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Transformation of C_{19}-steroids and testosterone production by sterol-transforming strains of *Mycobacterium* spp.

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ABSTRACT

Redox conversions of exogenic C_{19}-steroids (androstenedione (AD), androstadienedione (ADD), testosterone and 1(2)-dehydrotestosterone) were studied using a mutant strain of *Mycobacterium* sp. Et1 with high level of 17β-hydroxysteroid dehydrogenase (17-HSD) activity. Factors affecting target 17β-reduction and side reactions by resting and growing cells were estimated. The effects of glucose supplementation, pH, mode of substrate addition were identified. The results confirmed that double reduction of androstadienedione, both of 17-keto group and 1(2)-double bond, is more effective for testosterone formation than a single reduction of 17-keto group of AD. These findings argued for the application of the strain capable of sterol side chain degradation and expressed 17-HSD, 3-ketosteroid-1(2)-dehydrogenase and 1-ene-reductase activities, for testosterone obtaining from sitosterol. Under optimal conditions, the conversion of sitosterol (5 g/l) by *Mycobacterium* sp. VKM Ac-1816D in laboratory fermenter resulted in 50–55% molar yield of testosterone.

1. Introduction

Testosterone (17-hydroxyandrost-4-ene-3-one) is an important pharmaceutical androgen steroid. Its key role in human health, well-being and sexual functioning is well-known [1,2].

Among the primary raw materials used for testosterone production, natural sterols (β-sitosterol and cholesterol) are mainly used due to their availability and low cost [3]. There are a number of methods described in order to obtain testosterone (T) from sterols: (a) by a multistep chemical synthesis [2] or (b) via intermediate androstenedione (AD) or androstadienedione (ADD) production using a combination of different methods [4]. The latter includes microbial transformation of sterol to AD(D) using fast-growing saprophytic mycobacteria, or relative microorganisms with blocked steroid nucleus cleaving enzymes [4,5] (steroid structures are shown in Fig. 1). The obtaining of AD(D) using strains capable of cleaving sterol side chain (e.g., *Mycobacterium* spp. NRRL B-3805, NRRL B-3683, *Rhodococcus erythropolis*, etc.) is a well-described process [6–9]. The next step involves converting AD(D) to T, either by a four-step chemical synthesis, or using microbial 17β-reduction by yeasts (*Saccharomyces cerevisiae* [10]), fungi (*Acremonium strictum*, *Beauveria bassiana*, *Cephalosporium aphidicola*, *Cochliobolus lunatus*, etc. [11–14]), or bacteria (*Pseudomonas testosteroni* (syn. *Comamonas testosteroni*), *Mycobacterium* spp. NRRL B-3683, NRRL B-12472, etc. [15–17]).

An alternative way is via single-step biotechnological process based on microbial conversion of sterols to testosterone by using the strains capable of both sterol side chain cleaving and 17β-reducing of 3,17-diketoandrostene. In most cases, bacterial strains double-blocked in 9α-hydroxylase and 1(2)-dehydrogenase activities were applied (e.g., *Mycobacterium* spp. NRRL B-3805, NRRL B-12472, ST2, *Lactobacillus bulgaricus*) [17–20]. Very few publications have described β-sitosterol to testosterone conversion by strains lacking 9α-hydroxylase, but expressing 1(2)-dehydrogenase activity (e.g., *Mycobacterium* sp. NRRL B-3683, etc.) [16,21].

The biochemical pathway of testosterone formation from β-sitosterol includes the multienzyme oxidation of the side chain followed by a reduction of the 17-keto group of 3,17-diketoandrostene by 17β-hydroxysteroid dehydrogenase (17-HSD) (Fig. 1). Along with testosterone, other C_{19}-steroids (1(2)-dehydrotestosterone (1(2)-DT), AD, ADD) can be formed due to the presence of 3-ketosteroid-1(2)-dehydrogenase, 1-ene-reductase activities and redox reactions at C-17 by 17-HSD in whole cells [22].

