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Steroids 68 (2003) 415–421
Steroids
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Microbial conversion of pregna-4,9(11)-diene-17α,21-diol-3,20-dione acetates by *Nocardioides simplex* VKM Ac-2033D

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Received 5 June 2002; received in revised form 5 November 2002; accepted 8 January 2003

Abstract

The conversion of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate (**I**) and 17,21-diacetate (**VI**) by *Nocardioides simplex* VKM Ac-2033D was studied. The major metabolites formed from **I** were identified as pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate (**II**) and pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**IV**). Pregna-4,9(11)-diene-17α,21-diol-3,20-dione (**III**) and pregna-1,4,9(11)-triene-17α,20β,21-triol-3-one (**V**) were formed in minorities. Biotransformation products formed from **VI** were pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17,21-diacetate (**VII**), pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate (**II**), pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**IV**), pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17-acetate (**VIII**), pregna-1,4,9(11)-triene-17α,20β,21-triol-3-one (**V**). The conversion pathways were proposed including 1(2)-dehydrogenation, deacetylation, 20β-reduction and non-enzymatic migration of acyl group from position 17 to 21. The conditions providing predominant accumulation of pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate (**II**) from **I** and pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17-acetate (**VIII**) from **VI** in a short-term biotransformation were determined.

Keywords: Steroid; 9(11)-Dehydro-3-ketosteroids; 1(2)-Dehydrogenation; Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione; Bioconversion; *Nocardioides simplex* VKM Ac-2033D

1. Introduction

Microbial 1(2)-dehydrogenation is an important step in the organic synthesis of many pharmaceutical steroids. It was well investigated since the fifties mainly as a basis for commercial production of prednisteroids using a pathway starting from androstenedione (AD) being exploited by pharmaceutical companies still. The active strains with 3-ketosteroid-1(2)-dehydrogenase activity were described among *Arthrobacter*, *Corynebacterium*, *Bacillus*, and *Nocardia* [1–3].

Other applications of the process are less investigated. One of them is in a pathway of glucocorticoid synthesis starting from 9α-hydroxyandrostenedione (9-OH-AD). This pathway is an effective method to produce fluorocorticoids (dexamethasone, betamethasone, triamcinolone, etc.) and other modern steroids in shorter synthesis. It implies 1(2)-dehydrogenation of acetylated 9(11)-dehydrosteroids as one of the stages [4–7].

Chemical 1(2)-dehydrogenation of 9(11)-dehydro-3-ketosteroids was accompanied by undesirable spontaneous ring A aromatization which led to ineffective glucocorticoid synthesis. The yields of 1,4,9(11)-triene steroids at the chemical syntheses ranged from 11 to 63% [8,9]. Therefore, microbial conversion is of special interest.

Data on microbial conversion of 9(11)-dehydrosteroids are scarce [4,10]. Along with 1(2)-dehydrogenation, enzymatic destruction of steroid nucleus by living cells was observed and a special heat-drying procedure was applied towards the living cells to prevent steroid degradation.

The publications on microbial 1(2)-dehydrogenation of acetylated steroids are also limited and do not allow to estimate the effect of acetyl substitution on the process. For instance, unlike hydrocortisone, its 21-acetate was not dehydrogenated microbially at C-1 [11], while cortexolone 21-acetate was transformed to prednisone 21-acetate and prednisone by *Mycobacterium globiforme* 193 [12]. A mixture of hydrocortisone 17-acetate (86%) and hydrocortisone

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doi:10.1016/S0039-128X(03)00043-6

(10%) was converted by *Arthrobacter simplex* 6946 yielding a mixture of prednisolone 21-acetate (28%), prednisolone 17-acetate (66%), hydrocortisone 21-acetate (4%), and prednisolone (2%) [13]. The strain of *Corynebacterium simplex* converted two of five crystalline forms of cortisone 21-acetate to their 1(2)-dehydroanalogs [14]. Therefore, based on the literature data, the possibility of effective microbial production of pregna-1,4,9(11)-trienes from acetylated pregna-4,9(11)-diene substrates was not evident.

