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EXPERIMENTAL ARTICLES

The 1(2)-Dehydrogenation of Steroid Substrates by *Nocardioides simplex* VKM Ac-2033D

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Abstract—The bacterium formerly known as *Arthrobacter globiformis* 193 has high 1(2)-dehydrogenase activity toward pharmaceutically important steroids, 9(11)-dehydrocortexolone in particular. The complex analysis of the morphostructural, physiological, biochemical, and phylogenetic properties of this bacterium allowed us to reclassify it into *Nocardioides simplex* (*N. simplex* VKM Ac-2033D).

Key words: 1(2)-dehydrogenation, 3-ketosteroid 1(2)-dehydrogenase, *Nocardioides simplex*, 16S rDNA.

Microorganisms with 3-ketosteroid 1(2)-dehydrogenase activity are widely used in the manufacture of modern glucocorticoids [1]. The chemical methods of obtaining 1(2)-dehydrogenated steroids are multistage and may lead to spontaneous aromatization of the A ring of a steroid molecule and therefore provide a relatively low yield of the end products (from 11 to 63%) [2, 3]. At the same time, the microbial 1(2)-dehydrogenation of 3-ketosteroids is characterized by high rates, selectivity, and yield [1].

Reportedly, steroids can be converted by bacteria from the genera *Arthrobacter, Corynebacterium, Bacillus*, and *Nocardia* [1, 4, 5]. The strain known as *Arthrobacter globiformis* 193 has high 3-ketosteroid 1(2)-dehydrogenase activity toward a wide range of steroids, such as androstenedione, progesterone, hydrocortisone, 6α-methylhydrocortisone, cortexolone, and 21-acetylcortexolone [1, 6]. The microbial 1(2)-dehydrogenation of acetylated 9(11)-dehydro-3-ketosteroids is of great scientific and practical value because the products of this reaction serve as the key semiproducts for the manufacture of advanced 9-fluoro-substituted corticosteroid hormones (dexamethasone, betamethasone, triamcinolone, and others).

The mechanism of the microbial conversion of acetylated 9(11)-dehydrosteroids is far from being properly understood [5]. Moreover, the taxonomic position of *A. globiformis* 193 is in doubt. The culture was first isolated from soil samples and described as *Mycobacterium* sp. [7]. Later, the isolate was successively reclassified into *Mycobacterium globiforme* 193 [8], *Arthrobacter globiforme* 193 [9], and *A. globiformis* 193 [10]. Careful analysis showed that the cell wall of this organism contains L,L-diaminopimelic acid, which is not typical of bacteria from the genus *Arthrobacter*, whose cell wall contains peptidoglycan A4 with lysine as a component [11].

This prompted us to revise the taxonomic position of *A. globiformis* 193 and to study the 1(2)-dehydrogenation of 9(11)-dehydrocortexolone derivatives by this strain.

MATERIALS AND METHODS

Chemicals. Hydrocortisone was purchased from Russel-Uclaf (France) and prednisolone and cortisone acetate from Akrikhin (Russia). 9(11)-Dehydrocortexolone and its 21-acetate and 17,21-diacetate, as well as 16α-methylhydrocortisone and 9(11),16(17)-dienecortexolone 21-acetate, were obtained from G.S. Grinenko, All-Russia Chemical and Pharmaceutical Research Institute, Moscow. The antibiotics sagamycin and ceftasidim were manufactured in Japan and the United Kingdom, respectively. The other antibiotics and reagents used in the work were of Russian or Ukrainian production.

Bacterial strains. The strains *Nocardioides simplex* VKM Ac-1118^T, *N. simplex* VKM Ac-925, *N. jensenii* VKM Ac-1878^T, *N. albus* VKM Ac-805^T, *N. luteus* VKM Ac-1246^T, *Nocardioides* sp. VKM Ac-564, *Nocardioides* sp. VKM Ac-565, and *Nocardioides* sp. VKM B-806 were obtained from the All-Russia Collection of Microorganisms (VKM). The strain *Arthrobacter globiformis* 193 was obtained from the collection at the Laboratory for Microbial Transformation of Organic Compounds, Skryabin Institute of Biochemistry and Physiology of Microorganisms.