The strain of *Mycobacterium* sp. VKM Ac-1815D is deficient in 3-ketosteroid-1(2)-dehydrogenase activity, and accumulated AD is

Abbreviations: AD, androst-4-ene-3,17-dione; ADD, androsta-1,4-diene-3,17-dione; T, testosterone (17β-hydroxyandrost-4-ene-3-one); 1(2)-DT, 1(2)-dehydrotestosterone (17β-hydroxyandrost-1,4-diene-3-one); 17-HSD, 17β-hydroxysteroid dehydrogenase; mCD, randomly methylated β-cyclodextrin.

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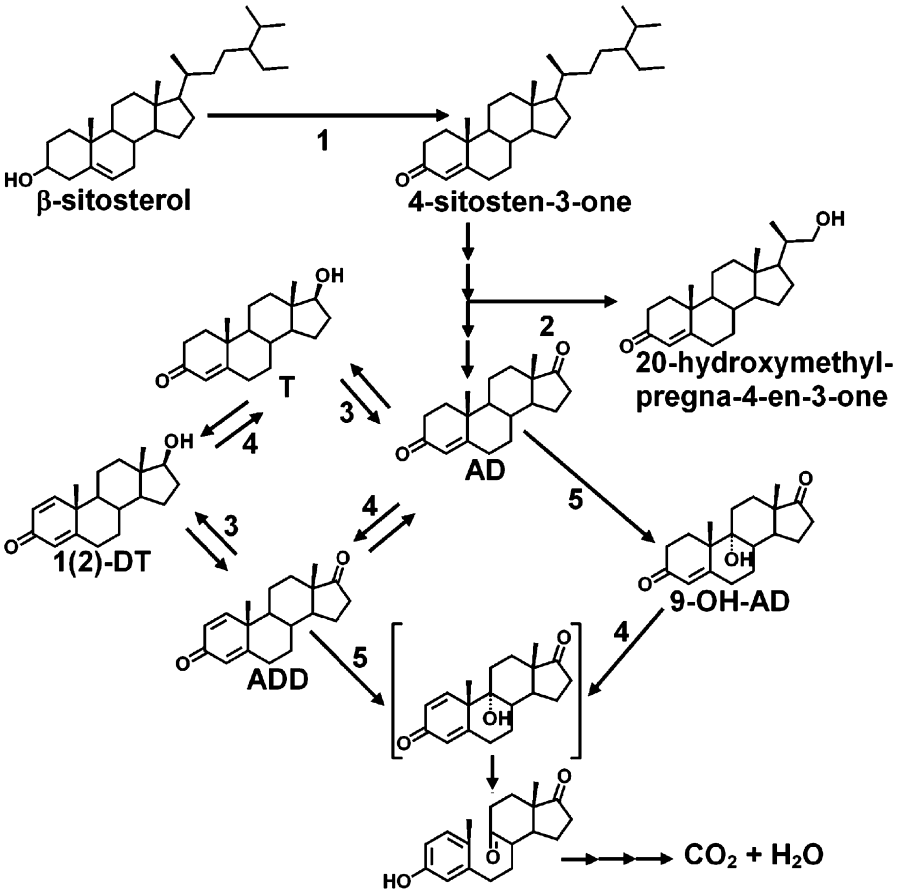

Fig. 1. β-Sitosterol transformation by *Mycobacterium* spp. (1) 3β-Hydroxysteroid oxidase; (2) sterol side chain oxidation enzymes; (3) 17-HSD; (4) 3-HSD3-ketosteroid-1(2)-dehydrogenase; (5) 9α-hydroxylase; 9-OH-AD, 9α-hydroxyandrost-4-ene-3,17-dione.

a major product from β-sitosterol, while ADD and T were formed in small amounts [23]. Its derivative, mutant *Mycobacterium* sp. Et1, expressed higher level of 17β-reducing activity, and retained the ability to degrade β-sitosterol side chain at C-17 [23]. The relative strain, *Mycobacterium* sp. VKM Ac-1816D converted sitosterol mainly to ADD due to the presence of high 1(2)-dehydrogenase activity [5].

