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Synthesis of Hydroxy-Androstane-1,4-Diene-3,17-Dione Derivatives by Biotransformations of Bile Acids with *Pseudomonas alcaliphila*

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Abstract

The synthesis of hydroxy-androstane-1,4-diene-3,17-dione derivatives (2a-d) by biotransformations with *Pseudomonas alcaliphila* of bile acids (1a-d) was reported. The scale-up of addition of deoxycholic acid (1a), cholic acid (1b), and chenodeoxycholic acid (1c) (1 g/L until 10 g/L) to the culture broth of *P. alcaliphila* switches the degradation process to the synthesis of 12β-hydroxy-androstane-1,4-diene-3,17-dione (2a, 95%), 7α,12β-dihydroxy-androstane-1,4-diene-3,17-dione (2b, 52%), and 7α-hydroxy-androstane-1,4-diene-3,17-dione (2c, 23%) Also hyodeoxycholic acid (1d) was transformed by *P. alcaliphila* to 6α-hydroxy-androstane-1,4-diene-3,17-dione (2d) with as much good yields (83%) but with lower concentration (1 g/L) in the culture broth.

Keywords: Hydroxy-androstane-1,4-diene-3,17-dione; *Pseudomonas alcaliphila*; Bile acids; Biotransformation

Introduction

Steroids constitute an important class of natural compounds that have various function in eukaryotic organisms but occur rarely in prokaryotic organisms. Nevertheless many bacteria are able to transform steroids and this ability is used for the production of steroids drugs [1-3]. On the other hand, although many bacteria are able to degrade completely naturally occurred steroids and use them as sources of carbon and energy, only one bacterial metabolic pathway for aerobic degradation, the 9,10-seco pathway, has been described in details [4-6] (Scheme 1). Biodegradation of bile acids: The 9,10-seco pathway of bile acids [4,6] starts with the oxidation of the A ring with the formation of 1,4-3-keto compounds. At the same time or subsequently, the acidic C_{17}-side chain of D ring is removed probably through two consecutive β-oxidation steps [7], leading to the androsta-1,4-diene-3,17-diones (ADDs) which are the central intermediates of the degradation pathway [6]. ADDs are further degraded by a monooxygenase-catalyzed hydroxylation at C_9, which causes the opening of the B ring concomitantly with the aromatization of the A ring [8,9]. The resulting 9,10-secosteroids are further degraded by the breakdown of the aromatic a ring [10,11]. The remaining C and D rings form acidic perhydroindane derivatives that are further degraded by as-yet-unknown reactions [12]. In this field the aerobic catabolism of bile acids by *Pseudomonas* spp. is well documented [13-21]. Various hydroxylated androsta-1,4-diene-3,17-dione from bile acids (i.e., cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA) and hyodeoxycholic acid (HDCA) were obtained in high yield by transposon mutants of *Pseudomonas putida* [22]. Recently the degradation of the acyl chain attached to C_{17} of cholic acid with *Pseudomonas* sp. strain Chol1 to afford 7α,12β-dihydroxy-androsta-1,4-diene-3,17-dione (12β-DHADD) has been studied [23]. In this paper the synthesis of hydroxylated androsta-1,4-diene-3,17-diones (2a-d) by the biotransformation of bile acids (1a-d) (23-95% yield) with *Pseudomonas alcaliphila* with high concentration of starting materials in the culture broth was reported.

Results and Discussion

Pseudomonas alcaliphila isolated from an environment [24] potentially adapted to the presence of bile acids, showed a high affinity to bile acids, in particular after 72 h of growth in ATT broth at 28°C the added sodium salt of deoxycholic cholic acid **1a** (1 g/L) was completely degraded in 24 h incubation. Another aliquot of **1a** (2 g/L) was added and the same result was obtained after 24 h incubation: no traces of **1a** and no products were detected. A third aliquot (10 g/L) was added and after 32 h incubation 12β-hydroxy-1,4-androstadiene-3,17-dione (12β-HADD, 2a) was obtained in 95% yield if calculated on the last addition (overall yield 73%) (Table 1) (Scheme 2). Synthesis of hydroxy-androstane-1,4-diene-3,17-dione derivatives (2a-d): A loopful of *Pseudomonas alcaliphila* was inoculated in ATT broth (100 mL). After 72 h at 28°C and 150 rpm (pH 8.5-9 of culture broth), the sodium salt of the selected bile acid (1) (0.1 g) was added (28°C, 150 rpm). After 24 h the bile acids (1a-c) (excluding hyodeoxycholic acid 1d) were completely degraded (TLC and HPLC). Another aliquot of the bile acid (1a-c) was added (0.2 g) that was also completely degraded in 32-76 h (see Table 1). So a third aliquot of 1a-c (1 g) was added. After the appropriate time (see Table 1), centrifugation (5.000 rpm, 15 min), extraction with ethyl acetate and chromatography, compounds 2a-d were obtained. The mechanism of the complete degradation of (1a) is described in Scheme 1. It was interesting that, although the complete degradation was repeated with the second addition of the substrate, the third process provided excellent yields of (2a) and no other products were detected by TLC and HPLC. Equally good results have been obtained so far only with mutant strains of *Pseudomonas putida* [22]. On the other hand, the adopted methodology (subsequent addition of (1a) after the complete degradation with a scale-up of concentration) showed to be necessary for obtaining 12β-HADD (2a), in fact the initial addition of (1a) in 10 g/L concentration inhibited the degradation process. In this case deoxycholic acid (1a) was recovered (80%) after 72 h incubation. It is possible to point out that the degradation pathway occurred easily with low concentration of (1a) while was inhibited with high concentration. On the other hand the activation of the