Cultivation conditions and induction of 3-ketosteroid 1(2)-dehydrogenase. The strains were cultivated and 3-ketosteroid 1(2)-dehydrogenase was induced as

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described earlier [6]. For chemotaxonomic analysis, cells were grown in a medium containing (g/l) peptone, 5; yeast extract, 3; K_2HPO_4, 0.2; and glucose, 5. Cultivation was carried out aerobically at 30°C in Erlenmeyer flasks on a shaker (180 rpm) for 17–20 h.

Transformation of steroids. Steroids were transformed in 750-ml Erlenmeyer flasks with 50–100 ml of 0.01 M Na phosphate buffer (pH 7.2) containing washed microbial cells in amounts from 0.1 to 10 g/l in dry weight and a powdered steroid in amounts from 1 to 5 g/l. The flasks were incubated at 28–30°C on a shaker (220 rpm) [9]. In some experiments, steroids were transformed in the presence of cyclodextrins, as described elsewhere [1, 12].

Analysis of transformation products. Samples (1 ml in volume), withdrawn from the transformation flask at regular intervals, were extracted with 5 ml of ethylacetate, and the extracts were analyzed by thin-layer chromatography on Silufol UV_{254} plates (Czech Republic). Aliquots (10–25 μl in volume) of the extracts and marker substances were applied to the TLC plates, and the plates were developed in a benzene–acetone (3 : 1, v/v) mixture. After separation, steroids were visualized in a Desaga HP-UV-VIS chemiscope at 254 nm. Marker substances (9(11)-dehydrocortexolone and its 21-acetate and 17,21-diacetate; 16α-methylhydrocortisone; 1(2),9(11)-dienecortexolone and its 21-acetate and 17,21-diacetate; and 16α-methylprednisolone) were applied to TLC plates in amounts varied from 1 to 10 μg. Some samples were analyzed spectrophotometrically.

Electron microscopy. A drop of a diluted culture was applied onto a formvar-coated grid (Serva). After incubation for 30 s, the drop was removed with a piece of filter paper, and the grid was dried in the air. Then the grid was placed in a JEE-4C coating device under an angle of 30–40° and coated with a platinum–carbon mixture for 5–6 s in a vacuum of about $\approx 4 \times 10^{-4}$ N/m^2. Specimens were examined in a JEM-100B electron microscope operated at 80 kV. Preparations for thin sectioning were fixed with 6.2% glutaraldehyde, refixed with 1% OsO_4, dehydrated with ethanol, and embedded in epon resin. Thin sections were obtained using an LKB-880 2A ultratome (Sweden) and examined in a JEM-7A electron microscope.

Light microscopy. Gram-stained cells were examined under an optical microscope at a magnification of 900× using an oil-immersion objective.

Antibiotic susceptibility tests. Bacteria were tested for susceptibility to antibiotics on a minimal Evans medium [13] containing 20 g/l agar and supplemented with 10 g/l each corn extract and glucose. The pH of the medium was adjusted to 7.8. After sterilization at 0.5 atm for 0.5 h, the pH spontaneously decreased to 7.2. Aliquots of cultures, containing 10^5–10^6 cells, were plated onto the agar medium, and the agar plates were incubated at 24°C for 48 h.

Utilization of carbon compounds. The ability of bacteria to utilize erythritol, arabinose, xylitol, adonitol, ribose, rhamnose, inositol, mannitol, mannose, sorbitol, glucose, fructose, lactose, melibiose, raffinose, sucrose, and cellobiose was assayed as described by Pridham and Gottlieb [14].

Hydrolase activity. The ability of bacteria to hydrolyze arbutin, esculin, casein, dextran, xanthine, hypoxanthine, adenine, gelatin, Tween-80, and tyrosine was determined by the standard method [15].

Chemotaxonomic analysis. Cell walls were purified by the Schleifer and Kandler method [11]. Diaminopimelic acid isomers were determined by the method of Becker *et al.* [16]. The amino acid composition of peptidoglycans was determined using a Hitachi amino acid analyzer. Isoprenoid quinones were isolated and purified as described by Collins and Jones [17]. Menaquinones were identified using an MAT 8430 mass spectrometer (Germany). Fatty acids were identified as described elsewhere [18].