In this work, we studied interconversions of C_{19}-steroids by *Mycobacterium* sp. Et1 and determined the factors affecting testosterone formation. Since the preference of double reduction of both 17-keto group and 1(2)-double bond was evidenced for testosterone production, the strain of *Mycobacterium* sp. VKM Ac-1816D was used for β-sitosterol conversion. The result was the development of an effective method for testosterone production.

2. Experimental

2.1. Microorganisms and cultivation

Mycobacterium sp. VKM Ac-1816D was obtained from All-Russian Collection of Microorganisms (VKM IBPM RAS). *Mycobacterium* sp. Et1 mutant strain was derived from the parent *Mycobacterium* sp. VKM Ac-1815D and maintained as reported previously [23]. The strains of *Mycobacterium* spp. were grown aerobically on glycerol mineral medium as described earlier [24].

2.2. Reagents

β-Sitosterol (of 91.4% purity) was purchased from Kaukas Co. (Finland), androst-4-ene-3,17-dione, androsta-1,4-diene-3,17-dione, testosterone were obtained from Sigma (USA). 17β-hydroxyandrost-1,4-diene-3-one (1(2)-dehydrotestosterone, 1(2)-DT) (98–99% purity, mass-spectrometry (*m*/*z* (%) M^+ 286(11), 271(1), 147(11), 122(100), 121(25), 91(23), m.p. 170–173 °C) was obtained from Laboratory MTOC (IBPM RAS), randomly methylated β-cyclodextrin CAWASOL® W7 M1.8 (mCD) was purchased from Wacker Chemie (Germany). Yeast extract and agar were obtained from Difco (USA); other materials were of reagent grade and were purchased from domestic companies.

2.3. Bioconversion of C_{19}-steroids by washed cells

The seed culture of *Mycobacterium* sp. Et1 (10%, v/v) was added to the glycerol mineral medium [23] and incubated on a rotary shaker (220 rpm) at 29–30 °C. 8–10 h after inoculation, the inducer of 17-HSD, AD, was added to a final concentration of 0.25 g/l as a solution in methyl alcohol. The content of methanol did not exceed 2% (v/v). After 23–24 h growth, the cells were harvested by centrifugation at 6000 × *g*, and washed twice with sterile 0.02 M potassium phosphate buffer (pH 7.0). The biomass obtained (2–6 g/l, wet weight) was re-suspended in 50–100 ml sterile 0.01 M potassium phosphate buffer (pH 7.0). The substrates, AD, or ADD (1.75–3.5 mM), were added as fine powders. The transformation

was carried out in 750-ml Erlenmeyer flasks at 29–30 °C on the rotary shaker (200 rpm) for 24–96 h. Glucose was added to a buffer medium to a final concentration of 0–40 g/l.

Different methods of substrate addition were assessed as follows: AD (3.49 mM) was added as a solution in dimethylformamide (DMF), dimethylsulfoxide (DMSO), or methanol. The solvent concentration did not exceed 2% (v/v). In some experiments, AD (3.45 mM) was added as an aqueous mCD (7.8 g/l) solution. In controls, fine powder of AD (3.45 mM) was used.

2.4. Bioconversion of C_{19}-steroids by growing cells

The seed culture of *Mycobacterium* sp. Et1 (10%, v/v) was inoculated in 100 ml of glycerol mineral medium [23] containing steroid substrate, and incubated in 750-ml Erlenmeyer flasks aerobically at 29–30 °C on the rotary shaker (200 rpm) for 96–170 h. In some experiments, mCD (0–7.8 g/l) was added to the medium. The concentration of substrates varied from 1.75 to 35 mM.

