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Steroid hydroxylations with *Botryodiplodia malorum* and *Colletotrichum lini*

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ABSTRACT

An improved procedure for the microbial hydroxylations of dehydroepiandrosterone (DHEA, **1**) and 15β,16β-methylene-dehydroepiandrosterone (**2**) was studied using whole cells of *Botryodiplodia malorum* and *Colletotrichum lini*. *C. lini* catalyzed 7α- and 15α-hydroxylation of **1** and 7α-hydroxylation of **2**, while *B. malorum* gave 7β-hydroxylation of both the substrates. The stability of the enzymatic activity was higher in the presence of co-substrates (i.e., glucose or mannitol) allowing for repeated batches of the biotransformations. The yields of 7α,15α-dihydroxy-**1** production were improved obtaining 5.8 g l^{-1} (recovered product) from 7.0 g l^{-1} of substrate. The structures of the hydroxylated products were assigned by a combination of two-dimensional NMR proton–proton and proton–carbon correlation techniques.

1. Introduction

Microbial modification of steroids is a long-established method for the preparation of a number of hydroxysteroids [1–3], but large scale transformations are sometimes limited by low yields due to low solubility of the substrates and by inhibition effects [4]. Microbial hydroxylations are among the most studied and useful transformations since they can be achieved under mild conditions with high chemo-, regio- and stereoselectivity [5]. Hydroxylation at positions 7 and 15 with controlled stereoselectivity allows for the production of important pharmaceutical intermediates [2]. Hydroxylation at positions 7 and 15 of dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one, DHEA, **1**) has been reported by using *Colletotrichum lini* allowing for the preparation of 3β,7α,15α-trihydroxy-5-androsten-17-one, while a process for preparing 3β,7β-dihydroxy-Δ^5-steroids is based on the hydroxylation of the position 7 of 15β,6β-methylene-3-β-hydroxy-5-androsten-17-one (**2**) catalyzed by *Botryodiplodia malorum* [7]. The products of these biotransformations (3β,7α,15α-trihydroxy-5-androsten-17-one and 3β,7β-dihydroxy-Δ^5-steroids) are key-intermediates for

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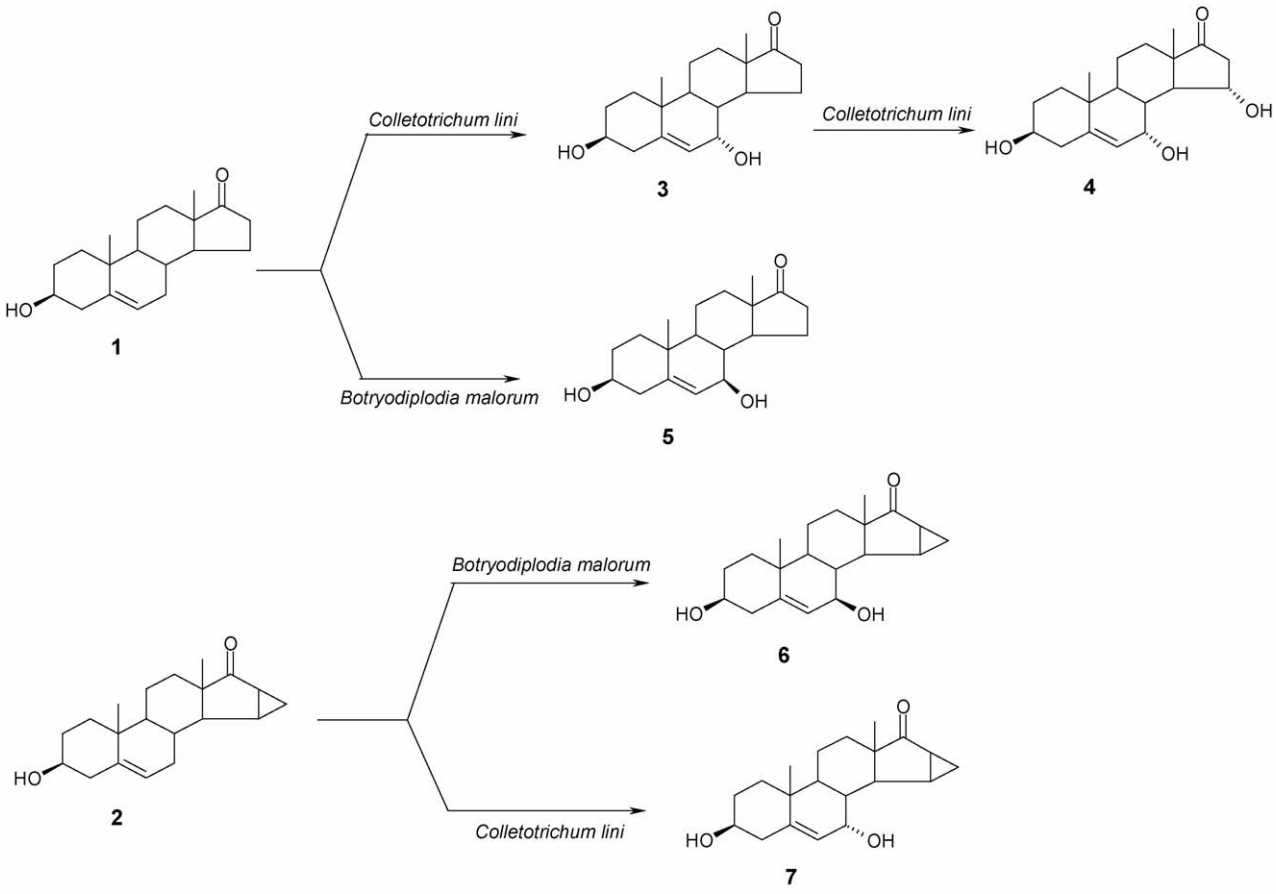

