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22-NBD-cholesterol as a novel fluorescent substrate for cholesterol-converting oxidoreductases

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ABSTRACT

Docking simulations and experimental data indicate that 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (22-NBD-cholesterol), a common fluorescent sterol analog, binds into active sites of bovine cytochrome P450scc and microbial cholesterol dehydrogenases (CHDHs) and then undergoes regiospecific oxidations by these enzymes. The P450scc-dependent system was established to realize *N*-dealkylation activity toward 22-NBD-cholesterol, resulting in 7-nitrobenz[c][1,2,5]oxadiazole-4-amine (NBD-NH_2) formation as a dominant fluorescent product. Basing on LC–MS data of the probes derivatized with hydroxylamine or cholesterol oxidase, both pregnenolone and 20-formyl-pregn-5-en-3β-ol were deduced to be steroidal co-products of NBD-NH_2, indicating intricate character of the reaction. Products of CHDH-mediated conversions of 22-NBD-cholesterol were defined as 3-oxo-4-en and 3-oxo-5-en derivatives of the steroid. Moreover, the 3-oxo-4-en derivative was also found to be formed after 22-NBD-cholesterol incubation with pathogenic bacterium *Pseudomonas aeruginosa*, indicating a possible application of the reaction for a selective and sensitive detection of some microbes. The 3-keto-4-en derivative of 22-NBD-cholesterol may be also suitable as a new fluorescent probe for steroid hormone-binding enzymes or receptors.

1. Introduction

Cholesterol (cholest-5-en-3β-ol) is an important lipid component of plasma membranes of most vertebrates. It is also a biosynthetic precursor of bile acids, vitamin D and steroid hormones, which are active participants of cell signaling and other regulation processes, including cell homeostasis, proliferation and apoptosis. Moreover, steroid hormones are involved in control of whole organism functions, such as sexual differentiation, reproduction, fertility and blood pressure [1,2]. Biosynthesis of steroid hormones is performed by two groups of oxidoreductases, namely, cytochromes P450 and hydroxysteroid-dehydrogenases. The first rate-limiting step of mammalian steroidogenesis is a conversion of cholesterol into pregnenolone catalyzed by P450scc (CYP11A1). This enzyme consists of a single polypeptide [3,4] and acts as a terminal oxidase of mitochondrial monooxygenative system, which includes additionally two electron-transferring proteins – adrenodoxin (Adx) and NADPH-dependent adrenodoxin-reductase (AdR). Pregnenolone has no hormonal activity. Like other 3β-hydroxy-5-ene, pregnanes and androstanes, it should be first converted into the corresponding 3-keto-4-en derivative by the $NAD(P)^+$-dependent 3β-hydroxysteroid dehydrogenase/Δ5–Δ4 isomerase (3β-HSD) to form the active steroid hormone [2]. On the other hand, cholesterol-metabolizing oxidoreductases also play essential roles in the cholesterol assimilation by some microbes (*e.g.*, genera *Pseudomonas*, *Nocardia*, *Mycobacterium*, *etc.*). Notably, that the pathogenicity and persistence of *Mycobacterium tuberculosis* – a pathogen causing about 2 million deaths annually – are closely associated with the utilization of host cell cholesterol. An initial step of the microbial degradation of the cholesterol and other 3β-hydroxy-5-ene steroids is their conversion into corresponding 3-keto-4-en derivatives. This step is catalyzed by either cholesterol oxidases (CHOXs, EC 1.1.3.6) or NAD^+-dependent cholesterol dehydrogenases (CHDHs, EC 1.1.1.145), more often referred in the literature as "microbial 3β-HSD" [5–11].

22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (22-NBD-cholesterol) is a fluorescent cholesterol analog, which has been used widely for investigations of the

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sterol traffic in cells, structural organization of lipid membranes and some cholesterol-binding proteins (sterol carrier protein-2, steroidogenic acute regulatory protein, *etc.*) [12,13]. To the best of our knowledge, the enzymatic conversion of 22-NBD-cholesterol into the corresponding 3-O-esters has been reported only [1,14,15]. However, there are practically no publications about interaction of the fluorescent steroid with cholesterol-converting oxidoreductases of the aforesaid pathways. Previously, we announced the ability of the bacterial CHOXs to convert 22-NBD-cholesterol into its 3-keto-4-one derivative [16].

In this article we show the results of computer simulations supported by further experimental data, demonstrating that the fluorescent steroid is a substrate of two cholesterol-metabolizing oxidoreductases, namely, the mammalian P450scc and the bacterial CHDH. We establish structures of the corresponding fluorescent products of these reactions and also propose possible applications of the newly discovered processes.

2. Materials and methods

2.1. Enzymes and chemicals

The following reagents were used: 22-NBD-cholesterol (Molecular Probes), cholesterol, pregnenolone, progesterone, hydroxylamine hydrochloride, NADPH and NAD^+ (Sigma), Polysorbate 80 and sodium cholate (Fluka). CHOX from *Brevibacterium sterolicum* and CHDH from *Nocardia* sp. ($\geq$18 U/mg solid) were obtained from Sigma. P450scc, adrenodoxin (Adx) and adrenodoxin-reductase (AdR) were purified from bovine adrenal cortex as described previously [4]. Spectral purity indexes for P450scc, Adx and AdR were A_{391}/A_{280} 0.83, A_{414}/A_{280} 0.83 and A_{272}/A_{455} 8.5, respectively. The P450scc preparation was more than 90% in high-spin form and contained less than 5% inactive P420 fraction.

