Speaker – Prof. Khodonov A.A.

Investigation of microenvironment selectivity in the bacteriorhodopsin chromophore binding site by the retinoid analogs application

Belikov N.E.[1], Melnikova I.A.[1,2], Demina O.V.[1], Lukin A.Yu.[2], Petrovskaya L.E.[3], Chupin V.V.[4], Varfolomeev S.D.[1], Khodonov A.A.[1]*

1 – N.M. Emanuel Institute of Biochemical Physics RAS, Moscow, Russia
2 – MIREA – Russian Technological University, Moscow, Russia
3 - Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia
4 – Moscow Institute of Physics and Technology, Dolgoprudny, Russia

**** khodonov@gmail.com***

Retinal based Proteins

The retinoid isomers play the key role in functioning processes in retinal based proteins — visual pigments; ion-pump bacteriorhodopsin (BRh), halorhodopsin (HRh), sensoric rhodopsins (SRhI, SRhII), tundra-rhodopsin (ESRh), and others, as well as in the retinoic acid nuclear receptors. Upon absorption of light quantum the isomerization of the definite double bond initiates a cascade of events needed for the generation of the physiological or chemical responses. During the evolution process this property of retinoid molecule became the basis for a number of light quantum energy transformation into chemical energy or some physiological response in biological systems, both in higher animals and microorganisms. Retinal based proteins contain a number of defined retinal isomers as part of their chromophoric groups bound via the protonated aldimine bond with the ε-amino group of the Lys residue.

Retinal proteins (Retinal based proteins) are chromoproteins that function either as sensors or as ion pumps in several species across all domains, Archaea, Eubacteria, and Eukarya. These light-sensitive proteins share a common fold of seven transmembrane (7TM) helices and bind a retinal chromophore through a protonated Schiff base (PSB) with a Lys residue located in helix seven. The absorption maxima of each retinal-based protein are modulated by the ionic environment of the PSB in the binding pocket. Several retinal based proteins with unexpected functions have been discovered and characterized recently.

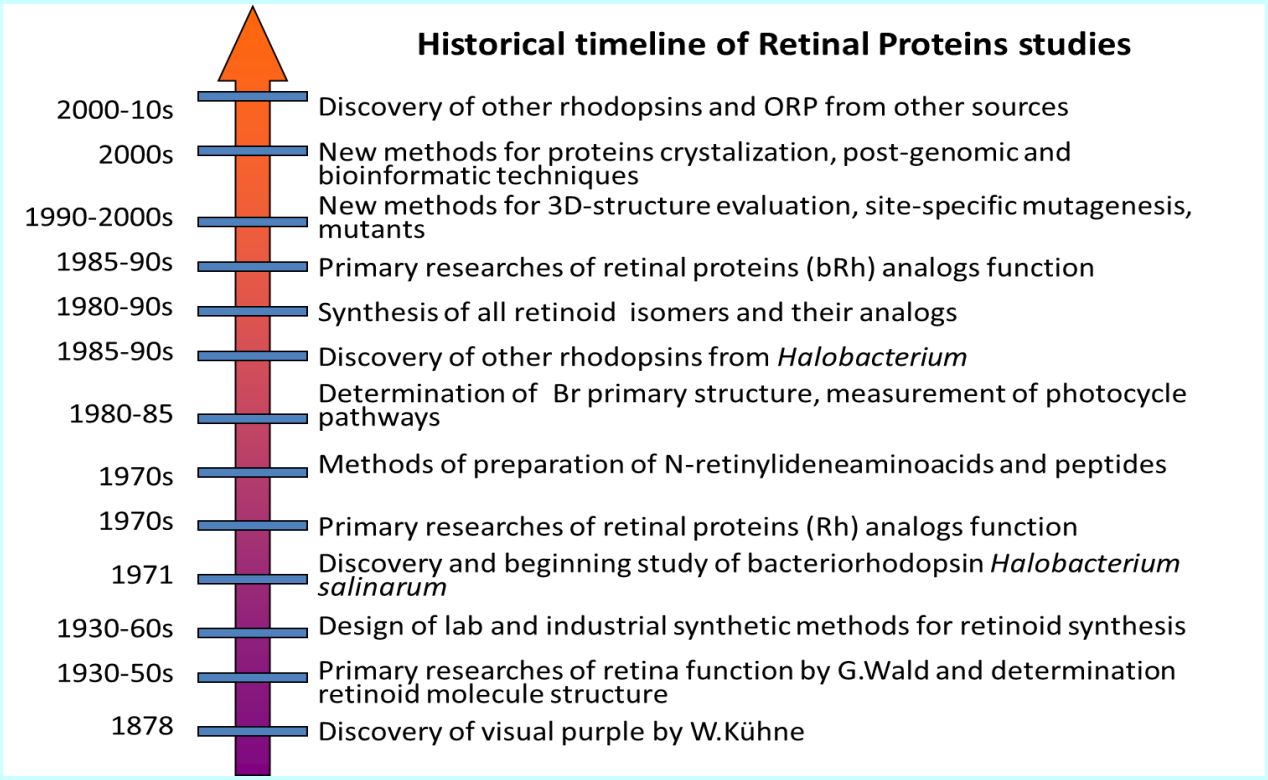
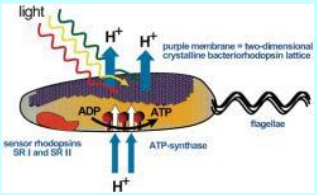