2.5. Bioconversion of sitosterol in fermenter

β-Sitosterol transformation by *Mycobacterium* sp. VKM Ac-1816D was carried out in 3 l fermenter (ANKUM, Russia) loaded with 1.5 l of medium contained (g/l): glucose, 30; KH_2PO_4, 1; K_2HPO_4, 4; $(NH_4)_2SO_4$, 3; $MgSO_4$, 0.2; $FeSO_4$, 0.01; $ZnSO_4$, 0.002; urea, 0.25; mCD, 13.3. Sitosterol (12.08 mM, 5 g/l) was added to the medium before sterilization as an aqueous suspension after ultrasonic treatment for 10 min (MSE-150B disintegrator (USA)). The fermentation was carried out at 29–30 °C; pH adjusted at 6.5–7.0; stirring rate, 220–500 rpm; dissolved oxygen concentration (DO), 10–25%. Glucose (15 g/l) was added to the medium 50 and 90 h after inoculation. The final volume of cultivation broth was 1.67–1.78 l. The duration of sitosterol to testosterone bioconversion was 135–140 h.

2.6. Isolation and purification of testosterone

After the completion of the transformation, the cultivation broth was heated to 60 °C for 15 min. The cells were separated by centrifugation at 6000 × *g* for 30 min. Cell-free cultivation broth was extracted by ethyl acetate (1.0:0.3, v/v) three times. Ethyl acetate extract was evaporated using rotary evaporator to 300 ml. The concentrate was twice washed by distilled water (200 ml) and the water phase obtained was re-extracted with ethyl acetate in the proportion of 1:0.5 (v/v). The extracts were combined (400 ml), dried over sodium sulfate for 12 h and then evaporated to 5 ml after filtration of sodium sulfate pellet. Testosterone was crystallized from the concentrate obtained, and re-crystallized from ethyl alcohol.

2.7. Steroid analyses

TLC: steroids were extracted with ethyl acetate, aliquots were applied to Sorbfil A-UV (Russia) or Kieselgel 254 (Merck) plates, developed in benzene/acetone (3:1, v/v), and visualized under UV light (254 nm). Sitosterol was visualized by spraying of the plate by 10% phosphoric–molybdic acid solution in ethanol.

HPLC: 3-ketosteroids were analysed by reversed-phase HPLC (Hp 1100) using C_{18}-column Serva Octadecyl-Dialtosil (250 mm × 4.6 mm; 5 μm) and UV detection at 240 nm, 30 °C. Mobile phase was composed of acetonitrile:water:glacial acetic acid (55:45:0.01, v/v/v), flow rate of 1 ml/min. AD, ADD, T and 1(2)-DT (98–99% purity) were used as standards.

MS: MS analysis was carried out using Finnigan MAT-8430 mass spectrometer (Germany) with direct inlet and ionization energy of 70 eV.

^1H NMR: ^{1}H NMR spectra recorded on a Unity + 400 (Varian) spectrophotometer at 400 mHz. The signal of resting protons D_6-acetone (δ 2.05) was used as an internal standard.

Product yield of the major conversion metabolites was estimated as:

$$\text{product yield}\,(\%) = \frac{\text{weight of product/MW of product}}{\text{weight of substrate/MW of substrate}} \times 100,$$

where MW is the molecular weight.

The yield of T during the conversion of β-sitosterol was calculated taking into account the increasing volume at the end of fermentation.

3. Results and discussion

3.1. Effect of glucose

Sterol-transforming strain of *Mycobacterium* Et1 was shown to catalyze reversible conversions of 3-keto-androstanes at C-17 due to the presence of 17-HSDs. The strain reduced 17-ketones (AD and ADD) to 17β-alcohols (T and 1(2)-DT, respectively), and oxidized 17β-alcohols to the corresponding 17-ketones [22,23].

Similar to other dehydrogenases, 17-HSDs play a significant role in the regulation of the pool of reducing equivalents in cells. The ratio of NAD^+/NADH is one of the factors influencing the direction of the reaction [4]. The ratio of the whole-cell biocatalysts can be regulated by carbon source supplementation.

We studied the effect of excess glucose on the bioconversion of 17-ketones and 17β-alcohols on washed *Mycobacterium* sp. Et1 cells. As shown in Table 1, the addition of glucose stimulated 17β-reduction of ADD: the yield of 1(2)-DT in the glucose medium increased 2.4–2.7 times compared to the control (without glucose). Trace amounts of T and AD were detected after 18–20 h bioconversion (data not shown) thus demonstrating the slight 1-ene-reducing activity of the strain. Therefore, unlike 17β-reduction, 1-ene-hydrogenation of ADD by *Mycobacterium* sp. Et1 was not stimulated by glucose supplementation.

