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22-NBD-CHOLESTEROL, A NEW FLUORESCENT SUBSTRATE OF BACTERIAL CHOLESTEROL-OXIDASES

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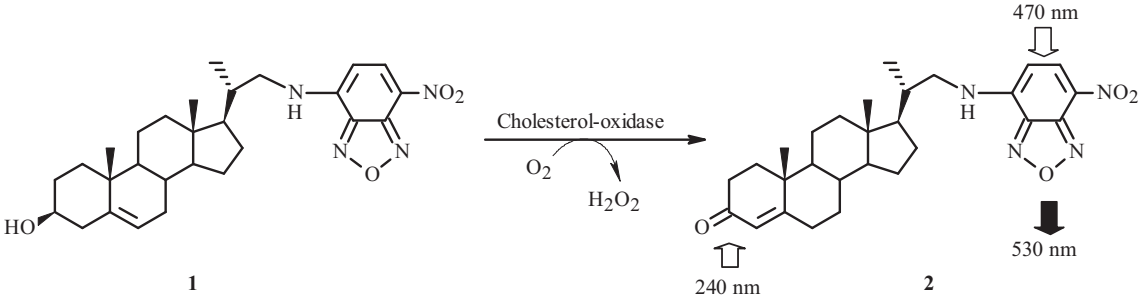
Fluorescent analogs of sterols are convenient agents for studying the transport, distribution, and protein—ligand interaction of natural sterols in cells [1]. However, the effect of the fluorescent moiety on the interaction parameters with cholesterol-transforming enzymes such as bacterial cholesterol-oxidases or mammalian steroid-hydroxylating cytochrome P450 has not been addressed in the literature. Herein we report data for the interaction of the fluorescent cholesterol analog 22-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-23,24-*bisnor*-5-cholen-3β-ol [22-NBD-cholesterol (**1**)] with cholesterol-oxidases (EC 1.1.3.6). Cholesterol-oxidases are FAD-dependent enzymes that catalyze the transformation of 3β-hydroxy-Δ^5-steroids and are widely used for laboratory and clinical cholesterol determinations [2].

We calculated the interaction of **1** with the enzyme based on the three-dimensional structure of *Brevibacterium sterolicum* cholesterol-oxidase (code PDB 1COY) using the Autodock 4.0 programs and MGLTools. It was found with the help of molecular docking that **1** can bind to the active center of cholesterol-oxidase such that its 3β-hydroxy group is situated in close proximity to the functionally important His447 and Glu361 residues [3] and the redox-active groups of the FAD molecule. The calculated binding energies for cholesterol and **1** were practically the same (–11.43 and –11.94 kcal/mol, respectively).

Next selective transformation of **1** was demonstrated using cholesterol-oxidase *in vitro*. After biotransformation of fluorescent substrate **1** with purified cholesterol-oxidase, the formation of **2**, which retained the fluorescent properties of **1** (Scheme 1) was detected by HPLC (Shimadzu LC10-AD chromatograph with an RF-10A×1 fluorimetric detector and SPD-10A spectrophotometric detector; CH_3CN:H_2O:*i*-PrOH, 84:16:5; LiChroCART C18 column, Merck, 250 × 4 mm).



Scheme 1. Enzymatic transformation of 22-NBD-cholesterol (**1**) into the 3-keto-Δ^4-derivative [22-NBD-cholestenone (**2**)] by the action of cholesterol-oxidase. White arrows designate absorption maxima; dark arrow, fluorescence emission maximum.

