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21-Acetoxy-pregna-4(5),9(11),16(17)-triene-21-ol-3, 20-dione conversion by *Nocardioides simplex* VKM Ac-2033D

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Abstract

The conversion of 21-acetoxy-pregna-4(5),9(11),16(17)-triene-21-ol-3,20-dione (**I**) by *Nocardioides simplex* VKM Ac-2033D was studied purposed selective production of its 1(2)-dehydroanalogues—value precursors in the synthesis of modern glucocorticoids starting from 9α-hydroxyandrostenes. 21-Acetoxy-pregna-1(2),4(5),9(11),16(17)-tetraene-21-ol-3,20-dione (**II**), pregna-4(5),9(11),16(17)-triene-21-ol-3,20-dione (**III**) and pregna-1(2),4(5),9(11),16(17)-tetraene-21-ol-3,20-dione (**IV**) were revealed as metabolites, and the structures were confirmed by mass spectrometry and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The metabolic pathways of **I** by *N. simplex* included 1(2)-dehydrogenation and deacetylation. The sequence of the reactions was shown to depend on the transformation conditions. The presence of both soluble and membrane associated steroid esterases in *N. simplex* was demonstrated using cell fractionation. Unlike inducible 1(2)-dehydrogenase, steroid esterase was shown to be constitutive. The conditions providing selective accumulation of **II** from **I** by whole *N. simplex* cells were determined.

Keywords: Steroid; 4(5),9(11),16(17)-Triene-3-ketosteroids; 1(2)-Dehydrogenation; Deacetylation; 21-Acetoxy-pregna-1(2),4(5),9(11),16(17)-tetraene-21-ol-3,20-dione; Bioconversion; *Nocardioides simplex*

1. Introduction

At present, 1(2)-unsaturated 3-ketosteroids with 16(17)-double bond as well as derivatives with 16α,17α-epoxy– and 17α-OH– groups are of great importance as immediate precursors in modern syntheses of 9α-fluorocorticoids: dexamethasone, betamethasone, etc. [1]. The presence of 9(11)-double bond in the precursor molecule facilitates their obtaining. As shown, chemical dehydrogenation of 9(11)-dehydro-3-ketosteroids was accompanied by undesirable spontaneous ring A aromatisation leading to ineffective glucocorticoid production [2,3]. Thus, microbial 1(2)-dehydrogenation is of special significance.

Data on bioconversion of acetylated 9(11)-dehydrosteroids with 16(17)-double bond are scarce [4]. Microbial 1(2)-dehydrogenation of pregna-4(5),9(11),16(17)-trienes was not investigated so far.

Protection of functional groups by means of acetyl substitution is widespread in the art. A further synthesis can include deacetylation. In this connection, study of microbial deacetylation in correlation with other steroid transforming activities of microorganisms is of special interest. However, literature data on the properties of microbial steroid esterases and the features of deacetylation by 1(2)-dehydrogenating cultures are contradictory and limited [5–8].

A strain of *Nocardioides simplex* VKM Ac-2033D was shown to express high 1(2)-dehydrogenase activity towards 3-ketosteroids [9]. In the recent work, we have reported an effective 1(2)-dehydrogenation of mono- and diacetylated pregna-4(5),9(11)-diene-17α,21-diol-3,20-diones by this organism [10]. Microbial 1(2)-dehydrogenation was accompanied by deacetylation with the formation of respective hydroxysteroids.

It was of interest to find out whether the introduction of 16(17)-double bond in 21-acetylated 4(5),9(11)-diene steroid would influence 1(2)-dehydrogenation and deacetylation by *N. simplex*. In order to explore full biocatalytic potential of this organism and clear structure/activity relationship in respect to acetylated 9(11)-dehydrosteroids, the

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conversion of 21-acetoxy-pregna-4(5),9(11),16(17)-triene-21-ol-3,20-dione (**I**) was investigated. The biotransformation of similar triene steroids has never been studied so far.

2. Materials and methods

2.1. Microorganism and cultivation

N. simplex VKM Ac-2033D was obtained from All-Russian Collection of Microorganisms (VKM RAS). If not otherwise mentioned, cultivation and 3-ketosteroid-1(2)-dehydrogenase induction were carried out as described earlier [10]. In some experiments, hydrocortisone (0.2 g/l) was used as an inducer added as an ethanol solution (20 g/l). After the cultivation for 20–24 h, biomass was separated by centrifugation at 7000 × *g* for 15 min, washed twice with 0.01 M sodium phosphate buffer (pH 7.2), and used for the bioconversion as described in 2.4. In some experiments, chloramphenicol (30 μg/ml) was used as a protein synthesis inhibitor.