Similarly, glucose had no significant effect on AD bioconversion. The molar yield of T from AD in glucose supplemented buffer was approximately the same as in the control (without glucose) and did not exceed 3–5%. The formation of ADD from AD in both cases was about 1–2% after a 90 h incubation (data not shown).

In the case of the reverse reaction, - oxidation of 17β-hydroxyl was notably suppressed in the presence of glucose. The addition of glucose led to a reduction in the formation of ADD from 1(2)-DT by 3.5–4.5 times, and the production of AD from T, by 1.7–10 times (Table 1).

The results indicated a glucose-mediated shift in 17-HSD activity of cells towards increased reduction and decreased oxidation. Clearly, glucose utilization promoted a 17β-reduction and inhibited the reverse oxidation of the product formed. This effect can be explained by a shift in the NADH/NAD^+ ratio provided by glucose uptake at the conditions of the competition for the respiratory chain.

Similar effects of glucose addition on 17β-reduction of AD(D) by *Mycobacterium* spp. have been reported [21]. Production of 1(2)-DT from ADD increased with the addition of sugars, while poor conversion of AD was observed. Glucose feeding during cholesterol conversion promoted testosterone formation by a mutant of *Mycobacterium* sp. NRRL B-3805 whereas AD mainly accumulated in the absence of glucose [18].

Table 1
Glucose effect on 3-keto-C_{19}-steroids conversion by *Mycobacterium* sp. Et1.

Substrate	Major product	Time (h)	Product molar yield (%)[a]	
			At the addition of glucose	Without glucose addition (control)
ADD	1(2)-DT	7	32	12
		21	86	36
		25	40	Traces
1(2)-DT	ADD	32	9	32
		48	11	52
		53	Traces	87
AD	T	72	3	3
		90	5	4
T	AD	26	12	20
		48	8	36
		72	6	68

Bioconversion was carried out in 0.01 M potassium phosphate buffer (pH 7.0); initial concentration of substrate, 1.75 mM; biomass, 4 g/l (wet weight); glucose, 40 g/l.
[a] Average values were obtained from at least three replicates.

Table 2
The effect of substrate addition modes on AD conversion by washed cells of *Mycobacterium* sp. Et1.

Substrate addition mode	Maximum molar yield of major products (%)[a]		Residual substrate (%)
	T	ADD	
Fine powder (control)	1.4	2.3	50–55
DMF solution	1.7	4.9	35–40
DMSO solution	1.7	4.3	35–40
Methanol solution	2.3	1.4	30–35
mCD solution	6	14.3	20–25

Bioconversion was carried out in 0.01 M potassium phosphate buffer (pH 7.0); initial substrate (AD) concentration, 3.49 mM; washed cells concentration, 6 g/l; mCD concentration, 7.8 g/l; final solvent concentration did not exceed 2% (v/v). Residual AD was estimated after 120 h conversion. The yield of T and ADD reached maximum level for 54 and 70 h, respectively.
[a] Average values were obtained from at least three replicates.

3.2. Effect of substrate addition modes

In contrast to ADD, exogenic AD was poorly transformed by mycobacteria. The solubility of AD (0.18 mM) was shown to be much lower that that of ADD (1.63 mM) [10,25]. We proposed that poor AD solubility can restrict its availability to the cell enzymes. To intensify AD bioconversion, different modes of addition to the incubation medium were tested.

As shown in Table 2, ADD was formed as a major product from AD at the addition of AD either as a fine powder, or solution in DMF or DMSO. Maximum levels of 0.15–0.17 mM were reached with the use of DMF or DMSO solutions. The formation of T slightly increased with the use of solvents, while the level of unconverted substrate decreased. This demonstrates a possible solvent-mediated steroid consumption (Table 2).