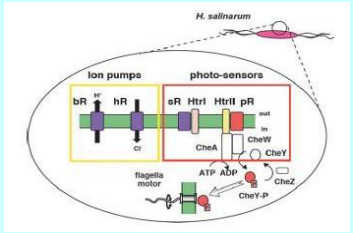
Fig. 1. Historical timeline of Retinal based proteins studies

A

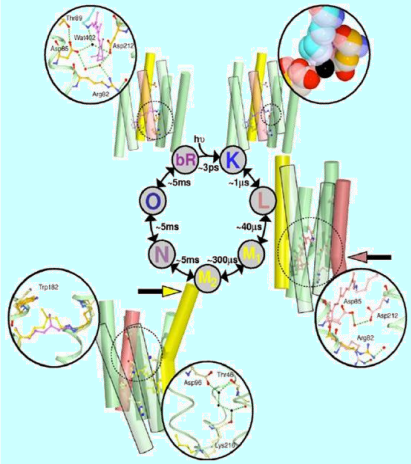
B



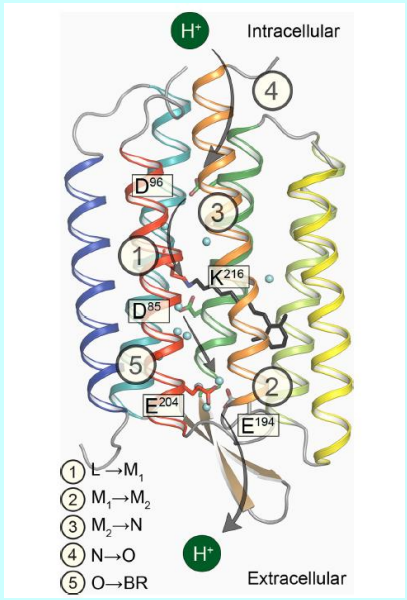
C



D



E



F

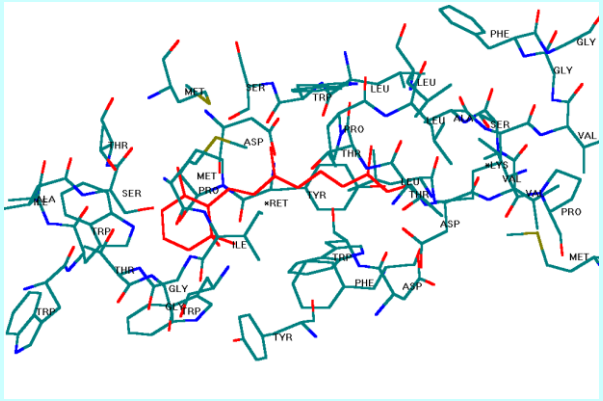


Fig.2.

A - habitats of extreme halophiles *Halobacterium salinarum* in sea bays or salt lakes with NaCl concentration more than 25%,

B, C - structural elements and components of *Halobacterium salinarum* cells (purple membranes and bacteriorhodopsin (BRh), halorhodopsin (HRh) sensory rhodopsins (SRhI, SRhII) and others);

D - BRh photocycle, main stages,

E - main stages in the BRh proton transfer channel (ID PDB file: 1C3W).