2.2. Reagents

21-Acetoxy-pregna-4(5),9(11),16(17)-triene-21-ol-3,20-dione (**I**) was obtained from Drug Chemistry Centre (Moscow), cortisone 21-acetate from Akrikhin (Russia). Hydrocortisone was purchased from Russel Uclaf (France), randomly methylated β-cyclodextrin (MCD) from Wacker Chemie (Germany), phenazine metasulphate (PMS) from "Calbiochem" (Switzerland). Other materials were of reagent grade and purchased from domestic companies (Russia).

2.3. Cell fractionation

All steps were performed at 4 °C. The biomass was separated by centrifuge at 7000 × *g* for 15 min, and washed twice with 0.01 M sodium phosphate buffer (pH 7.2). Frozen (−70 °C) cells (1.98 g wet weight) were disrupted by a single passage through French press, re-suspended in 250 ml of 0.01 M sodium phosphate buffer (pH 7.2). Cell debris was separated by centrifugation at 10 000 × *g* for 15 min thus obtaining fraction F1. The 10 000 × *g* supernatant was fractionated at 105 000 × *g* for 40 min. The 105 000 × *g* residue (F2) containing membrane fragments was separated and re-suspended in 20 ml of sodium phosphate buffer (pH 7.2). The 105 000 × *g* supernatant (F3) contained cytosol and solubilized membrane fragments.

2.4. Transformation of *I* by washed whole cells and cell fractions (F1, F2 and F3)

Steroid transformations by washed whole cells were carried out in 750 ml Erlenmeyer flasks containing 100 ml of 0.01 M sodium phosphate buffer on a rotary shaker (220 rpm, 30 °C) as described earlier [10]. Steroid substrate **I** (1 g/l) was added as a fine powder. The biomass varied from 33 to 330 mg/l (dry weight). Steroid transformations by cell fractions were carried out in 10 ml test tubes at 30 °C on a rotary shaker (220 rpm). The reaction medium contained: 0.01 M sodium phosphate buffer (adjusted to a final reaction volume of 5 ml), substrate **I** (1 g/l), MCD—7.1 g/l and one of the following components: F1, 200 μl; F2 suspension, 2 ml; or F3, 3.8 ml. In some experiments, PMS (0.4 mg/ml) was added.

2.5. Analyses

Steroids were extracted with ethyl acetate (1:5, v/v). The extracts were applied to Kieselgel 254 (Merck, Germany) or Sorbfil UV 254 (Russia) TLC plates, developed in benzene/acetone (3:1, v/v) and visualised under the UV light (254 nm). Metabolites were isolated by preparative TLC.

Mass-spectrometry analysis (MS) was carried out using a Finnigan MAT-8430 mass-spectrometer (Germany) by direct inlet with ionisation energy of 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 signal of $CHCl_3$ traces in the solvent (δ 7.24) was used as an internal standard.

3. Results

3.1. Identification of metabolites

Three major metabolites of **I** by *N. simplex* were revealed in this work upon examination by TLC (compounds **II**, **III**, **IV** with R_f-values: 0.861, 0.778 and 0.563, respectively).

The MS of **I** was characterised by a low signal of molecular ion M^+ 368 from which partly methyl and acetic acid functions were readily detached. The formation of fragment with *m*/*z* 295 was explained by β-rupture of C–C linkage at C-20 in a five-membered D-ring. MS of **II** (M^+ 366) repeated fragmentation of **I** with the major molecular ions reduced in 2 units (*m*/*z* 351, 306, 291, 263). Detection of ion with *m*/*z* 121 directly indicated 1(2)-double bond appearance in the A-ring. The presence of 1(2)-double bond determined sufficient energetic molecule stability.

The MS of **III** indicated the absence of acetyl group in a whole structure. The separation of both methyl group and 31-unit fragment (either $–OCH_3$ or $–CH_2OH$) from molecular ion M^+ 326 was supposed. Separated component of "$–CH_2OH$" structure was more evident for **III** meaning possible microbial hydrolysis of acetate group in **I**. MS of **IV** with M^+ 324 having fragmentation of **III** differed from the latter by molecular ion with *m*/*z* 121 indicating 1(2)-double bond in A-ring.

Based on MS and ^{1}H NMR data (Tables 1 and 2), the metabolites were identified as 21-acetoxy-pregna-1(2),4(5),9(11),16(17)-tetraene-21-ol-3,20-dione (**II**) (m.w.