Fig. 1 – Hydroxylations of dehydroepiandrosterone (DHEA, 1) and 15β,16β-methylen-dehydroepiandrosterone (2) with *Botryodiplodia malorum* and *Colletotrichum lini*.

the synthesis of pharmacologically active steroids, such as 6β,7β,15β,16β-dimethylene-3-oxo-17α-pregn-4-ene-21,17-carbolactone, an aldosterone antagonist [6,7].

The importance of these molecules led us to study methods for improving the performances of the microbial transformation, such as manipulation of the reaction medium or immobilization of the mycelium. We have also studied the possibility of expanding the applicability of these biotransformations for obtaining both the 7-OH epimers of **1** and **2**. The complete pattern of the modifications studied in this work is reported in Fig. 1.

2. Experimental

2.1. Microorganisms and microbial growth

B. malorum CBS 134.50 and *C. lini* CBS 112.21 were from Centraalbureau voor Schimmelcultures (Baarn, Holland) and were routinely maintained on PDA agar (potato infusion 200 g l^{-1}, glucose 20 g l^{-1}, agar 15 g l^{-1}, pH 5.6). *B. malorum* was cultured in 2.0 l Erlenemeyer flasks containing 200 ml of liquid medium (soy meal 10 g l^{-1}, glucose 10 g l^{-1}, pH 6.2) at 25 °C for 72 h under reciprocal shaking (120 strokes per minute, spm). The dry weights were determined after centrifugation of 100 ml of cultures. Mycelia were washed with distilled water and dried at 110 °C for 24 h. Dry weight biomass was in the range of 1.5–1.6 g l^{-1}. *C. lini* was grown using two cultivation steps; a vegetative phase using a liquid medium with glucose (30 g l^{-1}) corn steep liquor (10 g l^{-1}) and tap water at pH 7.0, 25 °C, reciprocal shaking (120 spm). The next phase was a fermentative phase where a richer liquid medium was employed (glucose 30 g l^{-1}, corn steep liquor 10 g l^{-1}, soy meal 10 g l^{-1}, $NaNO_3$ 2 g l^{-1}, KH_2PO_4 1 g l^{-1}, K_2HPO_4 2 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1}, KCl 0.5 g l^{-1}, $FeSO_4 \cdot 7H_2O$ 20 mg l^{-1}, pH 7.0). The liquid cultures were carried out in 2.0 l Erlenmeyer flasks containing 200 ml of medium at 25 °C for 72 h under reciprocal shaking (120 spm). Dry weight biomass was in the range of 8.9–9.1 g l^{-1}.