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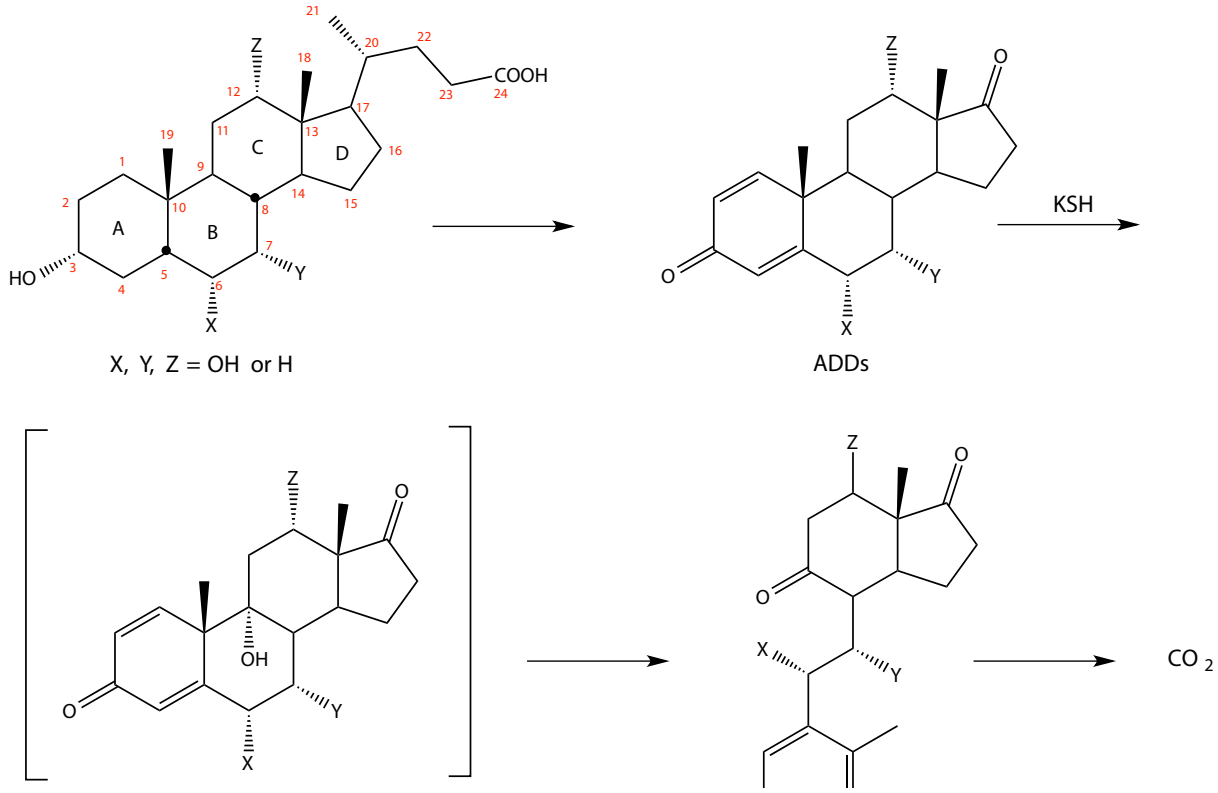
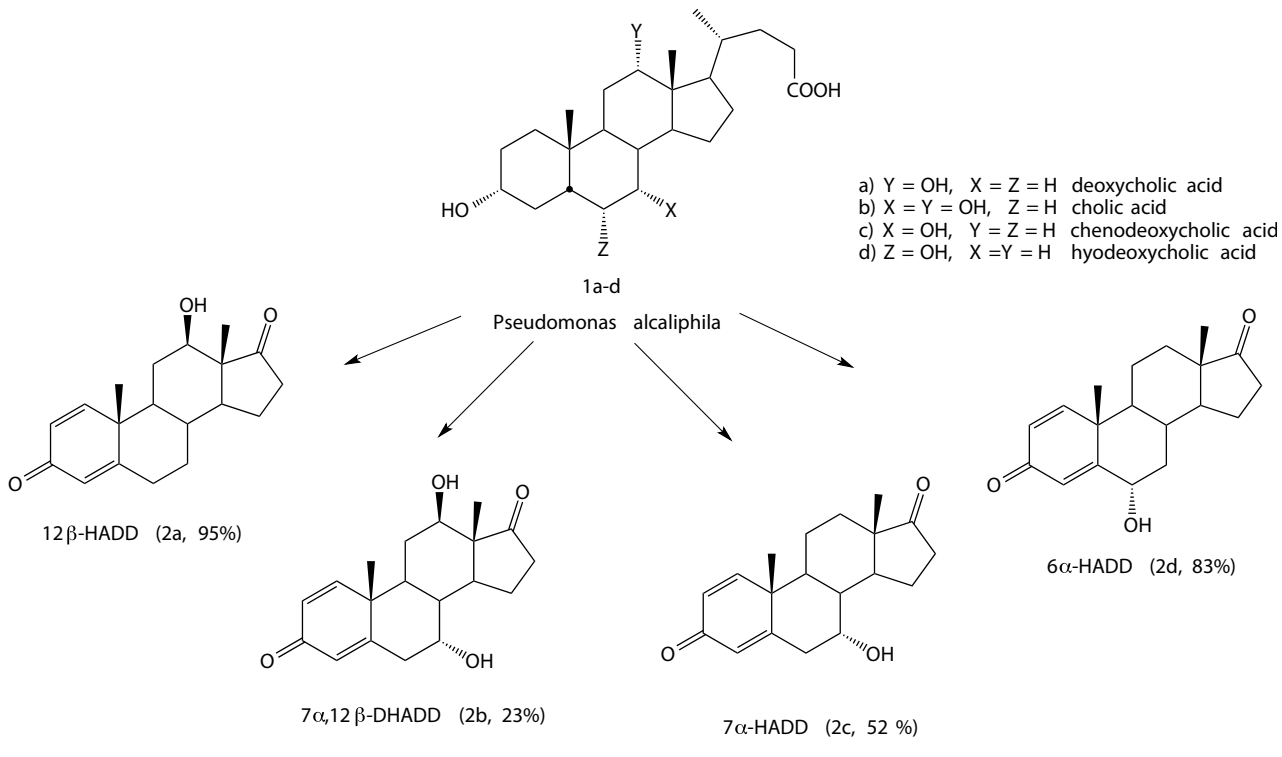