F - topography of the BRh chromophore cavity simulated using the HyperChem Pro v. 8.08.

α-helix 7TM are shown in the following colors: A, blue; B, greenish blue; C, green; D, light green; E, yellow; F, orange; G, red; chromophore - the protonated aldimine of the all-E isomer of retinal is depicted in the form of black rods, molecules of bound water in the form of blue balls. The numbers with arrows indicate the sequence of proton transfer stages;

proton transfer from RSBH + (PSB) to the primary proton acceptor Asp85;

release of a proton into the external extracellular environment from the proton-releasing complex - residues Glu194 and Glu204;

SB reprotonation from the primary proton donor Asp96;

reprotonation of Asp96 by proton capture from the cytoplasm;

transfer of a proton from Asp85 to a proton-releasing complex

the corresponding transitions between the photointermediates of the BRh photocycle are shown in the inset and in Fig. 2D.

Bacteriorhodopsin

This year marks the 50th anniversary of the discovery by D. Oesterhelt and W. Stockenius of a unique biophotochrome – light-dependent proton pump – bacteriorhodopsin from the extremely halophilic bacterium *Halobacterium salinarum* [1]. This chromoprotein is one of the first successful examples of a biological photochromic material developed by nature itself.

Bacteriorhodopsin (BRh) from *Halobacterium salinarum* is the first membrane protein whose structure was found to be composed of seven helices by electron microscopy, and was also the first membrane protein to have its amino acid sequence determined. As the best studied microbial rhodopsin, it serves as a paradigm of a light-driven retinal-binding ion pump and aids in studies of novel rhodopsins. BRh is the focus of our investigation. This protein is a unique natural photochrome acting as a light-driven proton pump. It is located in special areas of the cells, purple membranes (PM), consisting of BRh trimers embedded in the lipid bilayer. The chromophoric group of this protein is the protonated aldimine of all-*E*- and 13*Z*-isomers of vitamin A aldehyde (retinal). The purple membrane (PM) of *Halobacterium salinarum* is a natural 2D crystal honeycomb lattice of BRh trimers. The BRh protein contains a single polypeptide chain (248 aa) and converts light energy absorbed by the retinal chromophore covalently linked via a PSB to ε-amino group of Lys216 in helix 7 into a proton electrochemical gradient across the membrane.

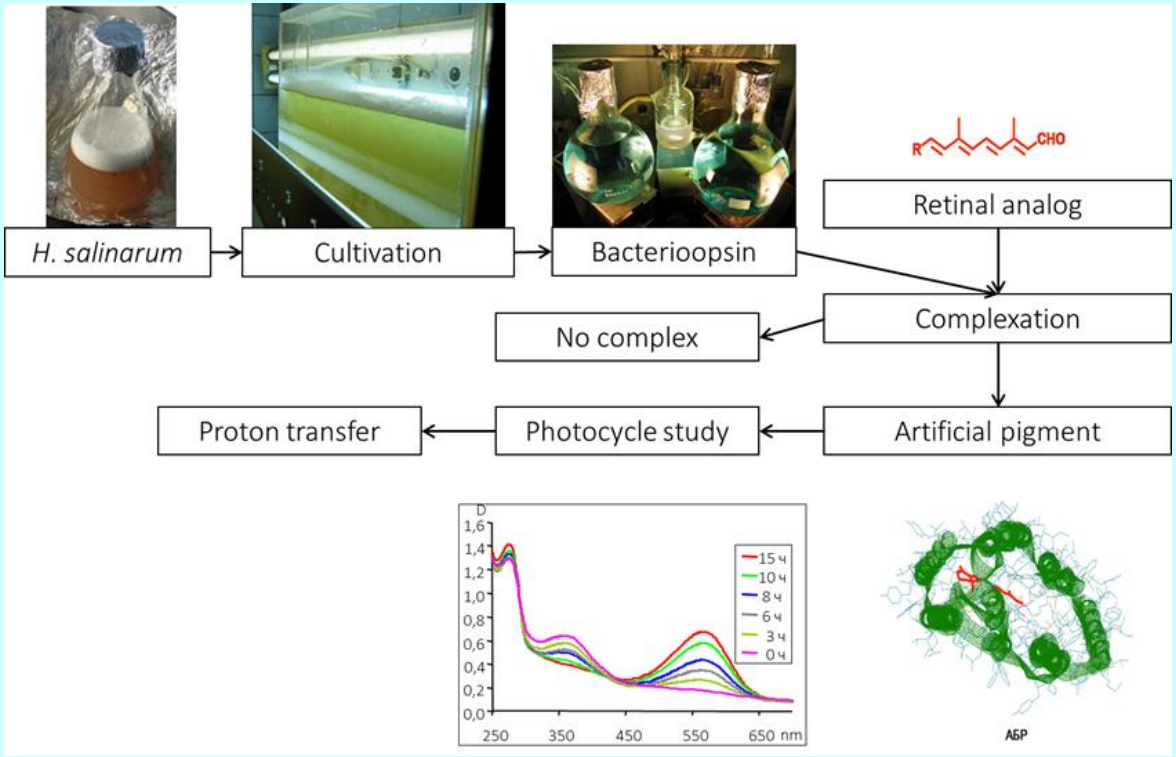
BRh undergoes cyclic photochemical reactions accompanied by the isomerization of the chromophore polyene chain and the deprotonation and reprotonation of the retinal aldimine moiety (Fig. 2D, 2E). The ground - B-state (λ_{max} 570 nm, ε 63,000 M^{-1} cm^{-1}) and the $M_{1,2}$-states (λ_{max} 412 nm, ε 45,000 M^{-1} cm^{-1}, Φ 0.64) are the key states. Fig. 2D depicts the photocycle of BRh with the species spectroscopically characterized, the wavelength at which each intermediate maximally absorbs light and their lifetimes.