2.2. Biotransformations

Substrates were kindly furnished by Industriale Chimica (Saronno, Italy). Biotransformations with submerged cultures were carried out in 2.0 l Erlenmeyer flasks containing 200 ml of the biotransformation suspension. Biotransformations with resting mycelia were carried out by harvesting the mycelium from submerged cultures by filtration on paper filters and by suspending the mycelium in aqueous buffers (200 ml). Reactions were started by addition of the substrates, followed by addition of co-solvents and/or co-substrates; the incubation was performed under reciprocal shaking (120 spm) and the reaction was followed by HPLC. The biotransformation

medium was then filtered and extracted with methyl isobutyl ketone. The organic extracts were dried over Na_2SO_4 and the solvent was removed; the crude product was purified by flash chromatography (hexane/acetone, 1/1, vanillin stained).

2.3. Immobilization

The mycelia obtained by filtration of the culture broths (200 ml) were suspended in 60 ml of sodium alginate (Sigma Chemicals) (3.0%, w/w) dissolved in distilled water. The resulting suspension was degassed and extruded through a thin injection needle into 50 ml of an iced calcium chloride solution (0.1 M). The bead size had an average diameter in the range of 2.5–2.8 mm. After hardening for 3 h at 4 °C, the beads were filtered under vacuum and used for the biotransformation or stored at 4 °C in fresh calcium chloride (0.01 M) solution. The amount of mycelium in the beads was estimated by dissolving 10 g of beads in 2% sodium poliphosphate solution; the suspension was then filtered through a pre-dried weighted filter paper (Whatman No. 1) and dried at 100 °C to a constant weight.

2.4. Repeated use of mycelium

Mycelium was reused in successive biotransformation cycles. At the end of 24 h, the mycelium was paper filtered and resuspended in fresh reaction mixture, and substrate was added. The reaction was discontinued when the yield was less than 80% after 24 h.

2.5. Analytical methods

Samples (0.5 ml) were taken at intervals and extracted with an equal volume of methyl isobutyl ketone; substrate and product concentrations were determined by HPLC using a Lichrospher 60 Select B column (Merck, Darmstadt, Germany), UV detection at 210 nm with a Merck-Hitachi 655-22 detector and acetonitrile as eluent with a flow rate of 1.0 ml min^{-1}. The mobility of substrates and products was: **1** = 4.1 min; **2** = 4.2 min; **3** and **5** = 5.7 min; **4** = 6.4 min; **6** and **7** = 5.6 min.

NMR spectroscopy. NMR spectra were performed at room temperature on a Bruker AMX-600 (Bruker Spectrospin, Rheinstetten, Germany), operating at 600.1 MHz frequency for the proton nucleus, using a 5 mm broad-band reverse probe equipped with gradients. Suitable amounts of sample (between 3 and 10 mg) were dissolved in acetone-d_6 (ISOTEC, USA) and transferred into 5 mm NMR tubes (type 535-PP, ALDRICH). Two-dimensional proton–proton (TOCSY and COSY-DQF) and gradient based carbon–proton chemical shift correlation experiments were performed using standard pulse sequences, present in the spectrometer library. One-bond (HMQC) and multiple-bond (HMBC) carbon–proton shift correlation experiments were performed assuming carbon–proton coupling constant values of 145 and 8 Hz, respectively. HMQC spectra were acquired with Broad Band-proton decoupling using a WALTZ-16 pulse sequence. Spectra were processed using XWINNMR software (Bruker) on a Silicon Graphics INDY workstation. Raw data were transformed to 2K × 2K real data points with a 90° shifted-sine bell squared weighting function and zero-filling. Baseline was corrected with a fifth degree polynomial. Chemical shifts were measured in δ (ppm), using the acetone residual signal as a reference and setting the methyl proton and carbon resonances at 2.1 and 29.8 ppm, respectively.

The $[\alpha]_D$ were measured in methanol (*c* 1.0) using a Perkin-Elmer Polarimeter (Model 343 Plus). Melting points were measured by differential scanning calorimetry analysis (Perkin-Elmer DSC 6).