High 3-ketosteroid-1(2)-dehydrogenase activity towards androstenedione, hydrocortisone, 6α-methylhydrocortisone, and progesterone was described for *Arthrobacter globiformis* 193 [15,16], recently re-identified as *Nocardioides* sp. [17].

In this work, we used *Nocardioides simplex* VKM Ac-2033D for bioconversion of 21-acetate and 17,21-diacetate of pregna-4,9(11)-diene-17α,21-diol-3,20-dione. The role of 1(2)-dehydrogenation, deacetylation and 20β-reduction in metabolic pathways of acetylated pregna-4,9(11)-dienes by *N. simplex* was estimated.

2. Experimental

2.1. Materials

2.1.1. Steroids

Pregna-4,9(11)-diene-17α,21-diol-3,20-dione, its 21-acetate and 17,21-diacetate, pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione, and its 21-acetate and 17,21-diacetate were obtained from the Drug Chemistry Center (Moscow). Other reagents were of analytical grade and purchased from Reakhim (Russia).

2.2. Microorganisms and cultivation

The bacterial culture of *N. simplex* VKM Ac-2033D was obtained from All-Russian Collection of Microorganisms (VKM IBPM RAS). Cultivation and 3-ketosteroid-1(2)-dehydrogenase induction were carried out as described earlier [18].

2.3. Steroid transformation

Steroid transformations were carried out by washed cells in 750 ml Erlenmeyer flasks containing 100 ml of 0.01 M phosphate buffer on a rotary shaker (220 rpm, 28 °C) as described earlier [15]. Steroid substrates (1–5 g/l) were added in powder form. The substrate/biomass ratio varied from 10:4 to 10:40, w/w (dry weight).

2.4. Analyses

Aliquots (1 ml) of the conversion medium were taken every 4 h. Steroids were extracted with ethyl acetate (1:5, v/v), analyzed by TLC using Silufol UV 254 (Czech Republic) or Kieselgel 60 F_{254} (Merck, Germany) plates, developed in benzene/acetone, 3:1 (v/v), and visualized under UV light (254 nm).

Mass-spectrometry analysis (MS) was performed using a Finnigan MAT-8430 mass-spectrometer (Germany) at 70 eV.

The proton (^{1}H) nuclear magnetic resonance (NMR) spectra were recorded on a Unity +400 (Varian) spectrometer at 400 MHz using $CDCl_3$ as a solvent; the $CHCl_3$ signal in the solvent (δ 7.24) was used as an internal standard.

3. Results

3.1. Bioconversion of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate

The compounds **II** and **IV** were detected as major metabolites during pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate (**I**) conversion by *N. simplex* (Fig. 1). Based on the correspondence of the Rf values and MS and ^{1}H NMR characteristics to the standards, these compounds were identified as pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate and pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione, respectively (Tables 1 and 2). Compounds **III** and **V** were fixed in small amounts and identified by MS as pregna-4,9(11)-diene-17α,21-diol-3,20-dione and pregna-1,4,9(11)-triene-17α,20β,21-triol-3-one, respectively.

The substrate (**I**) was actively converted by *N. simplex* (Fig. 1). After 28 h of bioconversion no residual substrate was detected in the medium indicating its whole conversion. The utilization of **I** was accompanied by accumulation of pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate (**II**) as a major bioconversion product. Its concentration

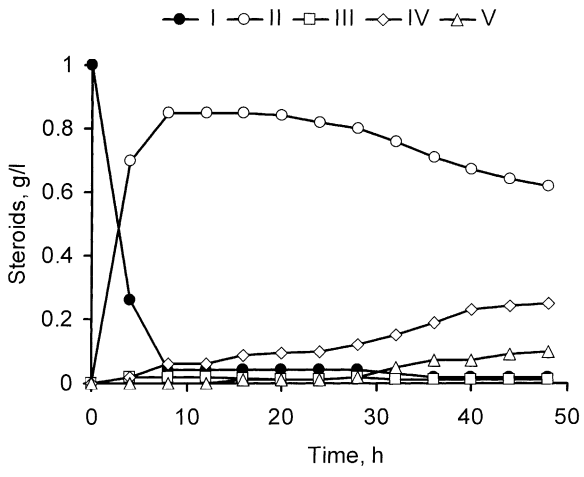

Fig. 1. Time course of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate conversion by *N. simplex* VKM Ac-2033D. Numeration of steroids: **I**—pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate; **II**—pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate; **III**—pregna-4,9(11)-diene-17α,21-diol-3,20-dione; **IV**—pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione; **V**—pregna-1,4,9(11)-triene-17α,20β,21-triol-3-one. Substrate concentration—1 g/l; substrate/biomass ratio—1:1 (w/w), pH 7.2.