2.2. Homology search and molecular docking simulations

The homology search was carried out by BLAST 2.2.22+ program using BLASTP algorithm [17]. The docking simulations were conducted with Autodock 4.0; the Graphical User Interface "AutoDockTools" (The Scripps Research Institute) was used to prepare, run, and analyze the docking simulations [18,19] with the 3D-structure of bovine P450scc (PDB ID: 3MZS) [20]. Gasteiger partial charges [21] were calculated and assigned to the atoms of heme and amino acid residues. The docking space was defined as a 60 Å × 60 Å × 60 Å box with its center close to redox-active heme of the protein. Small molecules were created and prepared using HyperChem 7.01 (Hypercube). The Lamarckian genetic algorithm with default parameters was applied for rigid docking calculations. The binding energy values were calculated automatically by Autodock.

2.3. 22-NBD-cholesterol conversion by P450scc enzymatic system

The stock solution of 22-NBD-cholesterol in propanol-2 was added to 50 mM potassium phosphate buffer (pH 7.4) with 0.02% sodium cholate, 0.1% Polysorbate 80 and then sonicated. The initial concentration of the fluorescent steroid was 2–20 μM. Then P450scc, Adx and AdR were added from stock solutions up to 0.2 μM, 2 μM and 0.2 μM, respectively, and mixtures were pre-incubated for 5 min at 30 °C. NADPH was then added from a stock solution up to 250 μM and incubation proceeded at 30 °C for 30 min. Control experiments were conducted without either P450scc or Adx and AdR as well as with hydrogen peroxide instead of NADPH. The conversions were stopped by ethyl acetate extraction. Each sample (1 ml) was extracted twice with 4 ml of ethyl acetate; corresponding organic phases were combined, evaporated to dryness and then dissolved in 0.5 ml of ethanol.

2.4. 22-NBD-cholesterol conversion by CHDH

The stock solution of the 22-NBD-cholesterol in propanol-2 was added to 50 mM potassium phosphate buffer (pH 7.4) with 0.05% sodium cholate, 0.2% Polysorbate 80 and then sonicated for 0.5 min at 35 kHz, resulting in 2–100 μM initial concentrations of the fluorescent steroid. NAD^+ was added up to 500 μM. Unless otherwise stated, enzymatic oxidation was initiated by the addition of the CHDH up to 0.04 U/ml. The conversion went at 30 °C for various time periods and stopped by ethyl acetate extraction. Each sample (1 ml) was extracted twice with 4 ml of ethyl acetate. The corresponding organic phases were combined, evaporated to dryness and then dissolved in 0.5 ml of ethanol. Further processing of the probes was carried out as described earlier (see Section 2.3).

2.5. 22-NBD-cholesterol incubation with growing opportunistic bacteria

Opportunistic strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were taken from the collection of the Republican Research and Practical Center for Epidemiology and Microbiology (Republic of Belarus). The bacteria were cultivated for 24 h at 37 °C on Petri dishes as described elsewhere [22] with 22-NBD-cholesterol added up to 10 μM into molten medium (45 °C) prior to the cultivation. After the cultivation corresponding zones (medium + bacteria) were cut out and placed into different tubes containing 10 ml ethanol. Then the tubes were sonicated (15 min at 35 kHz) and centrifuged (10 min at 3000 × *g*). The supernatant aliquots (1 ml) were transferred into vials and analyzed by HPLC with fluorescent detection (see Section 2.8).

2.6. Spectrofluorimetry

Fluorescence measurements were conducted with the spectrofluorimeter SM2203 (Solar). The slits of excitation and emission were set at 5 nm for all the fluorescence measurements. The fluorometric registration of the CHDH-mediated conversion of 22-NBD-cholesterol was performed using excitation at 350 nm (close to the NADH absorption maximum); fluorescence was registered at 470 nm and 530 nm (close to emission maxima of NADH and 22-NBD-cholesterol, respectively).

2.7. TLC

TLC was performed on silica aluminum foils (Fluka) with fluorescent indicator (254 nm). TLC plates were developed with benzene:acetone mixture (4:1 (v:v)). Spots of substances were monitored visually under 365 nm UV-light. Individual spots were cut out and extracted by ethanol in the necessary cases.

2.8. HPLC

HPLC was conducted using either the LC-10AT (Shimadzu) system with photodiode array detector SPD-M10A (operated at 200–600 nm range) or the LC10-AD (Shimadzu) system with fluorometric detector RF-10Axl (medium sensitivity, wavelengths of excitation and emission 466 and 530 nm, respectively). In both cases the column was LiChroCART C18 (250 mm × 4 mm, 5 μm) (Merck) with the elution conducted at a flow rate of 1 ml/min using either eluent A comprising acetonitrile:water:propanol-2 (84:16:5 (v:v:v)) or eluent B comprising acetonitrile:water:propanol-2 (80:20:5 (v:v:v)).

2.9. Purification of products

Definite reaction products were firstly separated from the reaction mixtures by TLC as described above. The corresponding TLC fluorescent yellow spots were cut out and the substances extracted with ethanol. The ethanolic extracts were concentrated to 200 μL by evaporation under argon stream and then applied on the HPLC column, which was equilibrated with 20% eluent A in water. Subsequently the column was washed by 20% eluent A in water followed by 100% eluent A. Fractions with absorbance maxima at 470 nm were collected. The recovery was 60–75% according to photometric data.

2.10. Liquid chromatography–mass spectrometry (LC–MS)

LC–MS measurements were performed using the LC-2020 (Shimadzu) system, equipped with a photodiode array detector and a mass spectrometer. The last one was operated in either electrospray (ESI) or atmospheric pressure ionization (APCI) mode at a detector voltage of 1.2 kV, a heat block temperature of 400 °C and a desolvation line temperature of 250 °C. Negative (*m*/*z* 150–650) or positive (*m*/*z* 250–450) ions were monitored. Nitrogen was used as a drying and nebulizer gas at flow rates 1.5 L/min and 15 L/min, respectively. The column was Shim-pack VP-ODS (2 mm × 150 mm, 5 μm). The elution was performed by the mixture of acetonitrile:water:formic acid (50:50:0.05 (v:v:v)) at 0.4 ml/min and at 40 °C.