Table 1
Identification of the metabolites of **I** conversion by *N. simplex* VKM Ac-2033D

Compound	R_f	Characteristics of the major fragments, *m/z* (%)
21-Acetoxy-pregna-4(5),9(11), 16(17)-triene-21-ol-3,20-dione (**I**)	1.00	M^+ 368 (4), 353 (18), 308 (100), 295 (33), 293 (48), 265 (12), 203 (10), 185 (30), 143 (21), 129 (20), 115 (15), 105 (22)
II	0.861	M^+ 366 (100), 351 (24), 306 (49), 293 (38), 291 (35), 278 (24), 263 (33), 185 (25), 143 (18), 129 (22), 128 (27), 121 (20), 115 (22), 105 (17)
III	0.778	M^+ 326 (63), 311 (100), 295 (82), 267 (8), 225 (9), 203 (12), 185 (12), 143 (13), 129 (14), 115 (12), 105 (17)
IV	0.563	M^+ 324 (100), 309 (55), 293 (70), 265 (18), 223 (13), 201 (10), 200 (13), 185 (15), 143 (21), 129 (29), 128 (33), 121 (21), 115 (34), 105 (25)

366), pregna-4(5),9(11),16(17)-triene-21-ol-3,20-dione (**III**) (m.w. 326) and pregna-1(2),4(5),9(11),16(17)-tetraene-21-ol-3,20-dione (**IV**) (m.w. 324).

3.2. Bioconversion of *I* by whole cells

As shown in Fig. 1a, compound **III** was formed as a major metabolite of **I** reached 40% for 5 h of incubation of **I** with *N. simplex* whole cells. Its further decrease was accompanied by the increase in the content of **IV**. Substrate (**I**) fully converted during 20 h.

To intensify bioconversion, the medium was supplemented with modified β-cyclodextrin, MCD. As shown in Fig. 1b, substrate (**I**) was fully converted for 2.5 h of incubation. Along with **III** and **IV**, the metabolite **II** was formed reached 75% for 1 h and then decreased. The decrease of **II** correlated with accumulation of **IV** yielding 90% for 5 h. The level of **III** did not exceed 10%.

Based on the results obtained, two pathways of **I** conversions by *N. simplex* were supposed (Fig. 2). The deacetylated 1(2)-dehydroderivative (**IV**) can be formed either by substrate deacetylation to **III** followed by its further 1(2)-dehydrogenation (pathway **I** → **III** → **IV**) or by a former 1(2)-dehydrogenation of **I** to **II** followed by deacetylation of **II** (pathway **I** → **II** → **IV**). Evidently, the pathway **I** → **III** → **IV** dominated in case of the absence of MCD, while MCD addition stimulated a pathway **I** → **II** → **IV**.

3.3. Influence of induction on bioconversion of *I* by whole cells

The influence of inducing effect of hydrocortisone and cortisone 21-acetate on 1(2)-dehydrogenase and esterase activities of *N. simplex* was studied. No significant differences were observed in the rate of formation or yield of **III**. Both compounds induced preferably 1(2)-dehydrogenase that confirmed by increased accumulation of **II**.

Non-induced cells converted **I** preferably to **III**. Its content reached 40% for 1 h of incubation. The compound **II** was detected in small amounts (∼5%) demonstrating low 1(2)-dehydrogenase activity towards **I**. The following decrease in the content of **III** was accompanied by accumulation of compound **IV** reached 85% for 5 h of conversion. The latter evidently indicated rise of 1(2)-dehydrogenase activity towards **III** due to its possible induction by **III** formed and/or preferable affinity of **III** as compared with **I** for the enzyme.

Both induced and non-induced cells formed **III** from **I**, thus expressed esterase activity (Table 3). Neither inducer, nor chloramphenicol influenced the deacetylation (**I** → **III**). Unlike non-induced, induced washed cells actively formed **II** from **I** after the substrate addition (Table 3, var. 1 and 2). The shortening of the induction time from 24 to 7 h resulted in some decrease in the accumulation rate and yield of **II**. An addition of protein synthesis inhibitor—chloramphenicol inhibited 1(2)-dehydrogenase activity—no **II** was formed in var. 3 during at least 40 min of **I** conversion. Interestingly, small amounts of **IV** were detected after 6 h incubation in the presence of chloramphenicol showing slight 1(2)-dehydrogenase activity towards **III** (Table 3, var. 3). At the use of non-induced cells (Table 3, var. 4) compound **II** was fixed after 2.5 h of the conversion, thus indicating possible inducing effect of **I** on 1(2)-dehydrogenase. The assumption is in accordance with the fact that no formation of **II** was observed at the addition of chloramphenicol to the non-induced cells (Table 3, var. 5).