The uniqueness of BRh – a natural photocontrollable photosynthetic system – for nanobiophotonics is defined by its following properties:

1) BRh is the most simple and surprisingly stable proton pump;
2) availability in high quantities, simplicity of isolation with relatively low cost;
3) stability in intensive light, oxygen, wide range of temperatures (–196 – 70°C), pH values (0–11), concentrations of salts, water-glycerol media;
4) the "primary act" after photon absorption (B→J) is an extremely fast process (0.5 ps);
5) high quantum yield (Φ 0.64);
6) possibility of making "dry" films as well as integrating BRh into polymer matrices of various compositions;
7) application possibilities both in optical and electronic devices, using either varying optical or electrical component of the response.

This chromoprotein is one of the first successful examples of biological photochromic material designed by the nature. One promising area of research on the retinal protein structure function relationship involves the replacement of the natural chromophore by analogs and the comprehensive study of the hybrid products. The photochemical properties of analogs BRh (ABR) can be controlled using the following approaches: 1) the substitution of one or more amino acid residues in certain positions of the BRh molecule by genetic engineering methods (using BRh mutant strains with slower photocycles); 2) the use of natural BRh incorporated into a polymer matrix, oriented Langmuir-Blodgett films, or oriented layers immobilized on a solid support; 3) the use of environmental conditions (low temperature, electric fields, humidity, pH level); 4) a combination of the above-mentioned approaches.

Fig. 3. Technology of the Bacteriorhodopsin analogs production



One of the approaches to studying the structure-function relationship in this protein as well as other retinal-based proteins is to replace the natural chromophore with its analogs and to comprehensively study the properties of new hybrid products.

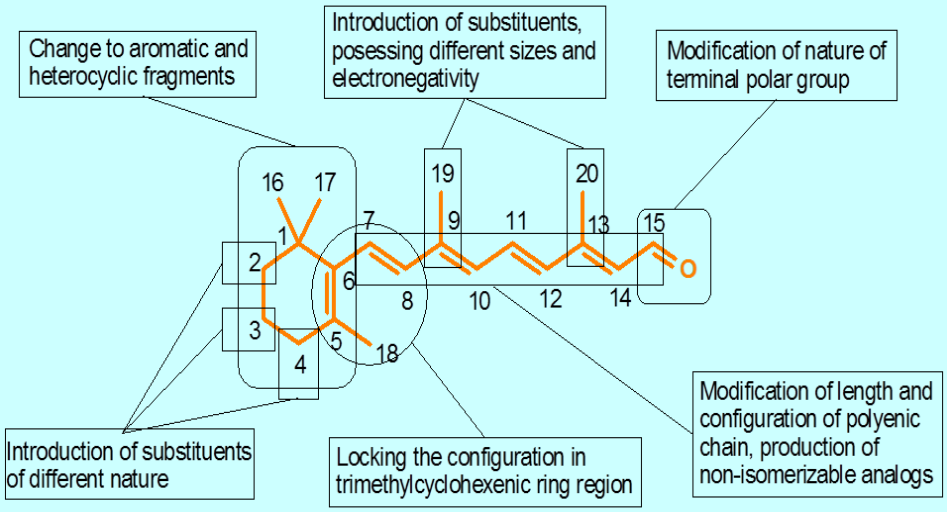
Several approaches to the preparation of ABRhs have been developed earlier based on the addition of polyenals to:

1) growing cells of retinal-deficient *H. salinarum* strains (for example, JW5);
2) to "white" membranes or membrane vesicles obtained from the retinal-deficient strains;
3) to so-called apomembranes containing bacterioopsin (BO) generated from purple membranes by hydroxylaminolysis at pH 7.0 and 0-5^{0}C under intense illumination (see Fig. 3).

We used the third approach in our investigations with an additional procedure for the removal of retinal oxime based on the treatment of BO with a saturated solution of β-cyclodextrin. Then a comprehensive study of the artificial pigments: the kinetic peculiarities of the formation of BRh analogs, the spectral properties (λ_{max}, the presence and type of the photochemical cycle, quantum yield, the adaptation to the light and darkness) and the efficiency of the proton transport were undertaken.

The synthesized retinal analogs were tested in recombination with bacterioopsin (BO), from apomembranes *H. salinarum* (strain ET1001). Apomembranes obtained from purple membranes by hydroxylaminolysis at pH 7.0 and 0 - 5^{o}C and intensive illumination. Resynthesis of pigments conducted by addition of a methanol solution of analog to a suspension apomembranes in a buffer (protein concentration - 2 mg/ml, 21^{0}C, pH 6.0, 5 mM MES). It was found, that the formation of pigments takes place from several min till 1 month period.

Fig. 4. Basic directions in the retinal molecule modification strategy



All retinal molecule modification variants were divided in next charts:

A. Natural chromophore — retinal and its isomers
B. Terminal polar group modification
C. Polyenic chain modification
D. Alteration of the bond types and its disposition in the chromophore polyenic chain
E. Alteration of the polyenic chain length and bond disposition and terminal group types
F. Alteration or locking of the bond configuration. Non-isomerizable analogs
G. Alteration of the trimethylcyclohexenic ring. Ring modification
H. Alteration of the trimethylcyclohexenic ring. Replacement ring to aromatic or heterocyclic fragments
I. Alteration of the trimethylcyclohexenic ring. Acyclic analogs
J. Miscellaneous modifications
K. Labelled BRh derivatives (radioactive, photo-affinic, fluorophoric, heavy-atom, paramagnetic (SL), ionophoric and photochromic probes)

We are presenting our database "**Properties of artificial bacteriorhodopsin analogs**. **Version 2, 2020. From 1975 to 2019**", which combines information from both our own and literature data sources published in period of 1975–2020 [2]. It includes the structural, spectral and photochemical parameters listed below as well as other information on the products of the interaction of more than 440 polyene compounds with the apoprotein – bacterioopsin [2]. The structures of all polyene compounds were classified based on their differences from the natural chromophore molecule (*all-E*-retinal); the **following series of retinal analogs (A-K)** were considered, which differ in certain types of functionally significant structural elements from the molecule of the natural chromophore group (see Fig. 4). The main descriptors were: the structure of the specific isomer of the polyene compound tested; λ_{max} of the starting compound; models (Schiff bases with n-butylamine in methanol, in non-protonated and protonated forms); non-covalent complex with bacterioopsin; pigment in an aqueous buffer (adapted to light and dark – LA and DA-forms); presence and type of photocycle, its main intermediates; efficiency of proton transport; isomeric composition of the chromophore (ratio of *all-E*- and *13Z*-isomers); the "opsin" shift; the stability of the interaction products to hydroxylamine and *all-E*-retinal; and other related data.

The comparative analysis of our database showed that, by diversifying the nature of the chromophore, it is possible to directly change λ_{max} in the spectra of bacteriorhodopsin analogs in a fairly wide range (from 412 to 830 nm), although not all of these new pigments are capable for cyclic photochemical reactions.