Additionally, aliquots of the probes of P450scc-catalyzed conversion of 22-NBD-cholesterol were analyzed by LC–MS as described above after additional derivatization with the cholesterol oxidase according to [16,23] (for 5-en-3β-ol steroids conversion into 4-en-3-oxo steroids) as well as with hydroxylamine according to [24] (for conversion of oxosteroids into oximes).

2.11. Calculations of kinetic parameters

Maximal velocity (V_{max}) and apparent Michaelis constant (K_m) values were estimated by hyperbolic regression of the data using Origin 7.0 software. Initial data were obtained from three independent experiments.

3. Results

3.1. Interaction of 22-NBD-cholesterol with cytochrome P450scc revealed by docking simulations

Docking simulations with the crystal structure of bovine P450scc [20] and 22-NBD-cholesterol demonstrated that the latter could be localized in the proximity of the protein's heme (Fig. 1). Two different enzyme–steroid complexes were predicted (Fig. 1A and B) with 22-NBD-cholesterol binding energy values of approximately −14.0 ± 0.2 kcal/mol, showing high affinity of the steroid to the protein. In these complexes the NBD-group of 22-NBD-cholesterol is primarily surrounded by the hydrophobic residues comprising Trp88, Leu102, Phe203, *etc*. Polar groups of Gln377 and Thr354 are close to the 3β-hydroxyl of the steroid, enabling of a direct or a water-mediated hydrogen bond formation [20]. In the complexes A and B, the distances from Fe to each atom of the C17—N23 fragment of the steroid side-chain are within 3.4–4.8 Å, but the complex A geometry favors the oxidation of C20 and C21 (Fig. 1A), whereas the complex B geometry – C22 and N23 (Fig. 1B). Taking previous data [25–27] into account, the predicted distances allowed us to propose the oxidation of 22-NBD-cholesterol by P450scc. It should be noted that P450scc oxidizes most of its steroidal substrates at positions 20 and 22; however, the oxidations at 17, 23 and 24 positions of substrates other than cholesterol were also reported [28–31]. Thus, our results confirm the potential ability of the 22-NBD-cholesterol to bind the active site of the P450scc, enabling the oxidation of the steroid side-chain.

3.2. P450scc-catalyzed conversion of 22-NBD-cholesterol

It was established, that the addition of 22-NBD-cholesterol to P450scc solutions decreased the pregnenolone-induced spectral changes ($A_{420} - A_{390}$), indicating a competitive binding of the steroids near the heme of P450scc [32]. An increase in 22-NBD-cholesterol fluorescence intensity after addition of P450scc was also monitored, suggesting that 22-NBD-cholesterol binds into a lipophilic site of the P450scc [12,13]. The TLC and HPLC analyses of the samples where 22-NBD-cholesterol had been incubated with the complete P450scc-dependent side-chain cleavage system (NADPH-AdR-Adx-P450scc) revealed a selective formation of a fluorescent substance as a dominant product (Fig. 2). There was no formation of this product when incomplete variants of the side-chain cleavage system were used as well as when the NADPH was substituted by a hydrogen peroxide. These data indicate that the substance formation is due to the specific P450scc enzymatic activity and that hydrogen peroxide, which could be generated in the P450scc-dependent system [33], does not seem to be a participant of the reaction monitored. According to TLC and HPLC mobility of the product, the substance was concluded to be more polar than the substrate (Fig. 2A and B). The LC–MS analysis of 22-NBD-cholesterol conversion by P450scc allowed us to establish that the fluorescent product is the 7-nitrobenz[c][1,2,5]oxadiazole-4-amine (NBD-NH_2, $[M-H]^-$ ion with *m*/*z* 179) (Fig. 3). The structure was additionally

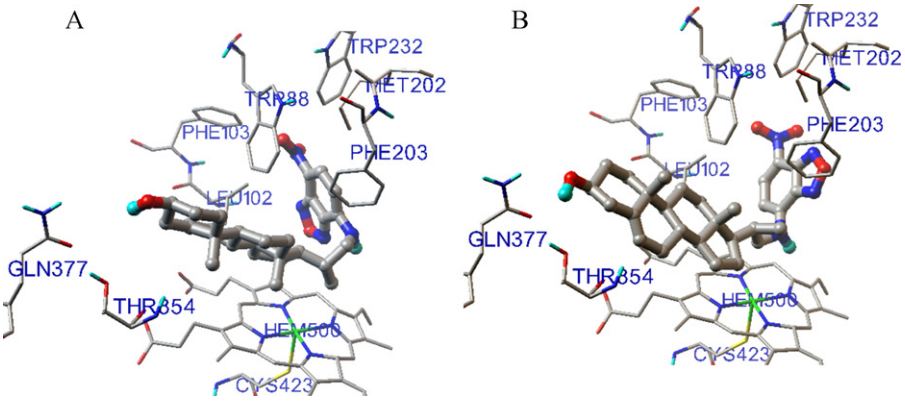

Fig. 1. Conformations of 22-NBD-cholesterol in the active site of bovine P450scc (PDB ID: 3MZS) predicted by docking simulations. The steroid is drawn with bold lines. Gray, blue, red, aquamarine and green fragments correspond to C, N, O, H and Fe atoms, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