Table 1
Identification of the major metabolites of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate conversion by *N. simplex* VKM Ac-2033D

Compound	Rf	Characteristics of the major fragments, *m*/*z* (%)
Pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate	1.00	M^+386(33),371(19),326(23),285(100),267(47),240(38),227(73),225(82)
Pregna-4,9(11)-diene-17α,21-diol-3,20-dione	0.64	M^+344(55),329(14),326(8),285(100),267(36),240(20),227(83),225(66)
Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate	0.85	M^+384(51),369(7),324(19),283(38),265(71),238(64),225(100),223(99),121(38)
Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione	0.54	M^+342(73),324(10),283(40),265(67),225(100),223(74),121(38)
II	0.85	M^+384(53),369(11),324(22),383(36),265(69),238(65),225(100),121(38)
IV	0.54	M^+342(62),324(8),283(52),265(75),225(100),223(86),121(31)
V	0.25	M^+344(12),326(10),265(100),225(10),171(14),143(22),129(26),121(20)

Table 2
Chemical shifts in ^{1}H NMR spectra of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate and 21-acetate and their bioconversion products (δ, ppm)

Compound	1-H	2-H	4-H	11-H	$C^{18}H_3$	$C^{19}H_3$	$C^{21}H_3$	17-OAc	21-OAc
Pregna-4,9(11)-diene-17α,21-diol-3,20-dione	1.1–2.8		5.73d	5.52d	0.62s	1.31s	4.67dd, 4.28dd	–	–
Pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate	1.1–2.8		5.72d	5.53d	0.62s	1.31s	5.06d, 4.82d	–	2.15s
Pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate	1.1–2.8		5.73d	5.53d	0.68s	1.32s	4.87d, 4.64d	2.08s	2.15s
Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione	7.12d	6.16dd	5.95t	5.46d	0.51s	1.32s	4.60d, 4.40d	–	–
Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17,21-diacetate	7.17d	6.27dd	6.06t	5.55d	0.71s	1.39s	4.85d, 4.62d	2.04s	2.15s
Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate	7.17d	6.26dd	6.05t	5.55d	0.65s	1.38s	5.03d, 4.81d	–	2.15s
Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17-acetate	7.18d	6.28dd	6.07t	5.57d	0.64s	1.40s	4.27s	2.03s	
II	7.17d	6.26dd	6.05t	5.55d	0.65s	1.38s	5.03d, 4.81d	–	2.15s
IV	7.12d	6.l6dd	5.95t	5.46d	0.51s	1.32s	4.60d, 4.40d		
VII	7.17d	6.27dd	6.06t	5.55d	0.71s	1.39s	4.85d, 4.62d	2.04s	2.15s
VIII	7.18d	6.28dd	6.07t	5.57d	0.64s	1.40s	4.27s	2.03s	

reached 0.80–0.85 g/l in 8 h and then gradually decreased. Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**IV**) was detected after 8 h and started to accumulate. The gradual increase of its amount correlated with a subsequent decrease in the content of pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate (**II**). Pregna-4,9(11)-diene-17α,21-diol-3,20-dione (**III**) was detected in traces since the first hours of the conversion and its amount did not change notably during at least 50 h. Pregna-1,4,9(11)-triene-17α,20β,21-triol-3-one (**V**) appeared after 24 h and then its content slightly increased to 0.08–0.10 g/l in 48 h.

Based on the results obtained, the following pathway of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate (**I**) conversion by *N. simplex* was proposed (Fig. 2). The substrate (**I**) underwent 1(2)-dehydrogenation forming compound **II**. In spite the reaction is obviously shifted towards dehydrogenation (**I** → **II**) the reverse reaction cannot be excluded since 1-ene-reductase activity was well documented for relative organisms [19].