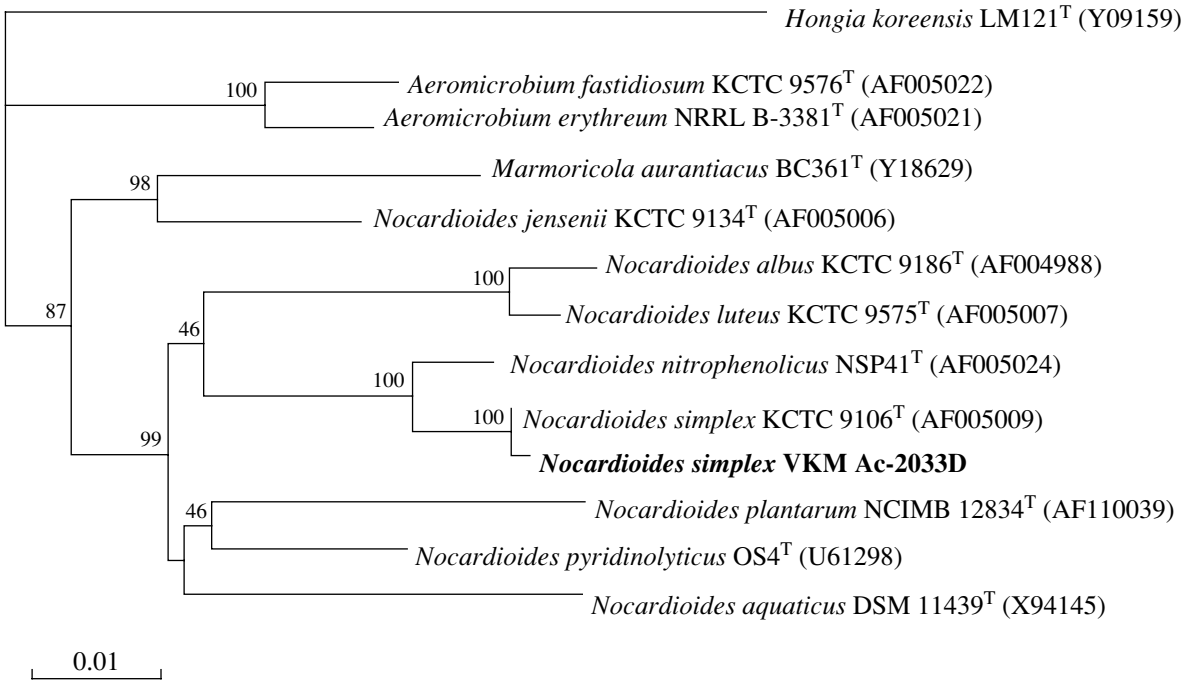
16S rRNA gene sequencing. 16S rRNA genes were amplified using the universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG) and 1522r (5'-AAGGAGGTGATCCARCCGCA). PCR products were purified on Promega columns and analyzed using an ABI-310 DNA sequencer (Perkin-Elmer) and the enzymatic Big Dye Terminator Kit (Perkin-Elmer) according to the manufacturer's protocol.

Phylogenetic analysis. The 16S rRNA gene sequence of the strain studied (1470 bp) was aligned manually with the sequences of reference strains from the genus *Nocardioides* and the order *Nocardioidaceae* with the aid of the CLUSTAL W program. Evolutionary distance was expressed as the number of substitutions per 100 nucleotides [19]. A phylogenetic tree was generated by the neighbor-joining method with the aid of the TREECON software package. Bootstrap values were derived by analyzing 100 alternatives.

RESULTS AND DISCUSSION

Cultural and morphostructural properties. The strain *A. globiformis* 193 grown on corn–glucose agar and bacterial agar with casein produced slightly glossy round colonies with even edges, which were grayish–white in color and had a diameter of 2–3 mm. Colonies grown on mineral agar with sucrose as the sole carbon source were ocher-colored, or yellowish when grown on mineral agar with arabinose, rhamnose, or cellobiose.