The application of mCD as a solubilizing agent promoted AD bioconversion. Testosterone and ADD content reached 0.21 and 0.5 mM, respectively, and the amount of unconverted substrate decreased by 2.2–2.5 times when compared to the control (without mCD addition). Thus, the application of mCD stimulated both 17β-reduction and 1(2)-dehydrogenation of AD by *Mycobacterium* sp. Et1.

At the same time, 17β-reduction of ADD seemed to be suppressed by mCD: no 1(2)-DT was formed in the presence of mCD, while almost full ADD to 1(2)-DT conversion was observed in a control (without mCD). It is of interest that addition of mCD stimulated reduction of 1(2)-double bond, - AD was formed as a major product from ADD (Table 3).

The intensification of 17β-reduction of AD to T by *S. cerevisiae* in the presence of chemically modified cyclodextrins was described [10]. Recently, we observed considerable effect of cyclodextrins on the growth, sterol-transforming activity and cell envelope features of sterol-transforming mycobacteria [24]. Cyclodextrin-mediated intensification of T production from cholesterol by *L. bulgaricus* was reported [20].

The examples of cyclodextrin-mediated shift in the direction of biocatalytic reactions are sparse. The positions of hydroxyl group introduction into 4(20)-taxadienes by *Absidia coerulea* were changed in the presence of cyclodextrins [26]. The selectivity of progesterone transformation by mycelium preparations

Table 3
Effect of mCD addition on ADD conversion by growing *Mycobacterium* sp. Et1 cells.

Time (h)	Product molar yield (%)[a]			
	Without mCD (control)		In the presence of mCD	
	1(2)-DT	AD	1(2)-DT	AD
4	40	0	0	4
70	71	0	0	24
100	95	Traces	Traces	32

The growth medium was supplemented with glucose (40 g/l). ADD (1.75 mM) was added as a solution in mCD. Final mCD content in the medium was 4.5 g/l.
[a] Average values were obtained from at least three replicates.

Table 4
Effect of pH on 17β-reduction of AD by *Mycobacterium* sp. Et1.

pH	Molar yield of 17β-OH product (%)[a]	
	T	1(2)-DT
5.7	4.0	1.2
6.5	2.9	1.2
7.1	6.0	0.3
7.5	4.9	0.57
8.1	5.2	4.6
8.4	4.6	1.4

Bioconversion of AD (3.49 mM) was carried out by growing cells in the presence of mCD (7.8 g/l) and 40 g/l glucose for 64 h.
[a] Average values were obtained from at least three replicates.

from *Aspergillus fumigatus* was increased in the presence of β-cyclodextrin [27]. The order of 1(2)-dehydrogenation and 21-deacetylation reactions at the bioconversion of 21-acetoxy-pregna-4(5),9(11),16(17)-triene-21-ol,3,20-dione by *Nocardioides simplex* VKM Ac-2033 changed depending on mCD supplementation [28].

Different mCD effects on 17β-reduction of AD and ADD, as well as mCD-mediated shift in ADD bioconversion towards preferred 1-ene-hydrogenation are not fully clear. The properties of AD and ADD inclusion complexes with mCD should be specially investigated. The concentration of the free form of every steroid in aqueous medium, and the properties of the corresponding enzymes should be specifically studied.

3.3. Effect of pH

The influence of pH on AD(D) bioconversion was studied ranging pH-value from 5.7 to 8.4. The formation of T from AD reached maximum level of 0.21 mM (6%) at neutral pH, and decreased at the acid or basic pH-values (Table 4). The highest yield of 1(2)-DT from AD was detected at pH 8.1 (4.6%) while considerably less 1(2)-DT (0.3–0.57%) was observed at pH 7.0–7.4. At the same time, regardless of pH-value, 15–20% AD remained unconverted with low yield of T (3–6%).

The formation of 1(2)-DT from ADD by washed cells reached a maximum level of 1.4 mM for 22–24 h at pH 7.1, and decreased at pH 5.7 and pH 8.4 by 3.3 and 1.8 times, respectively (data not shown).