The appearance of compound **III** since the first hours of the conversion allows to propose deacetylation of **I** at C-21. The similar reaction obviously resulted in the formation of compound **IV** from **II**. One can assume interconversions of compounds **III** and **IV** due to possible activities of 1(2)-dehydrogenase and 1-ene-reductase. Compound **V** seems to be formed from **IV** as a result of 20β-reduction. Since neither pregna-4,9(11)-diene-17α,20β,21-triol-3-one, nor any acetylated compounds reduced at C-20 were detected, the compound **IV** was assumed as an only substrate for 20β-reductase.

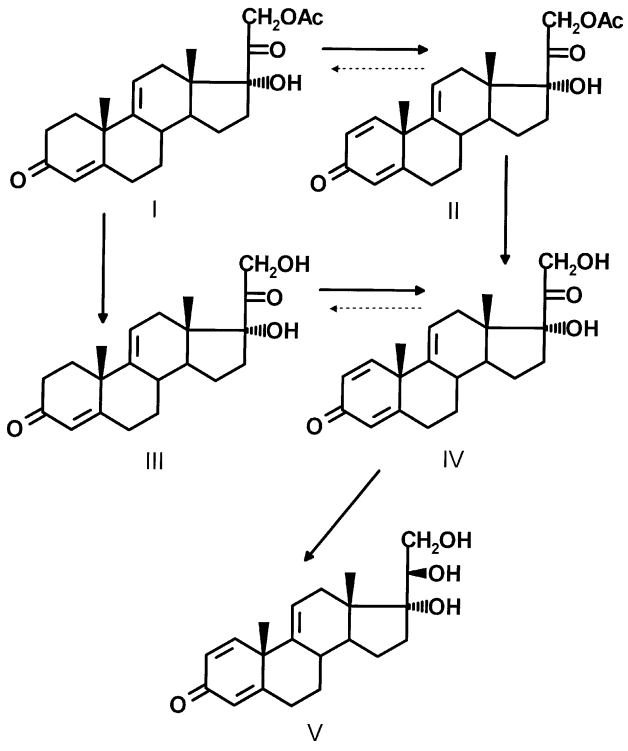

Fig. 2. Proposed scheme of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate (**I**) conversion by *N. simplex* VKM Ac-2033D.

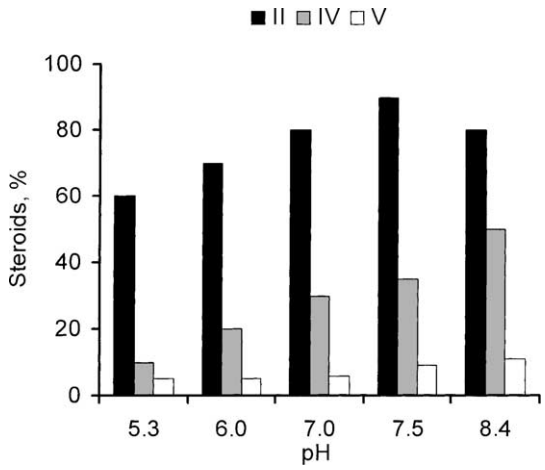

Fig. 3. Effect of pH on pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate (**I**) conversion by *N. simplex* VKM Ac-2033D.

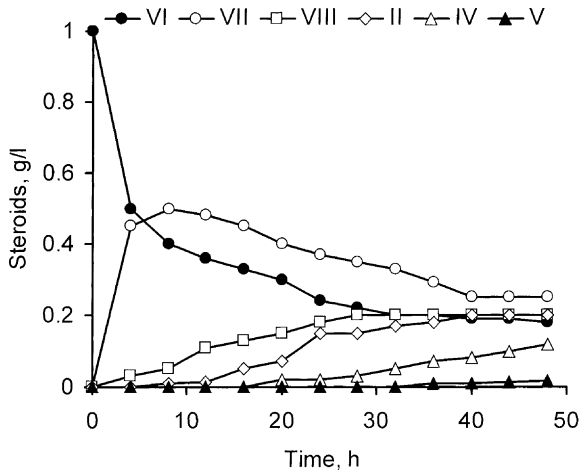

Fig. 4. Time course of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-di-acetate conversion by *N. simplex* VKM Ac-2033D. Numeration of steroids: **VI**—pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-di-acetate; **VII**—pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17,21-di-acetate; **VIII**—pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17-acetate; **II**—pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate; **IV**—pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione; **V**—pregna-1,4,9(11)-triene-17α,20β,21-triol-3-one. Substrate concentration—1 g/l; substrate/biomass—1:1, pH 7.2.