3. Results

3.1. Biotransformations with C. lini

C. lini showed no extracellular activity in the hydroxylation of **1**; therefore, the optimization of the biotransformation was performed using mycelium suspended in aqueous media. The optimization was carried out by simultaneously evaluating different parameters of the biotransformation (temperature, pH, type of buffer, substrate and mycelium concentration) using the Multisimplex experimental design [8]. The profile of the hydroxylation of **1** under optimized conditions (30 °C, concentration of the mycelium = 10 g l^{-1}, concentration of the substrate = 1 g l^{-1} in phosphate buffer at pH 6.4) is reported in Fig. 2.

The reaction occurred stepwise with formation of the 7α-hydroxy derivative (**3**, mp = 216–218 °C, $[\alpha]_D = -56.86$) before formation of the known compound **4**.

One of the main drawbacks in steroid biotransformation is the low solubility of the substrates in water, which diminishes reaction rates and overall productivity. Different methods for improving the solubility of the substrate were investigated, such as the use of co-solvents (acetone, dimethylformamide, dimethylsulfoxide, ethanol and mixtures of these solvents), and addition of different cyclodextrins. The use of dimethylformamide (2%, v/v) gave the highest conversion rates and yields, while cyclodextrins did not significantly improve the performance of the bioconversion. Table 1 shows the results of the biotransformation in phosphate buffer (pH 6.0) at 30 °C performed with different amounts of substrate dissolved in dimethylformamide.

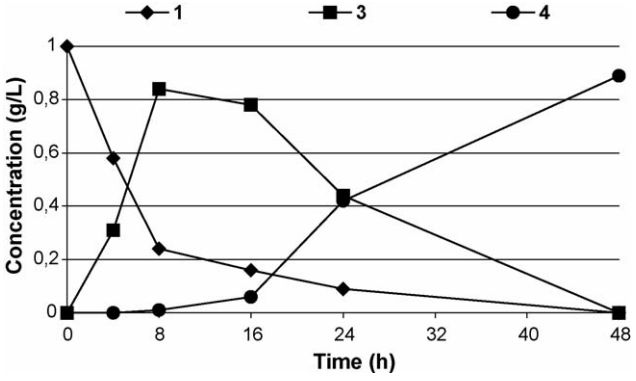

Fig. 2 – Hydroxylation of dehydroepiandrosterone (1, ●) into 3β,7α-dihydroxy-5-androsten-17-one (3, ■) and 3β,7α,15α-trihydroxy-5-androsten-17-one (4, ♦) with *Colletotrichum lini*.

Table 1 – Production of 3β,7α-dihydroxy-5-androsten-17-one (3) and 3β,7α,15α-trihydroxy-5-androsten-17-one (4) by biotransformation using different concentrations of dehydroepiandrosterone (DHEA, 1) with *Colletotrichum lini*

Time (h)	1 g l^{-1}		2.5 g l^{-1}		5 g l^{-1}	
	3	4	3	4	3	4
4	0.55	0	0.52	0	0	0
24	0	0.98	1.05	0.97	0.50	0
48	0	1.02	0	1.94	1.50	0
72	0	0.85	0	1.80	2.75	0

Starting from 2.0 g l^{-1} of substrate, complete conversion of **1** into **4** was obtained with little degradation of the product at prolonged times. The reaction was very slow with a substrate concentration above 3 g l^{-1}, and predominantly compound **3** was formed. When 5 g l^{-1} of substrate were employed, **3** was the only product observed even at prolonged times. The re-usability of the mycelium was tested, but the stability was quite poor since after the third addition, the activity was very slow. Two strategies were attempted for improving the operational stability of the enzymes involved: immobilization of the mycelium in Ca-alginate and addition of co-substrates (10 g l^{-1}) for maintaining the enzymes in a metabolically active state (Fig. 3).

The immobilization resulted in a lower production of the desired molecule and did not significantly improve the stability of the enzymatic activity, while the use of co-substrates, such as glucose and mannitol, proved effective as stabilizing agents.

The mycelium was also used in repeated batch reactions: after 24 h of reaction, the mycelium was filtered, the product was recovered from the aqueous solution and the mycelium was resuspended in fresh reaction buffer; at the beginning of each reaction cycle, 1 g l^{-1} of **1** and 10 g l^{-1} of glucose were added. The substrate was added in a dimethylformamide solution but was also finely ground and dispersed in a Tween 80 solution (0.1%, v/v); results are reported in Fig. 4. Glucose concentration was kept around 10 g l^{-1} by means of continuous addition.