As demonstrated in the literature, the intensity and course of bi-directional steroid conversion at C-17 extensively depended on pH value. In most cases, 17β-reduction of 3,17-diketosteroids reached an optimum at acid pH-values. For example, a maximum concentration of 17-hydroxysteroids was observed when ADD transformation by washed cells of *Mycobacterium* sp. NRRL B-3683 was carried out in K-phosphate buffer at pH 6.0 [21].

The regulation of pH can influence the selectivity of the process. For instance, the increase of pH-value at sterol bioconversion by mycobacteria resulted in the suppression of 17β-reduction and stimulation of 17β-oxidation [18]. At the same time, pH shifts in a range of 3–9 or 5–8.5 did not affected 17β-reduction of AD by *S. cerevisiae* or ADD by *A. strictum* [1,11]. The conversion of AD by *B. bassiana* was strongly regulated by pH: at pH 6.0, mainly 11α-hydroxylation of AD was observed, while at the neutral pH-values AD was reduced at C-17 [12].

Our experiments confirmed pH-dependency of steroid 17β-reduction by *Mycobacterium* sp. Et1 with optimum at pH 7.0.

3.4. Effect of substrate concentration

During first phase of ADD bioconversion, 1(2)-DT accumulated as a major product. After reaching a highest level, 1(2)-DT concentration decreased, while the content of T and AD increased. As shown in Table 5, an increase of substrate dose resulted in a prolonged period of transformation and a decline of the degree of ADD bioconversion. Full conversion of 0.87 mM ADD was reached after 96 h, while 40–45% of ADD remained unconverted even after 250 h when 34.9 mM ADD was initially added.

Maximum molar yield of 1(2)-DT (85%) was reached at ADD concentration of 3.49 mM. An increase of ADD concentration from 3.49 to 34.92 mM resulted in an increased AD yield from 0.2 to 49% (Table 5).

This effect can probably be attributed to ADD toxicity for mycobacteria. ADD was shown to inhibit the growth, respiratory activity and sterol side chain cleavage by *Mycobacterium* sp. NRRL B-3683 [8,29]. In the presence of exogenous ADD, a substantial depletion of ADD production from sterol by *Mycobacterium* sp. B-3683 and Ex4 was observed. The mutant strains resistant to androstanes were obtained from these organisms. An apparent reduction of ADD to AD and T was noticed [30].

3.5. β-Sitosterol to testosterone conversion by Mycobacterium sp. VKM Ac-1816D

Following from the data presented above, the attempts to promote AD to T conversion by *Mycobacterium* sp. Et1 did not result in considerable effects. The molar yields of T from exogenic AD did not exceed 10% even at low substrate loadings. The results demonstrate that AD was not suitable a substrate for conversion to testosterone.

Recently, we have isolated and purified two intracellular 17-HSDs from *Mycobacterium* sp. Et1. Close association of 17-HSD and 1-ene-reductase activities were revealed [22]. In the present study, the connection of 17β-reduction and hydrogenation of 1(2)-double bond in whole cells of *Mycobacterium* sp. Et1 was confirmed. The results correlated with the data published for *Mycobacterium* sp. NRRL B-3683 [16,21].

As compared with AD, ADD was shown to be an easier convertible substrate. We proposed that the pathway via sterol side chain oxidation to ADD followed by double reduction of both 17-keto group and 1(2)-double bond can be preferable for effective

Table 5
The effect of substrate concentration on ADD bioconversion by *Mycobacterium* sp. Et1.

ADD (mM)	Maximum product molar yield (%)[a]			Time of maximum of 1(2)-DT yield (h)	Substrate conversion (%)	Conversion period (h)
	1(2)-DT	T	AD			
0.87	20	39	30	48	95–100	96
1.75	60	5	7	54	95–100	154
3.49	85	11	0.2	74	90–95	178
6.98	52	0.4	36	96	75–80	198
17.46	48	0.4	42	124	65–70	220
34.92	45	7	49	148	55–60	250

The bioconversion of ADD was carried out by growing cells of Et1 without mCD. The maximum yields of T and AD are given reached at the end of conversion period.
[a] Average values were obtained from at least three replicates.