Table 2
Chemical shifts in ^{1}H NMR spectra of **I** and its metabolites (δ, ppm)

Compound	1-H	2-H	4-H	11-H	16-H	$C^{18}H_3$	$C^{19}H_3$	$C^{21}H_AH_B$	21-$OCOCH_3$
I	–	–	5.73d (1.8)	5.53dt (5.9, 1.9)	6.76dd (3.5, 2.0)	0.87s	1.34s	4.90d, 5.03d (16.0)	2.17s
II	7.19d (10.2)	6.25dd (10.2, 1.7)	6.04t (1.7)	5.53dt (6.0, 2.0)	6.74dd (3.3, 2.0)	0.89s	1.40s	4.87d, 5.01d (16.2)	2.15s
III	–	–	5.74d (1.7)	5.54dt (5.8, 1.9)	6.76dd (3.5, 2.0)	0.88s	1.35s	4.42d, 4.52d (17.8)	–
IV	7.20d (10.2)	6.27dd (10.2, 1.7)	6.06t (1.7)	5.56dt (6.0, 2.0)	6.74dd (3.4, 1.8)	0.91s	1.42s	4.42d, 4.51d (17.5)	–

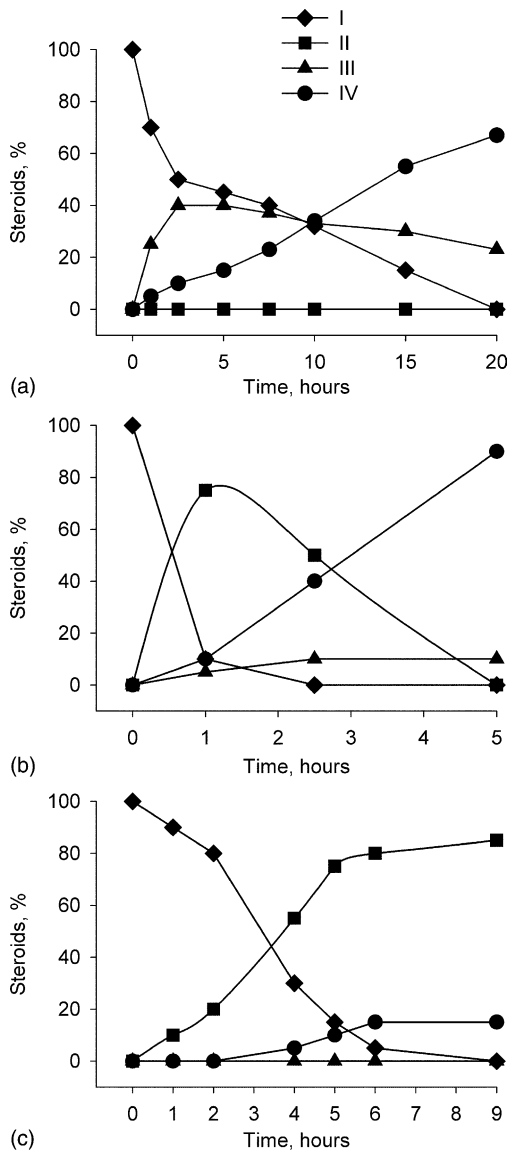

Fig. 1. Time course of **I** conversion by whole induced cells *N. simplex* without MCD (a) and in the presence of MCD (b, c). Numeration of steroids is as in the text. Substrate concentration, 1 g/l; biomass: 330 mg/ml (a, b) and 50 mg/ml (c).