Cells were irregular, sometimes curved, rods. Cells grown on corn–glucose medium varied from 0.3 to 1.3 μm in length and from 0.1 to 0.5 μm in diameter. The life cycle coccus–rod–coccus, which is typical of the genus *Arthrobacter*, was not observed. Cells had a polar flagellum. Intracytoplasmic membranes were represented by concentric lamellar and tubular–vesicular mesosomes. Electron-opaque inclusions found in most cells presumably represented volutin or poly-β-hydroxybutyrate granules. The cell wall was dense and homo-



Phylogenetic tree showing the position of *N. simplex* VKM Ac-2033D among close taxa. Bootstrap values, indicated on the tree, were derived by analyzing 100 alternatives. The scale bar represents 1 nucleotide substitution per 100 nucleotides.

geneous and had a thickness of 2 μm. Cell capsules were not observed.

Chemotaxonomic features. The cell-wall peptidoglycan contained alanine, glutamic acid, L,L-diaminopimelic acid, and glycine in molar ratios of 2 : 1 : 1 : 1. The major isoprenoid quinone of the respiratory chain was found to be menaquinone-8 (H_4). Fatty acids were dominated by saturated *iso*- and *anteiso*-branched acids and contained tuberculostearic acid. Mycolic acids were absent. Such a set of chemotaxonomic features is typical of the genus *Nocardioides* [20].

Phylogenetic analysis. The phylogenetic analysis of the 16S rRNA gene sequence (1470 bp) of *A. globiformis* 193 performed with the aid of the BLAST program showed its almost complete identity with the 16S rRNA gene sequence of *N. simplex* KCTC 9106^T (AF005009). As can be seen from the generated phylogenetic tree, which includes the type strains of all known species of the genus *Nocardioides* (see figure), strain 193 (VKM Ac-2033D) and *N. simplex* KCTC 9106^T are phylogenetically very close and form a cluster with *Nocardioides nitrophenolicus* NSP41^T.

Taken together, the morphological, chemotaxonomic, and phylogenetic characteristics of strain VKM Ac-2033D indicate that it belongs to the genus *Nocardioides*. The high degree of similarity of the 16S rRNA gene sequences of this strain and *N. simplex* KCTC 9106^T suggests that they belong to one species. This suggestion is in agreement with the results of the comparative study of strain VKM Ac-2033D with another *N. simplex* strain (VKM Ac-1118^T), which are presented below.

Comparison of the physiological and biochemical properties of strains VKM Ac-2033D and *N. simplex* VKM Ac-1118^T. The carbon and energy compounds utilized by the two strains were identical: both strains were found to be able to utilize arabinose, rhamnose, glucose, and cellobiose, but not erythritol, xylitol, adonitol, ribose, inositol, melibiose, and raffinose. Weak growth was observed on mannitol, mannose, sorbitol, lactose, and sucrose. Both strains hydrolyzed arbutin (weakly), esculin, casein, gelatin, Tween-80, and tyrosine, but not xanthine, hypoxanthine, and adenine.

Based on the following common characteristics—the absence of the life cycle coccus–rod–coccus, the presence of L,L-diaminopimelic acid in the cell wall peptidoglycan and of menaquinone-8 (H_4) as the major quinone in the respiratory chain, the prevalence of saturated *iso*- and *anteiso*-branched fatty acids and the presence of tuberculostearic acid in the cell wall fatty acids, and the very similar 16S rRNA gene sequences of strains VKM Ac-2033D and *N. simplex* KCTC 9106^T—the inference can be made that the former strain also belongs to the species *N. simplex*.

Susceptibility to antibiotics. As can be seen from Table 1, the strain *N. simplex* VKM Ac-2033D is resistant to ampicillin (500) (parenthesized are concentrations of particular antibiotics in μg/ml), nalidixic acid (50), phosphomycin (100), tetracycline (20), gentamicin (10), streptomycin (50), ceftasidim (100), and hetacillin (100). The antibiotic susceptibility pattern of