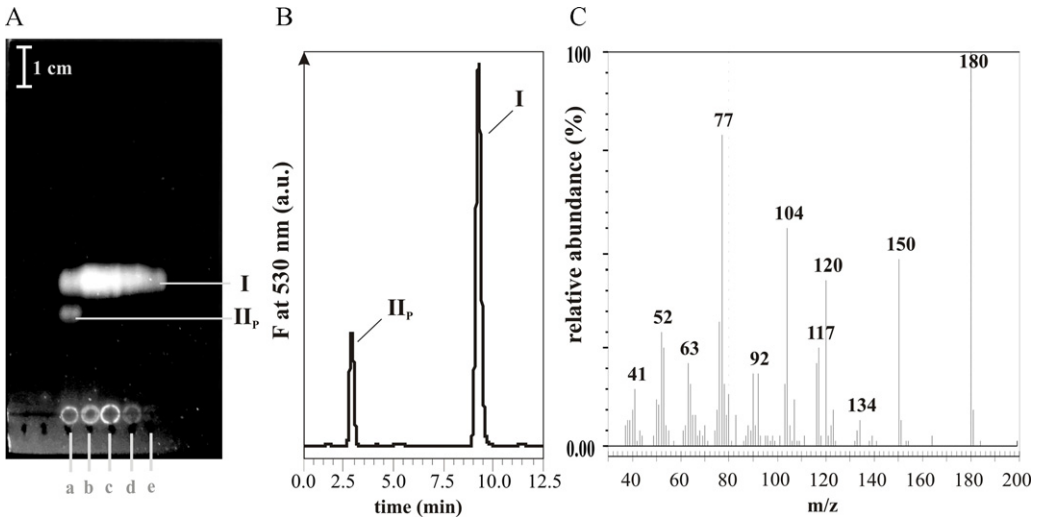

Fig. 2. Analysis of 22-NBD-cholesterol conversion by the P450scc-dependent side chain cleavage system. (A) TLC plate in 365 nm UV-light (shown as black and white), reflecting selective formation of the product **II$_P$** in the case of 22-NBD-cholesterol (**I**) conversion by complete P450scc-dependent system: a – P450scc + Adx + AdR + NADPH, b – Adx + AdR + NADPH, c – P450scc + NADPH, d – P450scc + Adx + AdR + H_2O_2, e – P450scc. (B) A representative chromatographic curve (fluorescence at 530 nm) showing the separation of **I** and **II$_P$** (eluent B) (see Section 2.10 for further details). (C) Mass spectrum with the electron impact ionization of the main fluorescent product **II$_P$**, showing the product is 7-nitrobenz[c][1,2,5]oxadiazole-4-amine (identity with the compound mass spectrum from Wiley8 library). The analysis was performed as described [34] with direct injection of the purified substance in our case.

confirmed by the mass spectrometry with the electron impact ionization of the purified product (M^+ ion with *m/z* 180) (Fig. 2C).

To determine a steroidal co-product, forming in pair with NBD-NH_2 during P450scc-dependent 22-NBD-cholesterol conversion, we first performed LC–MS analysis using APCI+ mode. This allowed registering the formation of compounds, giving cations with *m/z* values, which are attributable to supposed products of the reaction under consideration, namely, 20-formyl-pregn-5-en-3β-ol (molecular weight (MW) 330 Da; *m/z* 295, 313, 331) as well as pregnenolone (MW 316 Da; *m/z* 299, 317). To improve detection of steroidal analytes, derivatization with CHOX [16,23] or with hydroxylamine [24] was used. The CHOX treatment of the probes resulted in the appearance of three new compounds, which possessed absorption maximum at 242 nm. Two of the compounds were identified as the 3-keto-4-en derivative of 22-NBD-cholesterol [16] and progesterone (retention times (RT) 45.3 and 8.3 min, respectively) using comparison with authenticated standards. The third substance peak (RT 10.1 min) gave a mass spectrum consistent with MW 328 Da (*m/z* 311, 329, 370, *i.e.* $[M+H-H_2O]^+$, $[M+H]^+$ and $[M+H+CH_3CN]^+$) (Fig. 4A, C, D), which may be attributed to the 3-keto-4-en derivative of 20-formyl-pregn-5-en-3β-ol. Moreover, after derivatization of the probes with hydroxylamine, formation of two isomeric 20-oximes of pregnenolone (MW 331 Da) was detected (RT 4.8 and 5.4 min; *m/z* 332 and 373) as well as two additional more hydrophobic compounds (RT 6.3 and 6.8 min), which had identical mass spectra consistent with MW 345 (*m/z* 346, 369 and 387, *i.e.* $[M+H]^+$, $[M+H+CH_3CN-H_2O]^+$ and $[M+H+CH_3CN]^+$) of 22-oximes of 20-formyl-pregn-5-en-3β-ol (Fig. 4B, E, F). In total, our data is in accordance with the formation of NBD-NH_2, pregnenolone and 20-formyl-pregn-5-en-3β-ol during the P450scc-catalyzed conversion of 22-NBD-cholesterol. V_{max} and K_m values for 22-NBD-cholesterol conversion in the reconstituted enzymatic system was determined to be $1.0 \pm 0.3\ min^{-1}$ and $12.2 \pm 2.5\ \mu M$, respectively. For comparison, using the same conditions for natural cholesterol conversion yielded V_{max} and K_m of $8.0\ min^{-1}$ and 10 μM, respectively. Thus, V_{max}/K_m ratio for the artificial substrate was estimated to be approximately 10 times less than for the natural one.