Scheme : 1



Scheme : 2

Bile acid	1st addition[a] h	2nd addition[b] h	3rd addition[c] h	HADD (Yield %)	HADD (Overall yield %)[f]
1a	24	24	32	**2a** (95)[d]	**2a** (73)
1b	24	48	72-96	**2b** (23)[d]	**2b**(18)
1c	24	24-32	72	**2c** (52)[d]	**2c** (40)
1d	8-9 day	--	--	**2d** (83)[e]	

[a]Time of biotransformation with 1 g/L of bile acids (1a-d) as sodium salt; [b]Time of biotransformation with 2 g/L of bile acids (1a-c) as sodium salt; [c]Time of biotransformation with 10 g/L of bile acids (1a-c) as sodium salt; [d]Calculated on 10 g/L addition; [e]Calculated on 1 g/L addition; [f]Calculated on 13 g/L addition.

Table 1: Biotransformation of bile acids **1a-d** with *Pseudomonas alcaliphila*.

metabolic pathway with low concentration was necessary to overcome the inhibition due to high concentration. Moreover, it has been reported that the presence of insufficient amounts of oxygen, which make possible the 9α-hydroxylation, lowered the yields of HADD [25]. In our case the rate of formation and then the high concentration of 12β-HADD in the culture broth (deoxycholic acid was completely biotransformed after 32 h) probably makes insufficient the oxygen for the following degradation. The same approach (three consecutive addition) has been used with cholic acid (1b) and 7α,12β - dihydroxy-1,4-androstadiene-3,17-dione (7α,12β -DHADD) (2b) was obtained in 23% yield (overall yield 18%). The cholic acid (1b) is biotransformed less rapidly by *P. alcaliphila* (48 h to degrade 2 g/L) and the lower rate of formation of HADD (72-96 h) allowed less concentration of (2b) in the culture broth. In this case the amount of oxygen was sufficient to degrade partially the compound (2b) and lowered its yields: this is in agreement with the previous hypothesis. The competition between the rate of degradation and HADD production was confirmed by the biotransformation of chenodeoxycholic acid (1c). The degradation of (1c) by *P. alcaliphila* (24-32 h for 2 g/L) is more rapid with respect to (1b) and the following addition (10 g/L concentration) afforded, after 72 h incubation, 7α-hydroxy-1,4-androstadiene-3,17-dione (2c) in 52% yield (overall yield 40%). In both biotransformations of (1b) and (1c) no other products were detected while traces of 1b and 1c were yet present. Completely different was the biotransformation of hyodeoxycholic acid (1d). The incubation of 1g/L of (1d) with *P. alcaliphila* afforded 6α-hydroxy-1,4-androstadiene-3,17-dione (6α-HADD, (2d) in 83% yield after 8-9 days. The high yield in 6α-HADD, in spite of long time incubation, was probably due to the 9α-hydroxylation inhibition due to 6α-OH group. Moreover *P. alcaliphila* was not able to biotransform ursodeoxycholic acid and litocholic acid to give the corresponding hydroxyl-1,4-androstadiene-3,17-dione derivatives.

Experimental

General

Sodium salt of bile acids **1a-d** and have been supplied by ICE industry [24]. Melting points are uncorrected and were determined on a 510 Buchi melting point instrument. ^{1}H and ^{13}C NMR spectra were obtained with a Mercury Plus 400 spectrometer operating at 400 MHz (^{1}H) and 100 MHz (^{13}C), with Me_4Si as internal standard. TLC were performed on pre-coated Silica Gel plates (thickness 0.25 mm, Merck) with cyclohexane/ethyl acetate/acetic acid 60:40:1 as eluent and Silica Gel (Fluka, Kiesegel 60, 70-230 mesh) was used for preparative column chromatography. HPLC analysis were obtained with a Jasco Borwin LC 1500 instrument: detector RI-1530; column Agilent Zorbax SB-C18; flux 0.7 ml/min; temp. 23°C; isocratic conditions MeOH/CH_3CN/H_2O 53/23/24.

Isolation of *Pseudomonas alcaliphila*

The bacterium was sampled in external cockpit drainage of ICE industry. The sterilized physiological solution was conveniently diluted (about 10^{-4}) and put on Petri dishes containing PCA (Difco) to isolate the bacterium. The identification of bacterium *P. alcaliphila* was performed by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen).

Synthesis of hydroxy-androstane-1,4-diene-3,17-dione derivatives (2a-d)

A loopful of *Pseudomonas alcaliphila* from a culture on Columbia agar (Sigma) was inoculated in ATT broth (100 mL) containing tryptone (5 g/L), peptone (10 g/L) meat extract (8 g/L), starch (1 g/L) and NaCl (5 g/L). After 72 h at 28°C and 150 rpm (pH 8.5-9 of culture broth), the sodium salt of the selected bile acid 1 (0.1 g) was added and the culture was maintained in the same conditions. After 24 h (excluding hyodeoxycholic acid 1d) all the bile acid 1a-c were degraded (no product by TLC) and another aliquot of bile acids 1a-c was added (0.2 g). After 32-76 h (see Table 1) in the same conditions also this aliquot of bile acid 1a-c was completely degraded. So a third aliquot of 1a-c (1 g) was added. The reaction was monitored by TLC and HPLC and after the appropriate time (see Table 1) the biomass was removed by centrifugation (5.000 rpm, 15 min). The solution was extracted with ethyl acetate (3 × 100 mL), the organic layer dried over anhydrous Na_2SO_4 and the solvent evaporated. For deoxycholic acid (1a), the crude mixture was chromatographed (silica gel, ethyl acetate/cyclohexane/acetic acid 70/30/2 as eluent) to give 12β-hydroxy-1,4-androstadiene-3,17-dione (12β-HADD, 2a) (0.69 g): 95% yield (overall 73% yield); m.p. 222-224°C [18]; δ_H ($CDCl_3$)