As shown in Fig. 3, pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate (**II**) remained a major product of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 21-acetate (**I**) conversion by *N. simplex* in a range of pH values from 5.3 to 8.4 with maximum level of 85% reached in 8 h at pH 7.5. The formation of **IV** was enhanced with the increase of pH confirming expected pH-dependence of 21-deacetylation. 20β-Reduction of **IV** was slightly dependent on pH (Fig. 3).

After the optimization, the yield of acetylated 1(2)-dehydroanalog (**II**) of 90% in 8 h was achieved at a substrate loading dose of 5 g/l, a substrate/biomass ratio of 1:1 (w/w) and a pH of 7.2. The total content of by-products (**IV** and **V**, totally) did not exceed 8–10%.

3.2. Bioconversion of pregna-4,9(11)-diene-17α, 21-diol-3,20-dione 17,21-diacetate

At least five metabolites were detected during pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate (**VI**) conversion by *N. simplex* (Fig. 4). MS and ^{1}H NMR analyses confirmed the correspondence of compounds **VII**, **II**, and **IV** to authentic pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17α,21-diacetate, pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate and pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione, respectively, and also allowed for the identification of compound **VIII** as pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17-acetate (Tables 2 and 3). Compound **V** was found in minorities and identified as pregna-1,4,9(11)-triene 17α,20β,21-triol-3-one (Table 3).

As shown in Fig. 4, the uptake of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate (**VI**) by *N. simplex* was mostly active during the first 4 h (about 50% of **VI** was converted). Then, the rate of conversion decreased; only 35% of **VI** was transformed during the subsequent 44 h.

Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17,21-di-acetate (**VII**) accumulated as a major bioconversion product and reached a level of 0.5 g/l in 8 h. At the same time, pregna-1,4,9(11)-triene-17,21-diol-3,20-dione 17-acetate (**VIII**) was formed and stabilized after 28 h at a level of 0.2 g/l. 21-Acetate of pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**II**) was detected after 8 h, and its amount gradually increased to 0.25 g/l after 40 h. Pregna-1,4,9(11)-triene-17α,

Table 3
Identification of the major metabolites of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate conversion by *N. simplex* VKM Ac-2033D

Compound	Rf	Characteristics of the major fragments, *m/z* (%)
Pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate	1.00	M^+428(84),386(54),371(32),326(35),285(90),267(70),240(46),227(85),225(100)
Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17,21-diacetate	0.86	M^+426(5),366(20),266(24),265(100),237(12),223(12),157(22),121(20)
Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate	0.70	M^+384(53),369(11),324(22),383(36),265(69),238(65),225(100),223(99),121(38)
Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione	0.38	M^+342(73),324(10),283(40),265(67),225(100),223(74),121(38)
VII	0.86	M^+426(5),366(14),266(25),265(100),121(20)
VIII	0.49	M^+384(21),324(25),309(10),293(11),265(100),225(13),223(20),121(18)
II	0.70	M^+384(53),369(11),324(22),383(36),265(69),238(65),225(98),223(100),121(32)
IV	0.38	M^+342(56),324(18),283(78),265(72),225(90),223(74),121(38)
V	0.18	M^+344(5),326(10),265(100),251(34),237(11),225(9),223(11),121(16)