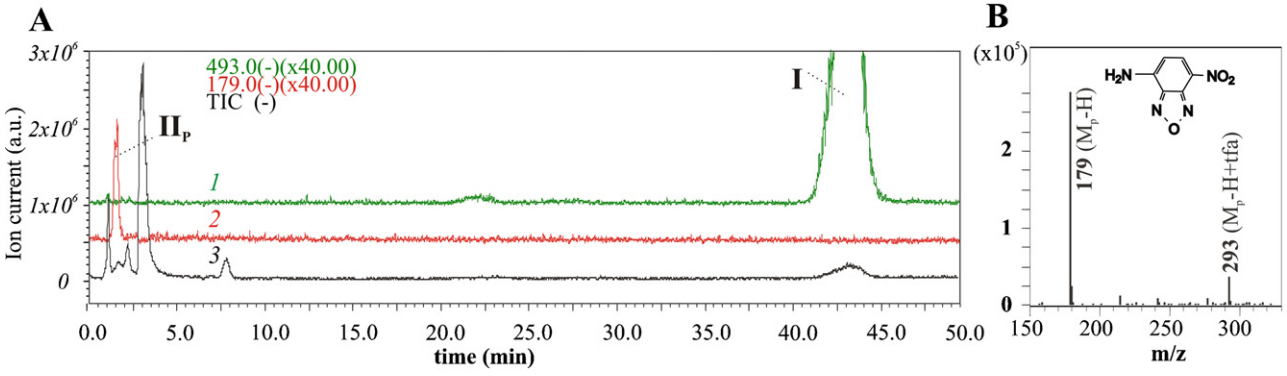

Fig. 3. 22-NBD-cholesterol conversion by complete P450scc-dependent system. (A) LC–MS chromatographic curves showing NBD-NH_2 (**II$_P$**) formation after 22-NBD-cholesterol (**I**) (retention times 2.6 and 42.3 min, respectively) conversion by complete P450scc-dependent system: 1 and 2 – 40-times multiplied signals of selected anions with *m/z* 493 and 179, respectively; 3 – the signal due to total anions with *m/z* 150–650. (B) The mass spectrum of the fluorescent product of P450scc-dependent conversion of 22-NBD-cholesterol, its interpretation (tfa is "trifluoracetic acid" – a concomitant from the solvents) and the established structure of the product.

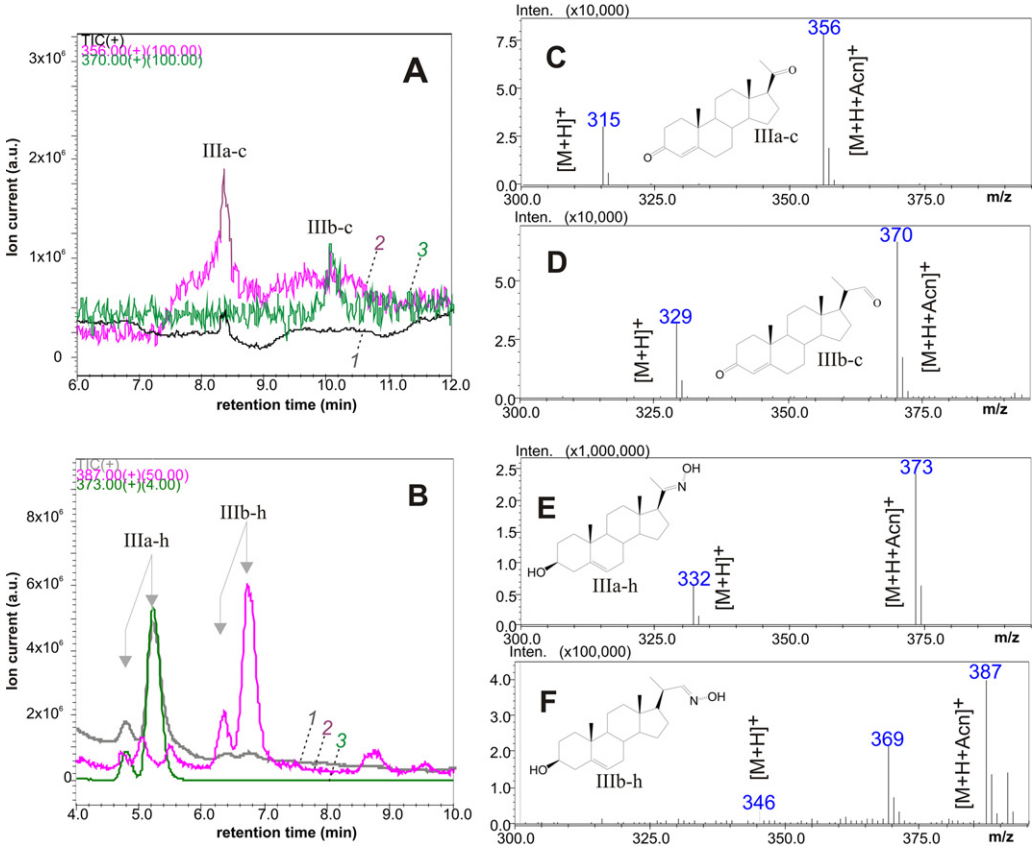

Fig. 4. 22-NBD-cholesterol conversion by complete P450scc-dependent system: detection of derivatives of non-fluorescent steroidal products. (A) LC–MS chromatographic curves (APCI+ detection mode) showing progesterone (**IIIa–c**) and another compound (**IIIb–c**) formation after the cholesterol oxidase treatment [16,23] of probes of the P450scc-dependent 22-NBD-cholesterol conversion: 1 – TIC, 2 and 3 – multiplied signals of selected cations with *m/z* 356 and 370, respectively. (B) LC–MS chromatographic curves (ESI+ detection mode) showing pregnenolone 20-oximes (**IIIa–h**) and two other compounds (**IIIb–h**) formation after the hydroxylamine treatment [24] of probes of the P450scc-dependent 22-NBD-cholesterol conversion: 1 – TIC, 2 and 3 – multiplied signals of selected cations with *m/z* 387 and 373, respectively. (C and D) Mass spectra of the **IIIa–c** and **IIIb–c** and their interpretation based on the progesterone and 20-formyl-pregn-4-en-3-one structures, respectively. (E and F) Mass spectra of the **IIIa–h** and **IIIb–h** and their interpretation based on structures of oximes of pregnenolone and 20-formyl-pregn-5-en-3β-ol, respectively. Acn is acetonitrile (CH_3CN).