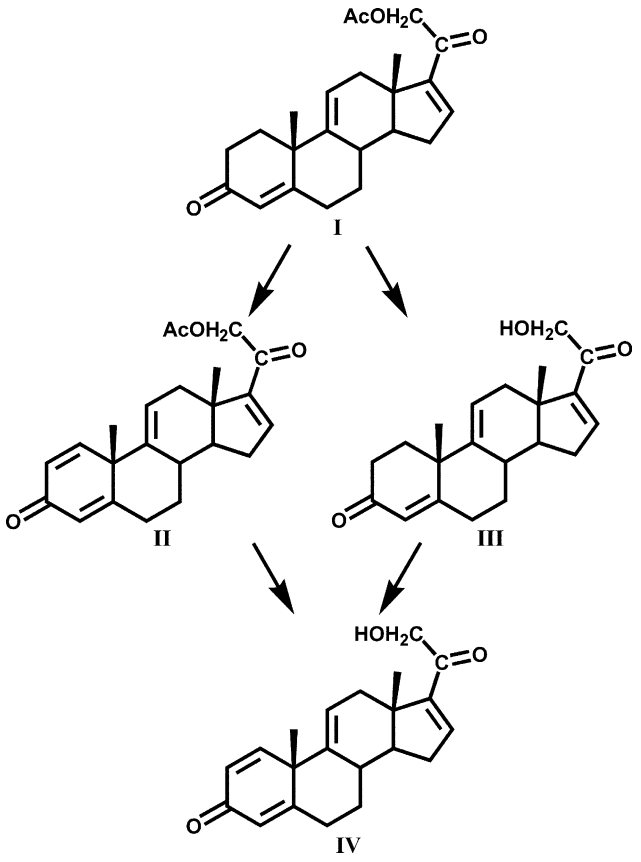

Fig. 2. Proposed scheme of **I** conversion by *N. simplex*. Numeration of steroids is as in the text.

Thus, the results confirmed inducibility of 1(2)-dehydrogenase and constitutive character of steroid esterase(s). Surprisingly, no formation of **II** was observed at the incubation of non-induced cells with **I** in the absence of MCD (Table 3, var. 6). Probably, induction of 1(2)-dehydrogenase system was hindered in the absence of MCD.

Since cortisone acetate induced 1(2)-dehydrogenase, but not esterase, we proposed the regulation of the process selectivity by biomass. As followed from Fig. 1c (compared to Fig. 1b), decrease of biomass from 330 to 50 g/l resulted in the accumulation of **II** as a major metabolite yielded 85%

Table 3
Influence of induced and non-induced *N. simplex* cells on **I** conversion

Variants	Content (%) of compounds **II**, **III** and **IV** for											
	20 min			40 min			2.5 h			6 h		
	II	**III**	**IV**	**II**	**III**	**IV**	**II**	**III**	**IV**	**II**	**III**	**IV**
Induced cells												
1. Induction during 24 h	40	5	8	62	10	16	8	10	70	0	0	88
2. Induction during 7 h	10	6	5	20	8	8	30	12	15	9	7	72
3. Induction during 7 h in the presence of chloramphenicol	0	5	0	0	8	0	6	40	7	6	20	62
Non-induced cells												
4. Bioconversion medium with MCD	0	6	0	0	10	0	6	40	8	4	16	67
5. Bioconversion medium with MCD and chloramphenicol	0	6	0	0	11	0	0	57	0	0	69	5
6. Bioconversion medium without MCD	0	6	0	0	11	0	0	60	0	0	67	5