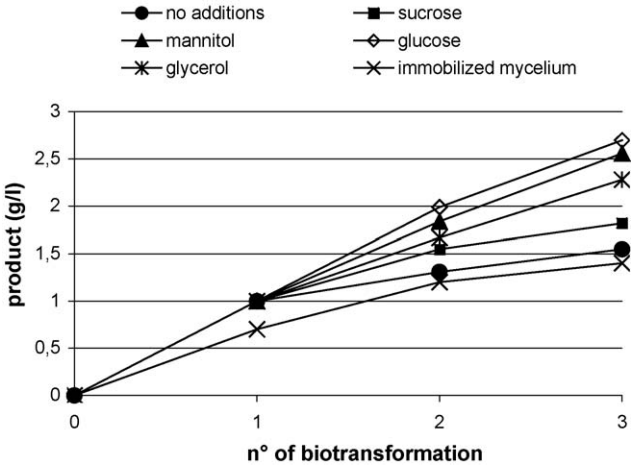

Fig. 3 – Re-usability of *Colletotrichum lini* as free and immobilized cells. Yields of 3β,7α,15α-trihydroxy-5-androsten-17-one (4) through three reaction cycles (72 h).

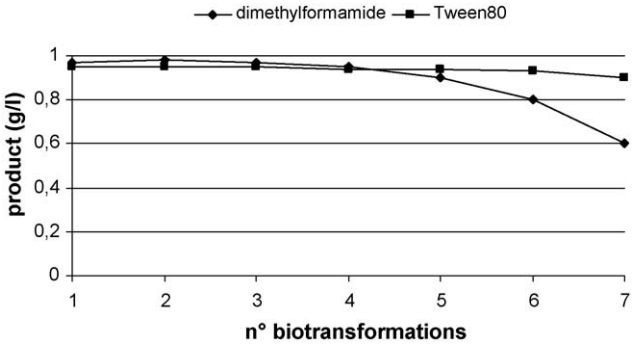

Fig. 4 – Repeated batches of hydroxylation of dehydroepiandrosterone (DHEA, 1) into 3β,7α-dihydroxy-5-androsten-17-one (3) with *Colletotrichum lini*.

Repeated-batch operation was maintained for seven cycles, before decrease of the enzymatic activity could be observed. The use of Tween 80 guaranteed higher operational stability of the catalyst. The overall product recovery was 5.8 g l^{-1} of **4** starting from 7 g l^{-1} of substrate.

Mycelium of *C. lini* was also used for the hydroxylation of **2** under the optimized conditions found for the biotransformation of **1**. The only product produced was identified as the 7α-derivative **7** (mp not determined because the compound decomposed before reaching melting point, $[\alpha]_D = -67.30$) with complete molar conversion within 72 h.

3.2. *Biotransformations with B. malorum*

The biotransformation of **1** was also studied with *B. malorum*. It was followed the same approach used for optimising the bioconversion with *C. lini* (influence of pre-treatment of the substrate, addition of co-solvents and co-substrates, effect of substrate concentration, immobilization of the mycelium). The best results were obtained by using free mycelium in the presence of dimethylsulfoxide (2%) starting from a substrate concentration of 1.0 g l^{-1}. The employment of these conditions yielded 70% molar conversion within 72 h, furnishing compound **5** (mp = 188–200 °C, $[\alpha]_D = -6.60$), as the only detectable product. No traces of hydroxylation in other position could be detected after 5 days.

The hydroxylation of **2** with *B. malorum* was carried out under the optimal conditions found for the biotransformation of **1**; the reaction gave the known 7β-hydroxy derivative **6** with complete conversion starting from 1.0 g l^{-1} of substrate in 72 h.