The found patterns are not only of purely theoretical significance, but will also make it possible in the future to carry out a directed search and prediction of the spectral properties of new ABRhs within the framework of the investigated series of modifications of the chromophore molecule. This circumstance is of considerable interest in the targeted production of new ABRhs with a given set of spectral and photochemical properties for the application in nanobiophotonics.

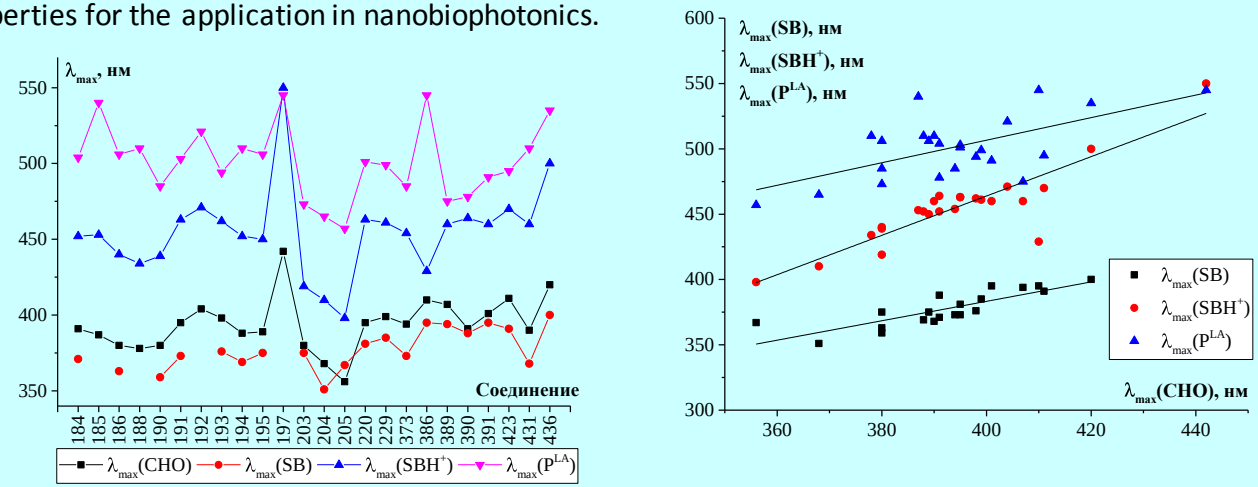


Fig. 5. **A** - Parameters of absorption spectra of aromatic retinal and ABRh analogs, their aldimines with n-butylamine and BO-based pigments. **B** - Influence of the structure of aromatic retinal analogs on the spectral properties of their aldimines with n-butylamine and BO-based pigments.

Research results

1. **Methods for the preparation, separation and analysis of the structure of retinoid derivatives have been developed.**
2. **Original novel methods for preparation of more than 150 new retinoids analogs were developed and the properties of new hybrid / chimeric photosensitive proteins based on them were studied.**
3. **Retinal-based proteins. Studies of structural and functional relationships in molecules of retinal-based proteins have been carried out. (Bacteriorhodopsin, visual pigments of animals). Published more than 50 articles, 2 patents, 200 conference reports**

Funding: The reported study was funded by RFBR, project number 20-03-00139.

References

[1]. Oesterhelt D., Stoeckenius W. Nature (New Biologist), 1971, vol. 233, pp. 149 – 151

[2]. Khodonov A.A., Belikov N.E., Demina O.V. Database **"Properties of artificial bacteriorhodopsin analogs. From 1975 to 2019". Editors: Chupin V.V., Varfolomeev S.D. Version 2, 2020. IBCP/MIPT, Moscow, Russia 186 pp.** http://biochemphysics.ru/assets/upload/documents/docs/BRDT_v2.pdf

[3]. https://www.researchgate.net/publication/340870452_Properties_of_artificial_bacteriorhodopsin_analogs_From_1975_to_2019_Editors_Chupin_VV_Varfolomeev_SD_Version_2_2020_IBCPMIPT_Moscow_Russia_186_pp

[4]. Khodonov A.A., Belikov N.E., Lukin A.Yu., Petrovskaya L.E., Chupin V.V., Demina O.V. Investigation of the selectivity of the environment of the chromophore of the binding site of the bacteriorhodopsin molecule using retinoid analogs. // ***Russian Journal of Biological Physics and Chemistry*****, 2020, 5 (1), pp. 91-100**