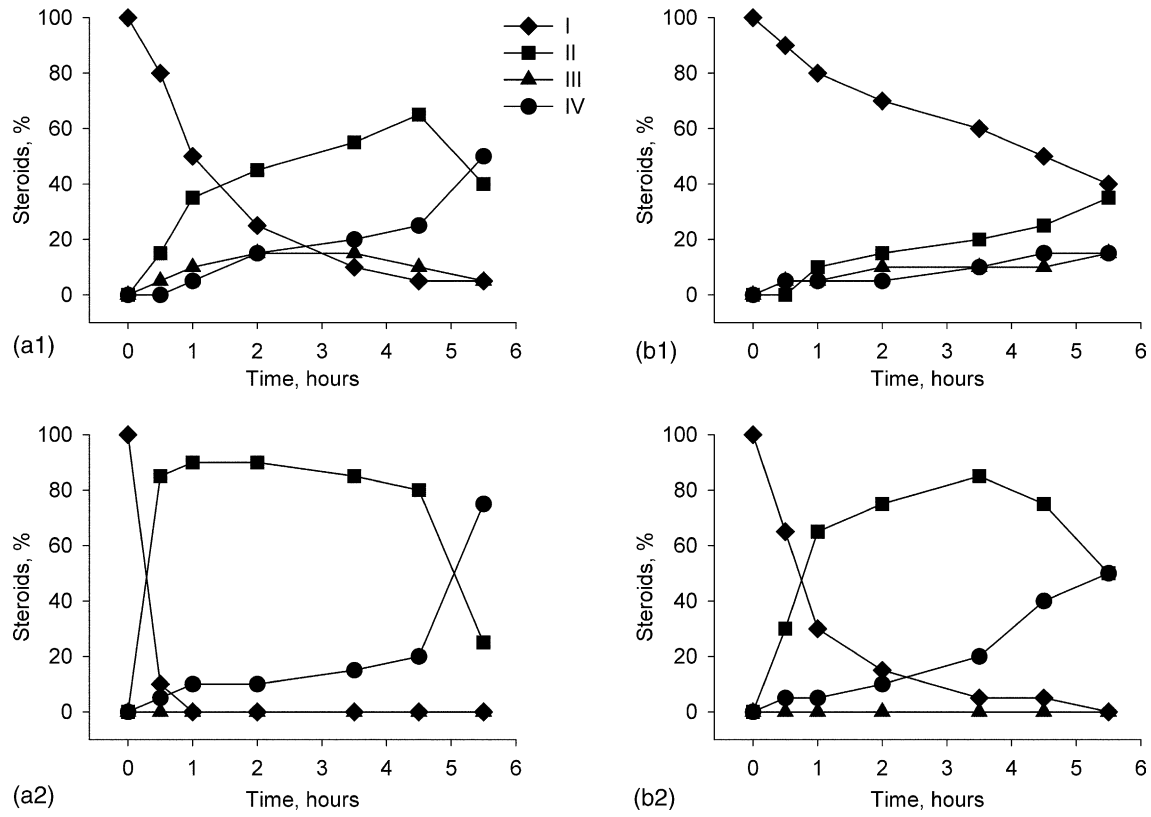

Fig. 3. Time course of **I** conversion by cell fractions F1 (a1 and a2) and F2 (b1 and b2) without PMS (a1 and b1) and with PMS (a2 and b2) in the presence of MCD. Substrate concentration: 1 g/l; biomass of F1 ≈ 2 g/l; biomass of F2 ≈ 500 mg/ml.

for 9 h and the decrease of the formation of deacetylated derivative **III**.

3.4. Bioconversion of *I* by cell fractions

The fractions of *N. simplex*: F1 contained mostly disrupted cells, F2 comprised of membrane fragments, and cytosol F3 were obtained as described in Methods.

As showed in Fig. 3(a1) and (b1), during the incubation of **I** with F1 or F2, metabolites **II**, **III** and **IV** were formed. The content of **II** reached 35 and 10% for 1 h, respectively.

1(2)-Dehydrogenation of **I** by F1 or F2 fractions was selectively stimulated in the presence of artificial electron acceptor, PMS (Fig. 3(a2) and (b2)). After 1 h of incubation, 85 and 65% of **II**, respectively, were accumulated. No formation of **III** in detectable amounts was fixed. At the further incubation the formation of **IV** was observed correlated with decrease in the content of **II**. Without PMS, F3 transformed **I** mostly to **III** (Fig. 4a). No formation of **II** was observed, while **IV** was detected in small amounts indicating slight 1(2)-dehydrogenase activity towards **III**.

The 1(2)-dehydrogenase activity of F3 towards **I** was slightly affected by PMS: about 5% of **II** was revealed after 5.5 h. PMS seemed to facilitate the formation of **IV** reached 80% in 22 h (Fig. 4b). The conversion of **I** by whole cells (33 mg/ml) in the presence of PMS (0.4 μg/ml) was carried out as a control experiment. The substrate **I** (1 g/l) was fully converted for 5.5 h with the yield of acetylated 1(2)-dehydroderivative (**II**) reached 92% and the content of **IV**: <5–7%.

The results indicated the presence of both soluble and membrane associated steroid esterases in *N. simplex*.

4. Discussion

In our recent publication, conversion of 21-acetate and 17,21-diacetate of pregna-4(5),9(11)-diene-17α,21-diol-3,20-dione by *N. simplex* was studied [10]. Along with 1(2)-dehydrogenation, deacetylation and 20β-reduction reactions were observed.

As follows from the present study, introduction of 16(17)--double bond into the structure of 21-acetate 9(11)-dehydrosteroid (with corresponding elimination of 17α-OH group) promoted conversion by *N. simplex*. Substrate (**I**) fully transformed during 20 h in the absence of MCD, while insufficient conversion of the corresponding 16(17)-saturated acetylated steroid—21-acetate of pregna-4(5),9(11)-diene-17α,21-diol-3,20-dione—was observed at the same conditions [unpublished data]. MCD supplement considerably enhanced bioconversions in both cases, and again, the transformation rate of 16(17)-dehydro steroid (**I**)