3.3. 22-NBD-cholesterol conversion by the CHDH from Nocardia sp.

During 22-NBD-cholesterol incubation with the CHDH in the presence of NAD^+, an increase in the absorbance at 340 nm (Fig. 5) as well as in the fluorescence emission at 470 nm were observed, pointing out NAD^+ conversion into NADH [35]. Initial slopes of the corresponding absorbance kinetic curves obtained during cholesterol and 22-NBD-cholesterol incubation with the CHDH and NAD^+ let us conclude that the natural substrate is about four times more preferable than the fluorescent substrate. In the case of 22-NBD-cholesterol conversion, the fluorescence emission at 530 nm increased 5 times as compared to emission at 470 nm, whereas in the case of natural cholesterol the increase of fluorescence emission at 470 nm was higher than at 530 nm. This was due to effective resonance energy transfer from NADH (emission maximum at 470 nm) to the NBD-fluorophore (excitation maxima at 470 nm and 350 nm (minor); an emission maximum at 530 nm) (data not shown). TLC and HPLC analysis of the samples after 22-NBD-cholesterol incubation with the CHDH in the presence of NAD^+ revealed the formation of a new fluorescent compound together with 22-NBD-cholest-4-en-3-one – the single product of the steroid oxidation by CHOXs [16]. This new product seemed to be more lipophilic than 22-NBD-cholest-4-en-3-one according to R_f values (0.62 and 0.46, respectively) and HPLC retention times (7.6 and 6.4 min, respectively) (Fig. 5B and C). The mass spectrum of the new product seemed to be identical to the mass spectrum of 22-NBD-cholest-4-en-3-one, whereas its absorbance spectrum was shown to be similar with the absorbance spectrum of 22-NBD-cholesterol. Thus, the dominant product of the CHDH-mediated 22-NBD-cholesterol conversion was concluded to be the 3-oxo-5-en derivative of the substrate (formally, "22-NBD-cholest-5-en-3-one"). V_{max} and K_m values for the 22-NBD-cholesterol conversion were estimated to be $2.1 \pm 0.4\ min^{-1}$ and $143 \pm 13\ \mu M$, respectively, whereas for the CHDH-mediated natural cholesterol conversion the parameters were $2.5 \pm 0.5\ min^{-1}$ and $49 \pm 5\ \mu M$, respectively.

3.4. 22-NBD-cholesterol conversion by opportunistic bacteria P. aeruginosa

A possibility of using the established activities of CHOXs and the CHDH toward the fluorescent substrate for the detection of some pathogenic microbes [5–11] represents an interest for medical analysis. To elucidate an applicability of CHDHs (microbial 3β-HSDs) and CHOXs as reporter enzymes for selective detection of some bacteria, opportunistic strains of *Escherichia coli*, *S. aureus* and *P. aeruginosa* were cultivated on the 22-NBD-cholesterol-containing medium. The HPLC analysis of the ethanolic extracts from the bacterial cells that had been cultured for 24 h demonstrated that the formation of the 22-NBD-cholest-4-en-3-one took place in the case of *P. aeruginosa* only (Fig. 6). The homology search allowed establishing that among all these bacteria only

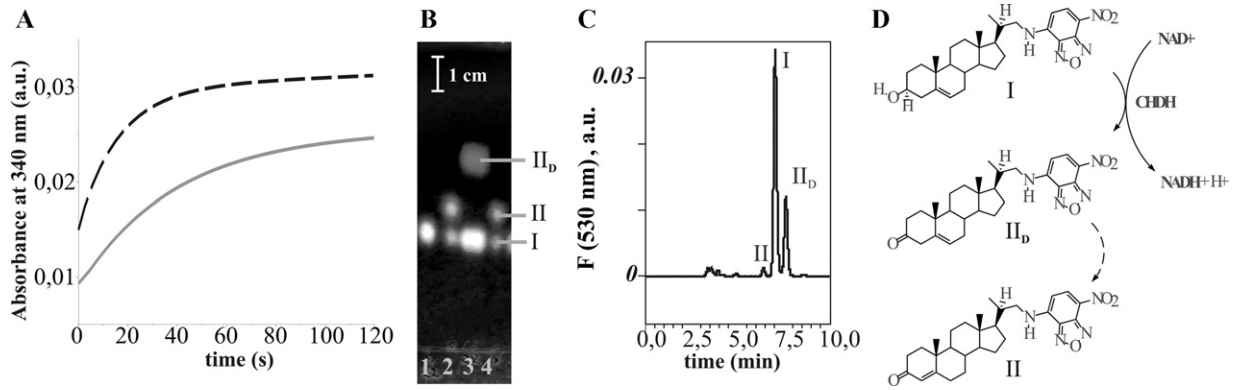

Fig. 5. CHDH-mediated conversion of 22-NBD-cholesterol. (A) Kinetic curves showing NADH formation during conversion of cholesterol and 22-NBD-cholesterol (**I**) (20 μM) (dashed and solid line, respectively) with the CHDH (2.5 U/ml) and NAD+ (200 μM) in buffer A. (B) TLC plate in 365 nm UV-light (depicted in black and white) showing separation of **I** and the products of its conversion by the CHDH – 22-NBD-cholest-4-en-3-one (**II**) and 22-NBD-cholest-5-en-3-one (**II$_D$**): 1 – **I** (standard), 2 and 4 – a mixture of **I** and **II**, 3 – a probe of the CHDH-mediated conversion of **I**. (C) a HPLC chromatogram, demonstrating separation of **I** and products of its oxidation by CHDH (**II** and **II$_D$**). (D) Proposed scheme of the CHDH-mediated conversion of 22-NBD-cholesterol.