(selected data) 1.02 (3H, s, C_{18}-CH_3), 1.25 (3H, s, C_{19}-CH_3), 3.75 (1H, dd, *J*=10.5 and 6.3 Hz, $C_{12\alpha}$-H), 6.1 (1H, s, C_4-H), 6.25 (1H, dd, *J*=10 and 1Hz, C_1-H), 7.02 (1H, d, *J*=10 Hz, C_2-H) ppm; δ_C ($CDCl_3$) 221.71 (C_{17}), 186.17 (C_3), 167.71 (C_5), 154.93 (C_1), 127.83 (C_2), 124.37 (C_4), 71.95 (C_{12}), 51.60, 50.29, 47.90, 43.18, 35.55, 34.19, 32.46, 31.60, 29.53, 21.84, 18.67, 8.31 ppm. For cholic acid (1b), the crude mixture was chromatographed (silica gel, ethyl acetate/acetic acid 50/1 as eluent) to give 7α,12β -dihydroxy-1,4-androstadiene-3,17-dione (7α,12β -HADD, 2b) (0.17 g): 23% yield (18% overall yield); m. p. 245-246°C [15]; δ_H (CD_3OD) (selected data) 1.01 (3H, s, C_{18}-CH_3), 1.30 (3H, s, C_{19}-CH_3), 3.65 (1H, br m, $C_{12\alpha}$-H), 4.18 (1H, s, fine splitting, $C_{7\beta}$-H), 6.11 (1H, s, C_4-H), 6.23 (1H, dd, *J*=10 and 1 Hz, C_2-H), 7.27 (1H, d, *J*=10 Hz, C_1-H) ppm; δ_C($CDCl_3$) 8.36, 18.78, 21.85, 30.77, 31.59, 36.30, 39.38, 41.87, 44.02, 44.92, 52.69, 68.58 (C_7), 72.23 (C_{12}), 127.32 (C_4), 127.91 (C_2), 158.47 (C_1), 168.98 (C_5), 188.10 (C_3), 222.01 (C_{17}) ppm. For chenodeoxycholic acid (1c), the crude mixture was chromatographed (silica gel, ethyl acetate/acetic acid 50/3 as eluent) to give 7α-hydroxy-1,4-androstadiene-3,17-dione (7α-HADD, 2c) (0.38 g): 52% yield (40% overall yield); m.p. 289-291°C [18]; δ_H ($CDCl_3$) (selected data) 0.91 (3H, s, C_{18}-CH_3), 1.22 (3H, s, C_{19}-CH_3), 4.17 (1H, s, fine splitting, $C_{7\beta}$-H), 6.12 (1H, s, C_4-H), 6.20 (1H, dd, *J*=10 and 1 Hz, C_2-H), 7.05 (d, 1H, *J*=10 Hz, C_1-H) ppm; δ_C ($CDCl_3$) 13.67, 18.41, 21.49, 21.92, 30.95, 35.71, 39.52, 41.05, 43.52, 44.42, 45.54, 47.52, 68.30 (C_7), 127.35 (C_4), 127.74 (C_2), 155.65 (C_1), 164.72 (C_5), 185.82 (C_3), 220.26 (C_{17}) ppm. For hyodeoxycholic acid (1d), the crude mixture was chromatographed (silica gel, acetone/hexane 2/1 as eluent) to give 6α-hydroxy-1,4-androstadiene-3,17-dione (6α-HADD, 2d) (0.06 g): 83% yield; m.p. 240-241°C [22]; δ_H ($CDCl_3$) (selected data) 0.96 (3H, s, C_{18}-CH_3), 1.25 (3H, s, C_{19}-CH_3), 4.65 (1H, m, $C_{6\beta}$ -H), 6.27 (1H, dd, *J*=10 and 1 Hz, C_2-H), 6.48 (1H, s, C_4-H), 7.01 (1H, d, J=10 Hz, C_1-H) ppm; δ_C ($CDCl_3$) 219.63 (C_{17}), 185.7 (C_3), 168.89 (C_5), 154.88 (C_1), 127.96 (C_2), 119.99 (C_4), 68.20 (C_6), 52.19, 50.30, 47.78, 43.51, 41.31, 36.68, 33.74, 31.15, 22.09, 22.02, 19.26, 13.91 ppm.

Conclusions

Pseudomonas alcaliphila, isolated from an environment potentially adapted to the presence of bile acids, produces by biotransformation of some bile acids hydroxy-androstane-1,4-diene-3,17-dione derivatives with good yields and in high concentration in the culture broth (10 g/L). This strain is a wild type and there are various possibility to scale-up and improve the process both regarding the yields and the concentration in the culture broth.

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