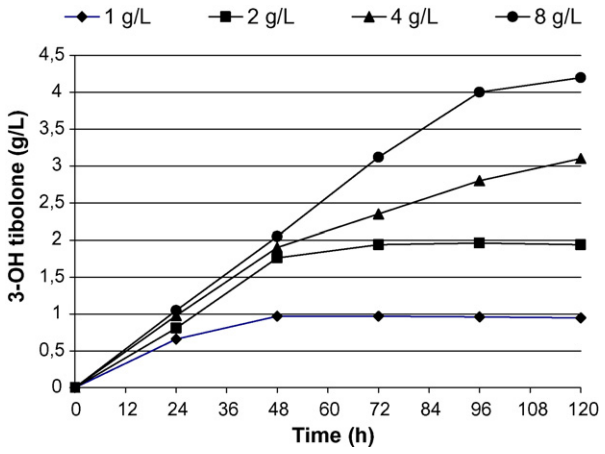

Fig. 3 – Influence of initial substrate concentration on the reduction of tibolone with *S. cerevisiae* CBS 3093.

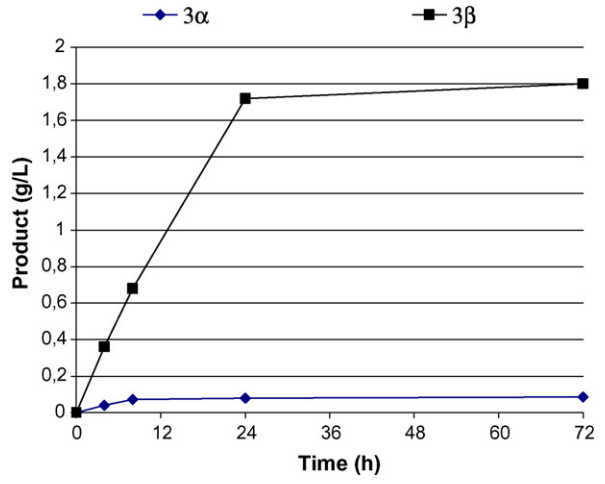

Fig. 4 – Time-course of the formation of 3α-hydroxy and 3β-hydroxy tibolone during the reduction of tibolone with *S. cerevisiae* CBS 3093.

were, therefore, carried out by adding the substrate as an ethanol solution at different temperatures and ethanol concentrations. The addition of the substrate in ethanol (5% v/v final concentration of ethanol in the biotransformation) at 65 °C strongly improved rate and molar conversion (96% after 24 h); an experiment where the whole cells were treated with ethanol at 65 °C before biotransformation showed that this pre-treatment did not affect the biotransformation.

Fig. 3 shows the results of the biotransformation performed with different amounts of substrate dissolved in hot ethanol (65 °C) and then added to the suspension of cells.

Almost complete molar conversion was achieved with initial substrate concentrations below 2 g/L, while marked inhibition was encountered above 8 g/L. The time-course of the epimeric ratio of the reaction performed with 2 g/L of substrate under optimised conditions is reported in Fig. 4.

The epimeric ratio β/α changed along the transformation, since the α-epimer was formed only during the first hours; the final β/α ratio was 96/4.

K. lactis CBS 2359 was used for the optimization of the biotransformation aimed at the obtainment of 3α-OH-tibolone **2**. The same strategy for the optimization of the biotransformation with *S. cerevisiae* was followed. The best results were obtained by adding micronized substrate to a yeast suspension in the presence of glucose (50 g L^{-1}) starting from a substrate concentration of 1.5 g L^{-1}. The employment of these conditions yielded 95% molar conversion within 60 h, furnishing an epimeric ratio α/β of 99/1.

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