3.3. *Determination of the hydroxylation positions and relative configurations by ^1H and ^{13}C NMR*

The structures of the hydroxylation products of **1** and **2** were determined by a combination of two-dimensional NMR techniques. Fig. 5 shows the expansions of the COSY-DQF and

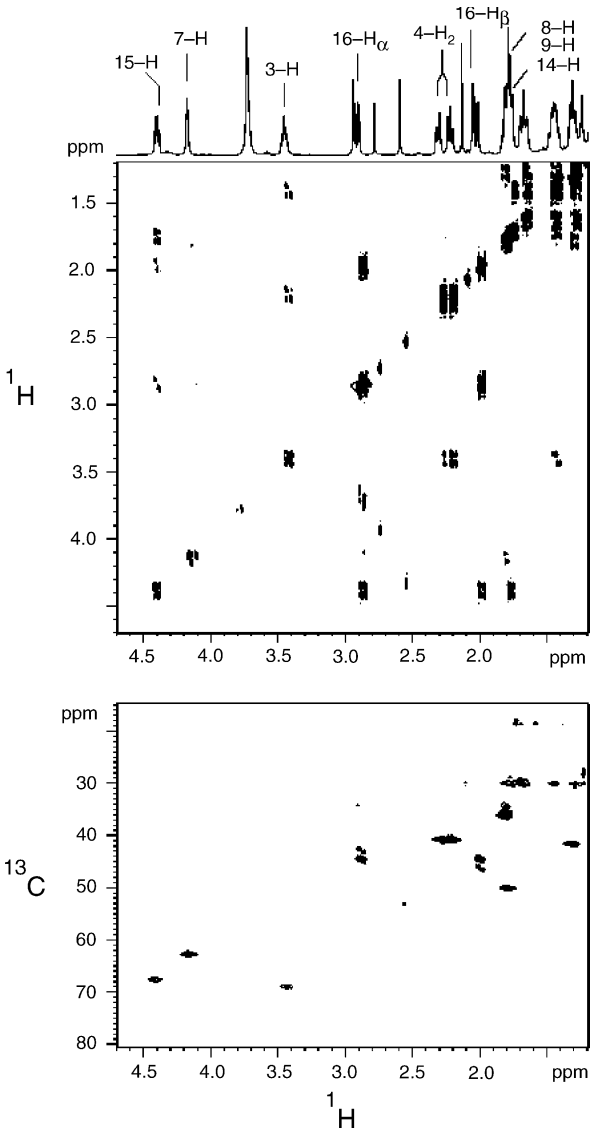

Fig. 5 – (A) Expansion of a COSY-DQF spectrum (600 MHz, performed on D_2O-exchanged) of 3β,7α,15α-trihydroxy-5-androsten-17-one (4) dissolved in acetone-d_6. The mono-dimensional spectrum is shown on top. (B) Expansion of a $^{13}C/^{1}H$ HMQC spectrum for the same compound.