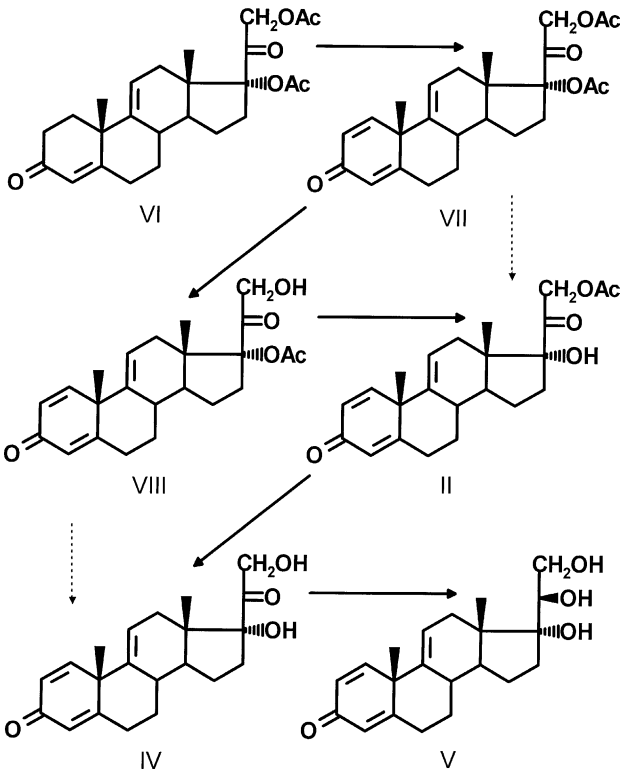

Fig. 5. Proposed scheme of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate (**VI**) conversion by *N. simplex* VKM Ac-2033D.

21-diol-3,20-dione (**IV**) slowly accumulated to 0.13 g/l within 48 h. Pregna-1,4,9(11)-triene-17α,20β,21-triol-3-one (**V**) was formed in small amounts (less than 0.02 g/l) during the conversion.

Based on the results obtained, the metabolic pathway of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate (**VI**) by *N. simplex* was proposed (Fig. 5). The compound **VII** was formed from **VI** as a result of 1(2)-dehydrogenation. Since no deacetylated metabolites saturated at C-1 were found, the substrate **VI** was proposed to be an only substrate for 1(2)-dehydrogenase of *N. simplex*. No evidence of deacetylation of the substrate (**VI**) was obtained, while its 1(2)-dehydroanalog (**VII**) obviously underwent deacetylation at C-21 forming pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17-acetate (**VIII**). Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate (**II**) possibly was formed from **VIII** by non-enzymatic migration of the acyl group from position 17 to 21.

To confirm a possibility of the acyl group migration, a model experiment was carried out using 17-acetate and 21-acetate of Reichstein compound S (pregn-4-ene-17α,21-diol-3,20-dione). These compounds were incubated in the transformation medium at the conditions used for bioconversion, but without microbial cells. No transformation occurred with 21-acetate of Reichstein compound S, while 17-acetate was converted to 21-acetate confirming non-enzymatic acyl migration at the conditions used. Expectedly, the non-enzymatic conversion of 17-acetate

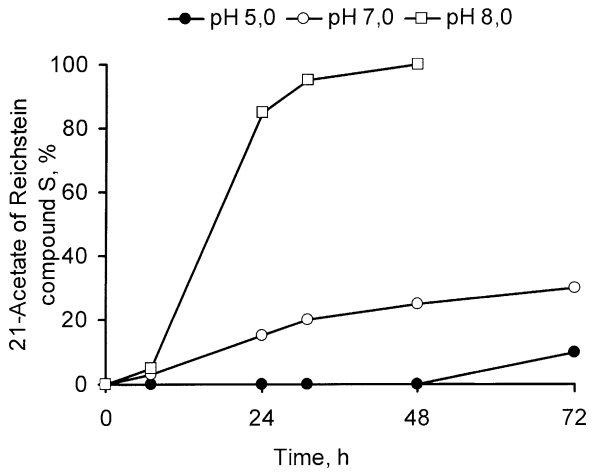

Fig. 6. Effect of pH on the non-enzymatic formation of 21-acetate of Reichstein compound S from 17-acetate of Reichstein compound S.

Reichstein compound S was strongly pH dependent (Fig. 6). As shown, it was slight at pH 5.8, enhanced at pH 7.0, and full conversion of 17-acetate to 21-acetate of Reichstein compound S was observed at pH 8.0. These results confirmed that formation of compound **II** from **VIII** during the bioconversion process by *N. simplex* can be due to non-enzymatic acyl group migration.