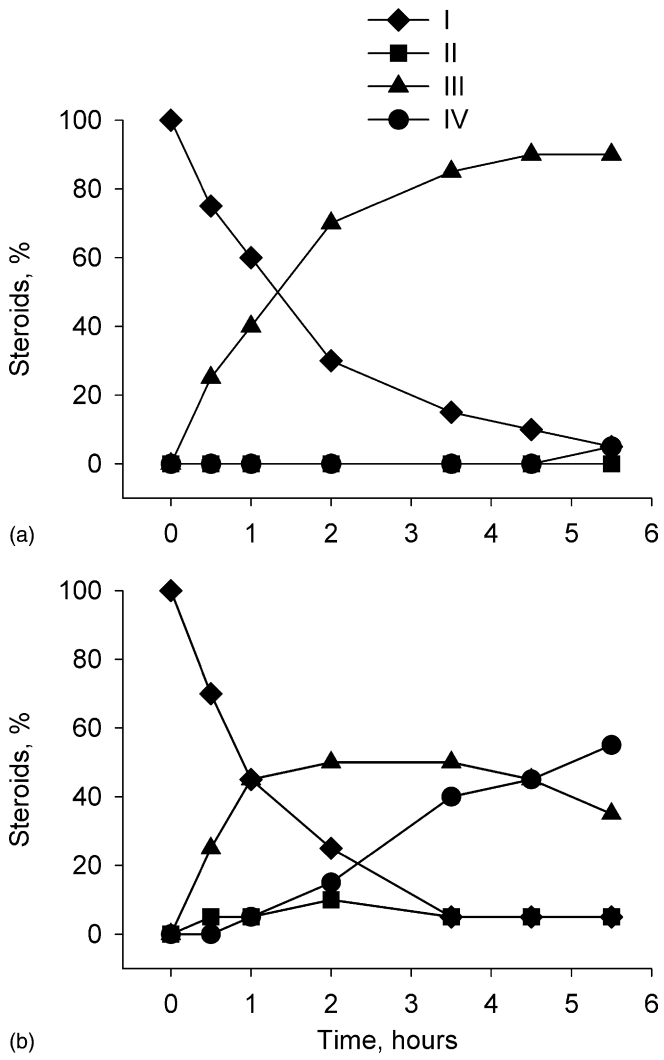

Fig. 4. Time course of **I** conversion by cell fraction F3 without PMS (a) and with PMS (b) in the presence of MCD. Substrate concentration: 1 g/l; biomass of F3 ≈ 1 g/l.

considerably preferred that of its 16(17)-saturated analogue [10].

Based on metabolite identification, 1(2)-dehydrogenation and 21-deacetylation were the major reactions in the pathway of **I** by *N. simplex*. The presence of 16(17)-double bond in **I** seemed to prevent 20β-reduction—no evidence of any 20β-reduced steroids formation from **I** was observed, while it was documented for the transformation of 16(17)-saturated 9(11)-dehydro steroids [10].

Two alternative pathways of **I** conversion by *N. simplex* were proposed: deacetylation of **I** followed by 1(2)-dehydrogenation (**I** → **III** → **IV**) and 1(2)-dehydrogenation followed by deacetylation (**I** → **II** → **IV**). The former was dominated at the conversion by non-induced cells or by induced cells in the absence of MCD. Evidently, steroid esterase expressed more affinity to **I** compared with 1(2)-dehydrogenase, while deacetylated steroid (**III**) was more available for 1(2)-dehydrogenase of *N. simplex* as compared with **I**. Similar tendencies were observed earlier for 16(17)-saturated steroid analogues [10].

Well-known inducibility of steroid 1(2)-dehydrogenase was confirmed in the present work. The presence of acetyl group in inducer did not influence the conversion of **I**. In addition to cortisone acetate and hydrocortisone, substrate **I** expressed inducing effect on 1(2)-dehydrogenase. The slight 1(2)-dehydrogenase activity towards **III** expressed in the presence of chloramphenicol can be attributed to the possible presence of constitutive 1(2)-dehydrogenase isoforms. The existence of few 1(2)-dehydrogenase isoenzymes was shown for relative organisms [11,12].

Unlike 1(2)-dehydrogenase, steroid esterases demonstrated a constitutive character. Deacetylation was influenced neither by inducer, nor protein synthesis inhibitor. Interestingly, in contrast to 1(2)-dehydrogenation, deacetylation process did not stimulated by MCD. Thus, decrease of biomass at the conversion of **I** by induced cells in the presence of MCD allowed almost selective production of acetylated 1(2)-dehydroanalogue (**II**).

Expectedly, cell fractionation showed 1(2)-dehydrogenase mostly associated with cell membrane and stimulated by exogenous artificial electron acceptor—PMS. The results are in accordance with current imagination on 1(2)-dehydrogenase localisation in bacterial cells [12].