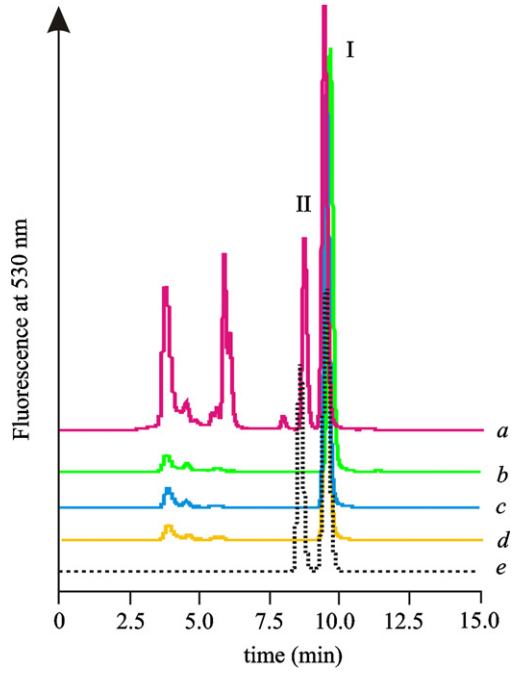

Fig. 6. The superimposition of the HPLC curves obtained by the analysis of 22-NBD-cholesterol incubation: a – with bacteria *P. aeruginosa*, b – with *E. coli*, c – with *S. aureus*, d – without any bacteria (negative control), e – with the pure CHOX from *B. sterolicum* (positive control). **I** – 22-NBD-cholesterol, **II** – 22-NBD-cholest-4-en-3-one. Peaks of others fluorescent substances with lower retention times may be attributed to further 22-NBD-cholesterol biodegradation by the bacteria.

P. aeruginosa possesses the 3β-HSD (GenBank ID: YP_001350609, AAM27844) and CHOXs (YP_002438395, ABJ13412, *etc.*). The obtained data, including established detection limits (about 0.2 pmol) of 22-NBD-cholesterol and 22-NBD-cholest-4-en-3-one, and the other common advantages of fluorescent substrates-based assays (selectivity, convenience, *etc.*) [14], may allow scientists to use this novel 22-NBD-cholesterol conversion for the precise detection of various microbes, expressing CHOXs and/or CHDHs.

4. Discussion

In this work a putative binding of 22-NBD-cholesterol into the catalytically active site of bovine P450scc has been shown for the first time. It should be noted that 22-NBD-cholesterol is a NBD derivative of 20(*S*)-aminomethylpregn-5-en-3β-ol – a strong reversible inhibitor of P450scc, which forms Fe—N coordination bond in the active site of the enzyme [36]. The NBD-substituent hinders such Fe—N coordination in the case of 22-NBD-cholesterol due to its planarity and electron-accepting properties. Quantum-chemical calculations, on the other hand, show that the negative partial charge on the N-atom of the fluorescent steroid is still sufficient for effective Fe—N coordination (data not shown). The ability of P450scc to incorporate steroids with bulky substituents into its active site was shown previously for 25-doxyl-27-nor-cholesterol [37] and (20R)-20-phenyl-5-pregnen-3,20-diol [38] (the substrate and the inhibitor of P450scc, respectively). Shape and volume of P450scc active site [39] also allow the binding of 22-NBD-cholesterol in it. In this work we showed that the 22-NBD-cholesterol is converted by reconstituted NADPH-AdR-Adx-P450scc system, resulting in oxidative C22—N23 bond cleavage and selective NBD-NH_2 formation. It should be noted that another artificial 23,24-bisnorcholesterol derivative, bearing a 7-hydroxyresorufin moiety at the C22 atom, has been previously reported to undergo P450scc-dependent conversion in Leydig tumor cell mitochondria [40], resulting in formation of pregnenolone and highly fluorescent 7-hydroxyresorufin. Pregnenolone formation was postulated based on radioimmunoassay (RIA) data only. Because of RIA assays may demonstrate cross-reactivity toward structurally related steroids and the reaction mentioned [40] is not in accordance with common P450-catalyzed O-dealkylation mechanism, another steroidal product may be also formed. We have supposed that the P450scc-catalyzed 22-NBD-cholesterol conversion proceed *via* a 22-hydroxy derivative rearrangement as common P450-mediated N-dealkylation [41]. Alternative ways of the P450scc-catalyzed NBD-NH_2 formation from 22-NBD-cholesterol, resulting in pregnenolone formation, are possible *via* oxidation of the presumed 20α-hydroxy intermediate at the 20-hydroxyl [39] or N23 (analogous to oxidation of sphingolipids described [34]). Indeed, basing on our data of the LC–MS analysis, pregnenolone was confirmed to be a major non-fluorescent steroidal co-product of NBD-NH_2. Another co-product of NBD-NH_2 was also detected and it was similarly deduced to be 20-formyl-pregn-5-en-3β-ol. Minor content of 20-formyl-pregn-5-en-3β-ol (10–12% from the pregnenolone formation level) in the probes may be attributed to natural specificity of the interaction between the P450scc and 22-NBD-cholesterol. Moreover, an insufficient extraction of the steroidal aldehyde due to Schiff bases formation with NH_2-groups of the proteins (P450scc and others) can take place. Additionally, an oxidative conversion (deformylation) of 20-formyl-pregn-5-en-3β-ol into pregnenolone and/or other steroids seems to be possible [42,43].