A characteristic difference of the absorption spectra of product **2** from that of the substrate was the presence in it of a distinct λ_{max} (EtOH) at 240 nm. The mass spectrum (Shimadzu QP2010+ spectrometer, EI, 70 eV) of starting **1** contained $[M]^+$ at *m*/*z* 494 whereas the mass spectrum of **2** had $[M]^+$ with *m*/*z* 492. Formation of **2** was confirmed definitively by a characteristic singlet for vinyl proton H-4 (5.74, br.s) in the PMR spectrum (Bruker, 400 MHz, $CDCl_3$) that is present for other steroids with a conjugated Δ^4-3-keto structure [4, 5]. Thus, the 3β-hydroxy-Δ^5-group of **1** was transformed into a conjugated

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3-keto-Δ^4-moiety to form **2** {20-methyl-21-[(7-nitro-4-benzofurazanyl)amino]-pregn-4-en-3-one or 22-NBD-cholestenone} during the enzymatic transformation (Scheme 1). For comparison, it was also found that another cholesterol-transforming enzyme, cytochrome P450scc, which cleaves the cholesterol side chain, caused only 20-hydroxylation of **1** without 22-hydroxylation and cleavage of the C20,22 bond owing to unique protein–ligand interactions and substrate specificity [6, 7]. The catalytic parameters for oxidation by cholesterol-oxidase of **1** (K_m 60.0 ± 4.4 μM and k_{cat} 0.6 ± 0.2 s^{-1}) and cholesterol (K_m 19.4 ± 2.0 μM and k_{cat} 6.4 ± 0.8 s^{-1}) were determined under identical conditions.

Thus, despite the similar calculated binding energies of cholesterol and 22-NBD-cholesterol (**1**) to the active center of cholesterol-oxidase according to docking data, the integral kinetic parameter k_{cat}/K_m was reduced by about 30 times in the experiment. This indicated that the bulky and polar fluorescent group of **1** affected the mechanism of enzymatic transformation by cholesterol-oxidase.

Inhibitory analysis in Dixon–Webb coordinates established that cholesterol is a competitive inhibitor of oxidation of **1** with Ki 45.1 ± 4.2 μM. Laboratory methods of cholesterol determination are sometimes modified to change the enzymatic reaction conditions and cause a special increase of the substrate K_m values [8]. Therefore, the increase of K_m found for **1** and the competitive nature of the inhibition will help to expand the range of determined cholesterol concentrations. The results enable the observed biotransformation of **1** into **2** to be used for selective and sensitive (the detection limits of **1** and **2** established by HPLC with fluorescent detection at signal-to-noise ratio 2:1 were less than 0.2 nmol) detection of several pathogenic microorganism strains that synthesize extracellular cholesterol-oxidase and as a basis for a new cholesterol determination method in biological media.

REFERENCES

1. R. Liu, P. Lu, J. W. Chu, and F. J. Sharom, *J. Biol. Chem.*, **284**, 1840 (2009).
2. J. Kreit and N. S. Sampson, *FEBS J.*, **276**, 6844 (2009).
3. Y. Ye, P. Liu, R. G. W. Anderson, and N. S. Sampson, *Arch. Biochem. Biophys.*, **402**, 235 (2002).
4. N. V. Kovganko, Zh. N. Kashkan, and V. M. Shkumatov, *Chem. Nat. Comp.*, **37**, 55 (2001).
5. V. M. Shkumatov, E. V. Usova, V. G. Radyuk, Zh. N. Kashkan, N. V. Kovganko, T. Juretzek, and S. Mauersberger, *Russ. J. Bioorg. Chem.*, **29**, 581 (2003).
6. V. M. Shkumatov, G. Smettan, O. Ristau, H. Rein, K. Ruckpaul, V. L. Chashchin, and A. A. Akhrem, *Chem. Biol. Interact.*, **68**, 71 (1988).
7. L. A. Novikova, Y. V. Faletrov, I. E. Kovaleva, S. Mauersberger, V. N. Luzikov, and V. M. Shkumatov, *Biochemistry (Moscow)*, **74**, 1482 (2009).
8. P. Sriwasdi, P. Jearanaikoon, M. H. Kroll, and P. H. Lolekha, *J. Clin. Lab. Anal.*, **19**, 247 (2005).