Table 1. The antibiotic susceptibility of *N. simplex* VKM Ac-2033D

Antibiotics, μg/ml	Susceptibility	Antibiotics, μg/ml	Susceptibility
Penicillins		Tetracycline	
Carbenicillin, 500	S	Tetracycline, 10	R
Carbenicillin, 1000	S	Tetracycline, 20	R
Ampicillin, 500	R	Aminoglycosides	
Ampicillin, 1000	SS	Gentamicin, 10	R
Augomentin, 50	S	Gentamicin, 20	SS
Augomentin, 100	S	Streptomycin, 50	R
Naphthyridine derivatives		Streptomycin, 100	SS
Nalidixic acid, 50	R	Kanamycin, 50	S
Nalidixic acid, 100	S	Kanamycin, 100	S
Macrolides		Sagamycin, 50	S
Erythromycin, 50	S	Sagamycin, 100	S
Erythromycin, 100	S	Cephalosporins	
Novobiocin, 50	S	Rocefin, 50	S
Novobiocin, 100	S	Rocefin, 100	S
Phosphomycin, 50	R	Claforan, 50	S
Phosphomycin, 100	R	Claforan, 100	S
Leicomycin, 50	S	Ceftasidim, 50	R
Leicomycin, 100	S	Ceftasidim, 100	R
Rifampicin		Other antibiotics	
Rifampicin, 50	S	Hetacillin, 50	R
Rifampicin, 100	S	Hetacillin, 100	R

Note: S, R, and SS stand for “susceptible”, “resistant,” and “slightly susceptible,” respectively.

N. simplex VKM Ac-2033D can be used as a phenotypic marker of this strain and for the prevention of possible contamination of large-scale cultures of this strain in various biotechnological processes.

The 1(2)-dehydrogenation of steroids by bacteria from the genus *Nocardioides.* The 3-ketosteroid 1(2)-dehydrogenase activity of representatives of the genus *Nocardioides* was assayed using hydrocortisone at a concentration of 5 g/l. As can be seen from Table 2, the strain *Nocardioides* sp. VKM Ac-806 and all three *N. simplex* strains tested (VKM Ac-925, VKM Ac-1118^T, and VKM Ac-2033D) were capable of the 1(2)-dehydrogenation of hydrocortisone into prednisolone, the 1(2)-dehydrogenase activity of strain VKM Ac-2033D being maximum.

It should be noted that, to the best of our knowledge, there is no data in the literature as to the ability of *Nocardioides* species to convert steroids. This may be related to the fact that the genus *Nocardioides* was first described only in 1976 [21]. At the same time, the possibility cannot be excluded that the application of modern taxonomic approaches may lead to the situation that some strains of the genus *Arthrobacter* with high 3-ketosteroid 1(2)-dehydrogenase activity will be reclassified into the genus *Nocardioides*, as is the case with *A. globiformis* 193 (presently *N. simplex* VKM Ac-2033D).

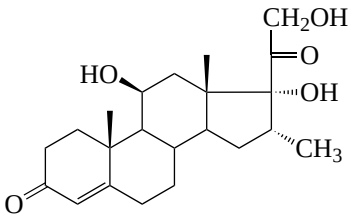
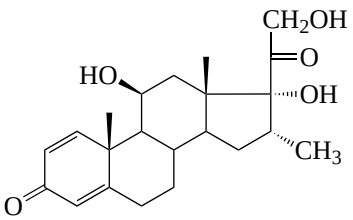
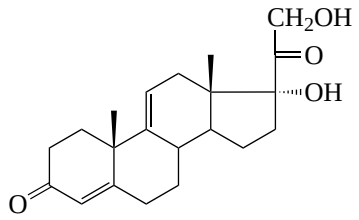
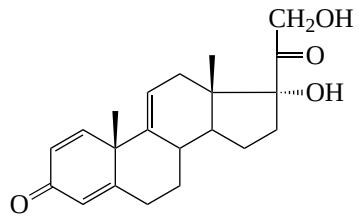
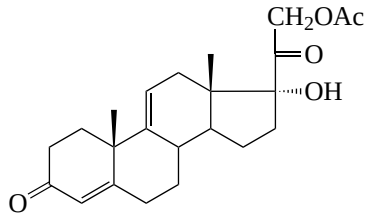
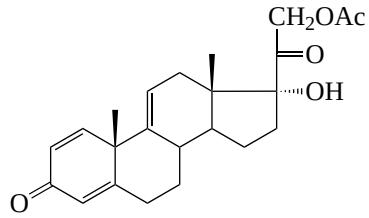
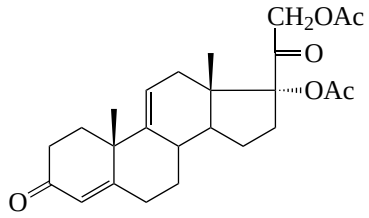
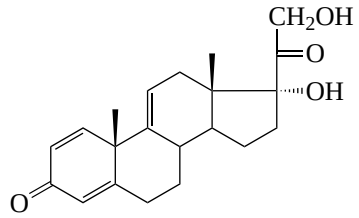
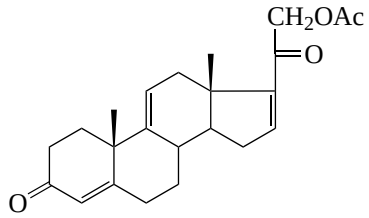
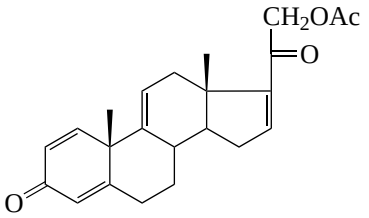
The 1(2)-dehydrogenation of 9(11)-dehydrocortexolones by *N. simplex* VKM Ac-2033D. In relation to the reevaluation of the taxonomic status of *A. globiformis* 193, we reexamined the capability of this organ-