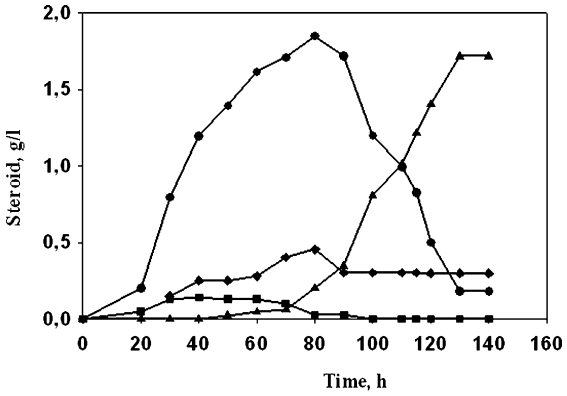

Fig. 2. Time course of metabolite concentration during sitosterol conversion to T using *Mycobacterium* sp. VKM Ac-1816D. (●) AD, (■) ADD, (▲) T, and (♦) 20-hydroxymethyl-pregna-4-en-3-one. The bioconversion of sitosterol by *Mycobacterium* sp. VKM Ac-1816D was carried out in the presence of glucose and mCD at 29–30 °C; stirring rate, 220–500 rpm; DO, 10–25%; pH 6.5–7.0. Extra amount of glucose was added after 50 and 90 h. Average values were obtained from at least three replicants.

T production from β-sitosterol. The *Mycobacterium* sp. VKM Ac-1816D strain uses this pathway. It can effectively produce of ADD from β-sitosterol [5] (Fig. 1). The level of 17-HSD activity in this strain was shown to be comparable with that in *Mycobacterium* sp. Et1 [31].

We used *Mycobacterium* sp. VKM Ac-1816D strain for sitosterol conversion using the conditions optimized in this study for highest expression of 17β-reducing activity. As shown in Fig. 2, at the first transformation stage, β-sitosterol was converted mostly to AD. Maximum levels of 1.8–1.9 g/l were reached 70–80 h after transformation. During a second transformation period (after 70 h) a decrease of AD was observed. This correlated with an increase in the accumulation rate of T. The yield of T reached a maximum of 1.6–1.7 g/l to 135–145 h after transformation. ADD and 20-hydroxymethyl-pregna-4-en-3-one were detected as by-products yielding 10–12%. The concentrations of 20-hydroxymethyl-pregna-1,4-diene-3-one and 1(2)-DT concentrations were less than 1% (of theor.). The level of non-converted sitosterol was estimated as 25–30% of initial concentration.

The crystalline powder of T (96–98% purity) was recovered from cell-free cultivation broth with the overall molar yield from sitosterol of 33–35%. It corresponded to the authentic standard with respect to its basic characteristics: needle crystals, m.p. 152.5–155.0 °C; mass-spectrometry: (*m*/*z* (%) M^+ 288(80), 246(41), 228(9), 203(19), 147(32), 124(100)); ^{1}H NMR ($CDCl_3$): δ 5.74 (4-H), 3.67 (17α-OH), 0.81 (18-CH_3), 1.21 (19-CH_3).

Production of T from natural sterols in a single biotechnological operation was described earlier [17–20]. The molar yield of crystalline T powder of 19% from β-sitosterol using *Mycobacterium* sp. NRRL B-12472 was patented [17]. A single step 100 h transformation of cholesterol (1 g/l) by *Mycobacterium* sp. NRRL B-3805 resulted in T molar yield of 42% (the content in fermentation broth, without recovery of crystalline product) [18]. The conversion of phytosterol (1 g/l) by a mutant strain of *Mycobacterium* sp. ST2 yielded 30% (mol/mol) testosterone in the fermentation broth (without crystalline powder recovery) [19].

Thus, the application of ADD-producing *Mycobacterium* sp. VKM Ac-1816D allowed us to obtain testosterone in a single biotechnological operation from sitosterol. A productivity of the method described exceeded the results obtained from previously reported approaches used for other sterol-transforming mycobacteria and related microorganisms.

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