The alternative possibility of compound **II** formation from **VII** as well as formation of **IV** from **VIII** due to enzymatic 17α-deacetylation (**VII** → **II** and **VIII** → **IV**) hardly can be fully excluded in spite of 17α-deacetylation of tertiary acyl group which is energetically and spatially hindered. The further metabolic conversions of **II** were evidently similar to those described in 3.1. Enzymatic 21-deacetylation forming **IV** was followed by 20β-reduction.

Fig. 7 demonstrates pH dependence of pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate (**VI**) conversion by *N. simplex*. As shown, the substrate (**VI**) uptake was slightly dependent on pH. Its content decreased almost twice during the first bioconversion period. No remarkable influence of pH on the accumulation of **VII** was observed, whilst the accumulation of **VIII** was definitely pH dependent. Pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 17-acetate (**VIII**) mainly accumulated at pH 6.2 with a yield of almost 90% reached in 50 h (Fig. 7a), while its accumulation decreased almost half with the rise of pH (Fig. 7b and c).

An increase of pH above 7.2 resulted in process shift towards the accumulation of pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**IV**) (Fig. 7b and c). It exceeded 80% after 48 h at pH 8.0 (Fig. 7c). At the same pH, the reduction at C-20 was activated resulting in the increase of compound **V** content.

By the optimization experiments, 17-acetate of pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**VIII**) yielded 90% from pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate (**VI**) by *N. simplex* in 20 h of transformation, at a substrate loading dose of 5 g/l, a substrate/

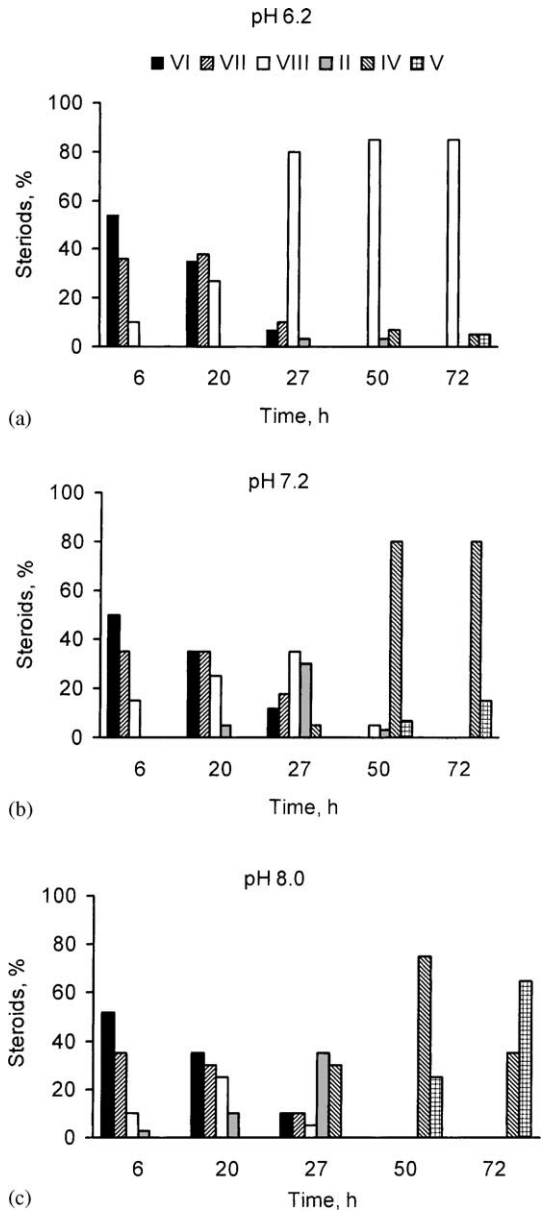

Fig. 7. Effect of pH on pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate (**VI**) conversion by *N. simplex* VKM Ac-2033D. Substrate concentration—1 g/l; substrate/biomass ratio—1:2 (w/w); (a) pH 6.2; (b) pH 7.2; (c) pH 8.0.

biomass ratio 1:4 (w/w), and a pH of 6.0. Under these conditions, the total content of other products, pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**IV**) and pregna-1,4,9(11)-triene-17α,20β,21-triol-3-one (**V**) did not exceed 7–10%.