Table 2. The ability of bacteria from the genus *Nocardioides* to convert hydrocortisone (5 g/l) into prednisolone

Strain	Conversion degree, %	Conversion time, h
N. jenseii VKM Ac-1878^T	0	96
N. albus VKM Ac-805^T	0	96
Nocardioides sp. VKM Ac-564	0	96
Nocardioides sp. VKM Ac-565	0	96
Nocardioides sp. VKM Ac-806	12	96
N. luteus VKM Ac-1246^T	0	96
N. simplex VKM Ac-925	87	4
N. simplex VKM Ac-1118^T	90	4
N. simplex VKM Ac-2033D	92	2

Note: In all cases, the steroid–cell dry weight ratio was 2 : 1.

Table 3. The conversion of 3-ketosteroids (5 g/l) by *N. simplex* VKM Ac-2033D (formerly *A. globiformis* 193)

Starting steroid	Conversion product	Conversion degree, %	Conversion time, h	Steroid/cell dry wt ratio
 16α-Methylhydrocortisone	 16α-Methylprednisolone	92	1–2	1 : 2
 9(11)-Dehydrocortexolone	 1(2),9(11)-Dehydrocortexolone	92	6–8	1 : 1
 9(11)-Dehydrocortexolone 21-acetate	 1(2),9(11)-Dehydrocortexolone 21-acetate	85	8–12	1 : 1
 9(11)-Dehydrocortexolone 17,21-diacetate	 1(2),9(11)-Dehydrocortexolone	85	20–24	1 : 4
 9(11),16(17)-Dehydrocortexolone 21-acetate	 1(2),9(11),16(17)-Trienecortexolone 21-acetate	90	7–9	25 : 1

ism for the 1(2)-dehydrogenation of 3-ketosteroids: 9(11)-dehydrocortexolone and its 21-acetate and 17,21-diacetate, 16α-methylhydrocortisone, and 9(11),16(17)-dienecortexolone 21-acetate.

As is evident from Table 3, *N. simplex* VKM Ac-2033D possesses high 1(2)-dehydrogenase activity toward all of these 3-ketosteroids, the conversion rate and product yield depending on the structure of the particular steroid and on the presence, number, and position of acetoxy- and methyl-substituents and double bonds. Unlike the methyl group at position 6α [6], the presence of a methyl group at position 16α insignifi-

cantly influenced the rate of 1(2)-dehydrogenation. Indeed, 16α-methylhydrocortisone was 1(2)-dehydrogenated by 92% in 1–2 h. The presence of the 9(11)-double bond did not prevent 1(2)-dehydrogenation, whereas the mono- or diacetylation of 9(11)-dehydrocortexolone at positions 21 and 17,21, respectively, considerably diminished the rate and the degree of conversion of this steroid (the mono- and diacetylation of 9(11)-dehydrocortexolone increased the conversion time of this steroid from 6–8 to 8–12 and 12–24 h, respectively). In contrast, the presence of a double bond in the D ring of 9(11),16(17)-dienecortexolone 21-acetate enhanced the rate and the degree of conversion of this steroid.

Thus, the data presented in this paper provide evidence that *N. simplex* VKM Ac-2033D has high 3-ketosteroid 1(2)-dehydrogenase activity with a wide substrate specificity and hence is of great biotechnological importance.

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