Thus, these circumstances can bring about diminishing of final content of the 20-formyl steroid in the probes.

The N-dealkylation of arylalkylamines has been well characterized primarily for drug-metabolizing P450s [41], although an ability of P450scc to catalyze the cumene hydroperoxide-driven N-dealkylation of N,N-dimethylaniline was previously described [44]. It is known that a P450-catalyzed N-dealkylation proceeds according to either hydrogen-atom transfer (HAT) or single electron transfer (SET) mechanisms [40,41]. It should be mentioned, that the SET variant of the 22-NBD-cholesterol oxidation may lead to a non-fluorescent NBD-derivative formation *via* the N23-centered radical rearrangement described [45] for NBD-labeled cardiolipin. Ability of P450scc to catalyze various oxidations at C22 of such "unusual" substrates as 25-doxyl-27-nor-cholesterol, vitamin D3 and ergosterol (pro-vitamin D2) [28–31] confirms possibility of formation a 22-hydroxylated 22-NBD-cholesterol as an intermediate during P450scc-catalyzed conversion of the artificial steroid.

In this work 22-NBD-cholesterol was also shown to undergo the NAD^+-dependent oxidation of its 3β-hydroxy group, catalyzed by the bacterial CHDH, followed by the Δ5–Δ4 isomerization. Similar reaction was previously shown for two bacterial CHOXs–FAD-containing enzymes, using oxygen as an oxidative co-substrate [16]. The catalytic properties of the CHDH and in particular CHOX enzymes have revealed practical applications for (1) the measurement of cholesterol in clinical analysis and (2) biotechnological synthesis of the fluorescent 3-keto-4-en-steroid, which may be interpreted as a fluorescent 3-ketosteroid hormone analog – a new probe for steroid hormone-binding enzymes or receptors. Upon exhaustive bioconversions of 22-NBD-cholesterol by CHOX or CHDH the corresponding 3-keto-4-en-derivative (22-NBD-cholest-4-en-3-one) was purified with high yields (70–80%). It should also be noted that both CHOX and 22-NBD-cholesterol have been used separately as molecular tools for investigations of cholesterol's role in structure-function properties of biological membranes. In membrane bilayer the fluorophore of 22-NBD-cholesterol was reported to be partially exposed to aqueous media, suggesting lateral or even "up-side down" orientation of the fluorescent steroid in comparison with cholesterol [12,13]. In the latter orientation, the CHOX-mediated oxidation of 22-NBD-cholesterol in membranes seems to be strongly hindered. Thus, the reactions established may provide an additional approach to elucidate differences in orientations of the artificial sterol in biological membranes.

Another interest in this newly discovered reaction is related to the circumstance, that the CHDH from *Nocardia* sp. shares high homology with the 3β-HSD of *M. tuberculosis* (76% identity with 95% of sequence coverage) – a severe human pathogen. Such enzymes may be useful as markers of infection in biological fluids (blood, secrets) and similar probes. In this regard, we show that 22-NBD-cholesterol conversion to the 3-keto-4-en derivative is suitable for the detection of the CHOX- or CHDH-expressing bacteria, including pathogenic (genera *Gordonia*, *Frankia*, *Vibrio cholerae*, *M. tuberculosis, etc.*). It is noteworthy that our experimental results were in agreement with a database search of CHOX- or CHDH-coding genes of the bacteria tested. Also, cholesterol degradation by the microbe was shown to be strongly associated with its ability to utilize cholesterol, and therefore some enzymes of the pathway represent possible new types of targets for anti-tubercular drugs. For example, inhibition of the mycobacterial 3β-HSD by some 4-aza-steroids was studied [10]. A fluorescent substrates-based assay would be very suitable for the large-scale *in vitro* sensitive screening of the enzyme's inhibitors. Also these microbial 3β-HSDs share homology with some mammalian 3β-HSDs (identity are 30–32% with coverage 90–95%). Note, that mammalian 3β-HSDs types 1 and 2 cannot convert cholesterol and oxidize 3β-hydroxyls of pregnanes and androstanes only, whereas microbial 3β-HSDs are able to convert cholesterol and other sterols with long aliphatic chains. Because real 3D-structures of 3β-HSDs have not fully revealed yet, the established properties of 22-NBD-cholesterol may provide opportunities to study structural features of the enzymes' active centers using fluorescence-based techniques.

In summary, the conversion of 22-NBD-cholesterol by three sterol-converting oxidoreductases, namely, CHOX, CHDH and cytochrome P450scc, as well as structures of the specific fluorescent products have been established. Our results unambiguously prove the substrate-like localization of 22-NBD-cholesterol in the active sites of the enzymes, which provides opportunities for their investigations using fluorescence-based techniques. This is particularly interesting in the cases of the cholesterol dehydrogenase or homologous 3β-HSDs with partially undefined 3D-structures and P450scc – with slightly uncertain catalytic mechanism. Moreover, the established conversions of 22-NBD-cholesterol by microbial enzymes create prerequisites for creation of new selective and sensitive methods for detection of the CHOX- or CHDH-expressing microbes. The 3-keto-4-en derivative of 22-NBD-cholesterol was obtained and, being interpreted as a fluorescent 3-ketosteroid hormone analog, it proposed to be useful for structure-function investigations of various steroid hormone-related enzymes and receptors.

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