4. Discussion

Current interest in the synthesis of 1,4,9(11)-triene steroids is explained by their importance in corticosteroid chemistry. Stereoselective chemical synthesis of pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione 21-acetate from 3β-acetoxy-pregna-9(11),16-dien-20-one included seven stages and yielded 9–11% of the goal product [9]. In another procedure, chemical dehydrogenation of 16α,17-epoxy-16β-methyl-5α-pregn-9(11)-ene-3,20-dione gave 63% of 16α,17-epoxy-16β-methylpregna-1,4,9(11)-triene-3,20-dione [8]. Within the context of this paper, bioconversion looks more attractive because by using *N. simplex* VKM Ac-2033D, 1(2)-dehydrogenated analogs of pregna-4,9(11)-diene-17α,21-diol-3,20-dione acetylated derivatives were obtained in single step without any special pretreatment of the biomass.

The substrates (**I** and **VI**) were fully converted by *N. simplex*. 1(2)-Dehydrogenation was a major bioconversion process resulting in effective formation of pregna-1,4,9(11)-triene compounds. The rate of 1(2)-dehydrogenation of acetylated substrates (**I** and **VI**) exceeded that of deacetylation and resulted in predominant formation of acetylated pregna-1,4,9(11)-triene-17α,21-diol-3,20-diones at the early transformation stage. Almost 90% and over 55–60% molar yield of 21-acetate and 17,21-diacetate of pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**II** and **VII**), respectively, were obtained in 8 h.

Along with 1(2)-dehydrogenase activity, esterase and 20β-reductase activities of *N. simplex* were observed. Esterases seem to be responsible for deacetylation in position 21. Evidently, these enzyme(s) showed more affinity towards 1(2)-dehydrogenated compounds as compared with 1(2)-saturated steroids. Introduction of the second acetyl group at position 17 affected the rate of deacetylation and lead to a more complicated metabolic pathway (Fig. 5). Acetyl group in position 21 obviously is a good target for esterase attack due to its energetic and spatial proximity compared to tertiary 17α-acetyl group. As a result, the 21-hydroxy compound (**VIII**) is formed.

The most questionable point is the possibility of enzymatic 17α-deacetylation of 17,21-diacetate of pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**VII**) forming 21-acetate of pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**II**) and 17-acetate of pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**VIII**) forming pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**IV**). The ability to hydrolize tertiary 17α-acetate of C-21 steroids and 17β-acetate of C-19 steroids was reported for *Flavobacterium dehydrogenans* [2]. No other evidence of microbial 17α-deacetylation of diacetate, or three-enol-acetates of steroids was found in the literature. Primary and secondary steroid acylates underwent enzymatic hydrolysis, but not tertiary functions [20,21].

The compound **II** can be formed from **VIII** as a result of non-enzymatic migration of acetyl group from position 17 to position 21. The formation of prednisolone 21-acetate at the conversion of hydrocortisone 17-acetate by *A. simplex* 6946 was documented without any explanations [13]. No deacetylation at position 17 was registered in this work. The suggestion is in good correspondence with our results

obtained on the non-enzymatic formation of 21-acetate of Reichstein compound S at the incubation of its 17-acetate without microbial cells.

As a result of step-by-step deacetylation, pregna-1,4,9(11)-triene-17α,21-diol-3,20-dione (**IV**) became the major intermediate in pregna-4,9(11)-diene-17α,21-diol-3,20-dione 17,21-diacetate (**VI**) bioconversion, yielding over 80% at pH of 7.0 during 72 h of incubation at biomass/substrate ratio 2:1.

Therefore, the strain of *N. simplex* VKM Ac-2033D has been shown to effectively convert acetylated 9(11)-dehydro-3-ketosteroids to their 1(2)-dehydroderivatives during short-term transformation period. Both 21-acetate, and 17, 21-diacetate of pregna-4,9(11)-diene-17α,21-diol-3,20-dione can be recommended as substrates for 1(2)-dehydrogenation by *N. simplex* in the commercial production of 9-halogenated steroids.

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