Esterase activity towards **I** and **II** was found both in cytosol and membrane fractions as indicated by the formation of **III** and **IV**, respectively. The localisation of steroid esterases in *Nocardioides* or relative organisms was hitherto unreported, while exogenous and endogenous steroid esterases of other microbial groups were described [13,14].

The results allow to recommend microbial conversion of **I** by *N. simplex* for selective production of 21-acetoxypregna-1(2),4(5),9(11),16(17)-tetraene-21-ol-3,20-dione—an immediate precursor in the synthesis of fluorocorticoids.

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References

[1] A.M. Turuta, T.M. Fadeyeva, A.V. Kamernitsky, Transformed steroids. Part 188. Modes of synthesis of 11β-hydroxysubstituted 16-dehydro 17β-hydroxy and 16α-, 17α-epoxypregnanes based on 17α-ethynyl-11β,17β-dihydroxyandrostanes, Khim.-Pharm. Zhurn. 26 (3) (1992) 84–87 (in Russian).

[2] G.M. Cirilo, R.L. Jorge, J.N. Pedro, Synthesis and ^{1}H and ^{13}C nuclear magnetic resonance of 16-methylene-17α-hydroxypregna-1,4,9(11)-triene-3,20-dione, Steroids 58 (9) (1993) 396–399.

[3] N.M. Castro, J.A. Ruiz, Synthesis of 17α,21-dihydroxy-pregna-1,4,9(11)-triene-3,20-dione 21-acetate, Rev. CENIC Cienc. Quim. 25–26 (1–3) (1994–1995) 9–12.
[4] L.A. Kominek, H.J. Wolf, Process for preparing 1,2-dehydro steroids, US Patent 4-524-134 (1985).
[5] H.P. Schmauder, D. Schlosser, T. Gunther, A. Hattenbach, J. Sauerstein, F. Jungnickel, H. Augsten, Application of immobilised cells for biotransformations of steroids, J. Basic Microbiol. 31 (6) (1991) 453–477.
[6] K.A. Koshcheyenko, E.A. Borman, T.G. Baklashova, Dehydrogenation of cortisone acetate to prednisone acetate by the culture of *Mycobacterium globiforme* 193, Prikl. Biokhim. Mikrobiol. 10 (3) (1974) 376–384 (in Russian).
[7] G. Spassov, R. Krutzfeldt, W.S. Sheldrick, W. Wania, R. Vlahov, G. Snatzke, Crystallographic monitoring of microbiological steroid transformations, Eur. J. Appl. Microbiol. Biotechnol. 17 (1983) 80–84.
[8] Y. Catroux, J. Fournier, H. Blachere, Importance de la forme crystalline de e'acetate de cortisone pour la dehydrogenation en C-1 par *Arthrobacter simplex*, Can. J. Biochem. 46 (1968) 537–542.
[9] G.V. Sukhodolskaya, M.V. Donova, V.M. Nikolaeva, K.A. Koshcheyenko, D.V. Dovbnya, S.M. Khomutov, S.A. Gulevskaya, Method for production of 1,2-dehydroderivatives of 4-delta-3-ketosteroids. RF Patent 2-156-302 (2000) (in Russian).
[10] V.V. Fokina, G.V. Sukhodolskaya, B.P. Baskunov, K.F. Turchin, G.S. Grinenko, M.V. Donova, Microbial conversion of pregna-4,9(11)-diene-17α,21-diol-3,20-dione acetates by *Nocardioides simplex*, Steroids 68 (5) (2003) 415–421.
[11] B. Wagner, P.G. Atrat, J.E. Clark-Curtiss, M. Wagner, Localization of the steroid 1-dehydrogenease in *Rhodococcus erythropolis* IMET 7030 by immunoelectron microscopy, J. Basic Microbiol. 32 (1) (1992) 65–71.
[12] R. Van Der Geize, G.I. Hessels, L. Dijkhuizen, Molecular and functional characterization of the *kstD2* gene of *Rhodococcus erythropolis* SQ1 encoding a second 3-ketosteroid Δ^1-dehydrogenase isoenzyme, Microbiology 148 (10) (2002) 3285–3292.
[13] A.A. Akhrem, Yu.A. Titov, Steroids and microorganisms. Moscow (1970) 272–277 (in Russian).
[14] N.N. Lestrovaya, G.B. Ksandopulo, Zh.G. Karasevich, The effect of steroids on esterase activity of *Mycobacterium album Jensen*, Mikrobiologiya 40 (3) (1971) 461–465 (in Russian).