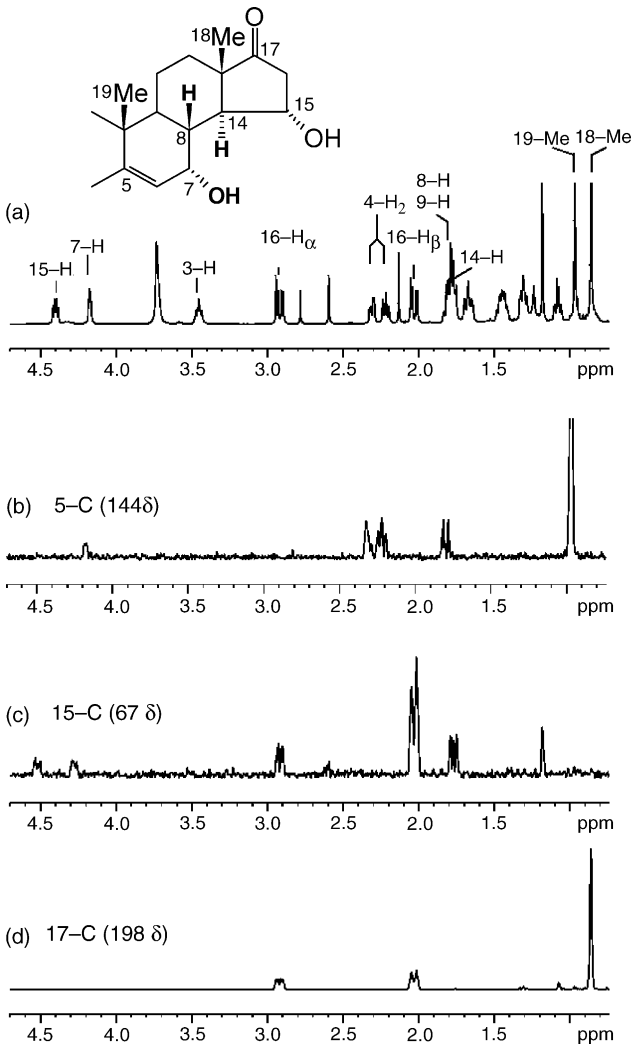

Fig. 6 – Selected traces of the $^{13}C/^{1}H$ HMBC spectrum (600 MHz) performed on a D_2O-exchanged sample of 3β,7α,15α-trihydroxy-5-androsten-17-one (4), dissolved in acetone-d_6. Trace a: 1D spectrum.

HMQC spectra for 7α,15α-dihydroxy **1**. For the same molecule, traces of the HMBC spectrum are reported in Fig. 6 for the most significant carbon resonances. The HMQC experiment clearly identifies, in the 60–70 ppm ^{13}C chemical shift region, all protonated carbons directly attached to the hydroxyl groups. The hydroxylated carbon at position 3 was already present in **1** and other related steroids, such as cholesterol; thus, its resonance was readily assigned by analogy with literature data [9]. An independent assignment was made by observing the long-range connectivity between the unique quaternary sp^2 carbon at position C-5 (144 ppm) and the methylene protons CH_2-4 (2.2–2.3 ppm, Fig. 6b), which, in turn, correlated by vicinal couplings with the proton CH-3 in the COSY-DQF experiment (Fig. 5). C-5 had another long-range interaction with proton CH-7, whose resonance was assigned at 4.2 ppm, and with CH_3-19 (0.95 ppm). The hydroxylation at position 7 was also proved by a TOCSY connectivity (not shown) between CH-7 and the olefinic CH-6 (5.5 ppm). The last proton at 4.4 ppm should, therefore, be assigned to CH-15. This position was confirmed by the connectivity observed in the COSY-DQF experiment between CH-15 and the methylene protons CH_2-16 (2.05 and 2.90 ppm, Fig. 5) and by the two-bond carbon–proton interactions involving the same methylene protons and C-15. The assignment of the geminal protons at 2.05 and 2.9 ppm to the CH_2-16 group was confirmed by the two-bond carbon–proton connectivity with C-17 (198 ppm, Fig. 6d). The same trace showed the correlation of C-17 with protons CH_3-18 (0.85 ppm), providing a cross-check for the assignments. The relative configurations of the hydroxylated carbons were determined by means of the proton–proton vicinal coupling constant values. The $^{3}J_{HH}$ value between protons CH-7 and CH-8 was measured less than 4 Hz (confirmed by the weak interaction displayed in the COSY-DQF spectrum), and such

a low value indicated that the relative orientation must have been *sin*. Since CH-8 was known to be β, CH-7 must also have been β and, consequently, OH-7 was α. In the same compound, OH-15 was found to be α because of the large vicinal coupling constant value with H-14 ($^3J_{14,15}$ = 9.7 Hz, $^3J_{15,16}\alpha$ = 7.9 Hz, $^3J_{14,15}\beta$ = 6.2 Hz). Such values were measured from the analysis of the H-15 multiplet in a resolution-enhanced 1D spectrum and the corresponding COSY-DQF cross-peak.

With the above described reasoning, the relative configurations of the hydroxylation sites in the isolated derivatives of **2** were similarly determined.

4. Conclusions

The hydroxylation of dehydroepiandrosterone **1** and 15β,16β-methylene-dehydroepiandrosterone **2** by microbial means has been optimized in terms of yields and selectivity. A suited combination of substrates and whole-cell biocatalysts allowed for the production of different hydroxylated compounds (**3**–**7**), as optically pure epimers. The structures and relative configurations of the hydroxylated products were assigned by a combination of two-dimensional NMR proton–proton and proton–carbon correlation techniques.

The production of 3β,7α,15α-trihydroxy-5-androsten-17-one was performed under repeated batch mode allowing for the recovery of 5.7–5.8 g l^{-1} of product with 75–80% molar conversion.

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