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СИНТЕЗ И ИССЛЕДОВАНИЕ СПЕКТРАЛЬНЫХ СВОЙСТВ КОНЪЮГАТОВ BODIPY С АМИНОКИСЛОТАМИ

К.В. Ксенофонтова, А.А. Ксенофонтов, И.А. Ходов, Е.В. Румянцев

Ксения Витальевна Ксенофонтова*

Ивановский государственный химико-технологический университет, Шереметевский пр., 7, Иваново, Российская Федерация, 153000
E-mail: kvk@isuct.ru*

Александр Андреевич Ксенофонтов

Институт химии растворов им. Г.А. Крестова РАН, ул. Академическая, 1, Иваново, Российская Федерация, 153045
E-mail: ivalex.09@mail.ru

Илья Анатольевич Ходов

Институт химии растворов им. Г.А. Крестова РАН, ул. Академическая, 1, Иваново, Российская Федерация, 153045
Институт физики, Казанский федеральный университет, ул. Кремлевская, 18, Казань, Российская Федерация, 420008
E-mail: ilya.khodov@gmail.com

Евгений Владимирович Румянцев

Ивановский государственный химико-технологический университет, Шереметевский пр., 7, Иваново, Российская Федерация, 153000
Ивановский государственный политехнический университет, Шереметевский пр., 21, Иваново, Российская Федерация, 153000
E-mail: naturer@yandex.ru

В настоящей работе описан направленный одностадийный синтез двух новых конъюгатов типа аминокислота – борфторидный комплекс дипиррометена (BODIPY), в которых остатки гистидина и тирозина связаны с остовом флуорофора через аминогруппу. Синтезированные вещества были полностью охарактеризованы посредством ^{1}H и ^{11}B спектроскопии ЯМР-, ИК-спектроскопии, времяпролетной масс-спектрометрии с матрично-активированной лазерной десорбцией/ионизацией. Все полученные данные находятся в соответствии с предполагаемыми структурами. Исследуемые конъюгаты BODIPY с аминокислотами были изучены методами спектрофото- и спектрофлуориметрии. Были получены спектры поглощения и испускания веществ в смеси диметилсульфоксид (ДМСО) – буферный раствор (БР) (1:1). Кроме того, был рассчитан ряд фотофизических характеристик конъюгатов, а именно: относительный квантовый выход флуоресценции, молярный коэффициент экстинкции, относительное время жизни возбужденного состояния, константы скорости излучательной и безызлучательной дезактивации. Были получены 3D спектры флуоресценции веществ в смеси ДМСО – БР (1:1). Было проведено спектрофото- и спектрофлуориметрическое титрование флуорофоров. Результаты титрования позволили нам дополнительно подтвердить стехиометрический состав конъюгатов BODIPY с аминокислотами. Был проведен квантовохимический анализ структурных и спектральных

свойств веществ в основном состоянии. Результаты квантовохимических расчетов позволили понять природу взаимодействий BODIPY с аминокислотой в конъюгатах. Более того, было обнаружено, что синтезированные конъюгаты BODIPY с аминокислотами обладают высокой растворимостью в полярных растворителях. Таким образом, ряд уникальных свойств объектов настоящего исследования обеспечивает возможность их использования для визуализации как биоактивных макромолекул, так и биохимических процессов в живых клетках.

Ключевые слова: BODIPY, аминокислоты, конъюгация, маркеры, флуоресценция

SYNTHESIS AND STUDY OF SPECTRAL PROPERTIES OF AMINO ACIDS – BODIPY CONJUGATES

K.V. Ksenofontova, A.A. Ksenofontov, I.A. Khodov, E.V. Rumyantsev

Ksenia V. Ksenofontova*

Ivanovo State University of Chemistry and Technology, Sheremetevskiy ave., 7, Ivanovo, 153000, Russia
E-mail: kvk@isuct.ru*

Alexander A. Ksenofontov

G. A. Krestov Institute of Solution Chemistry of the RAS, Akademicheskaya st., 1, Ivanovo, 153045, Russia
E-mail: ivalex.09@mail.ru

Ilya A. Khodov

G. A. Krestov Institute of Solution Chemistry of the RAS, Akademicheskaya st., 1, Ivanovo, 153045, Russia
Institute of Physics, Kazan Federal University, Kremlyovskaya st., 18, Kazan, 420008, Russia
E-mail: ilya.khodov@gmail.com

Evgeniy V. Rumyantsev

Ivanovo State University of Chemistry and Technology, Sheremetevskiy ave., 7, Ivanovo, 153000, Russia
Ivanovo State Politechnical University, Sheremetevskiy ave., 21, Ivanovo, 153000, Russia
E-mail: naturer@yandex.ru

The present work describes direct one-pot synthesis of the two novel amino acids – borondipyrromethene (BODIPY) conjugates with histidine and tyrosine residues bound to fluorophore via amino group. The synthesized compounds were fully characterized by means of ^{1}H and ^{11}B nuclear magnetic resonance spectroscopy, infrared spectroscopy, time-of-flight mass spectrometry with matrix-activated laser desorption / ionization. All the data are in accordance with the proposed structures. The amino acids – BODIPY conjugates under study were investigated by means of absorbance and fluorescence spectroscopy. Absorption and emission spectra of the compounds in dimethyl sulfoxide (DMSO) – buffer solution (BS) mixture (1:1) were obtained. Moreover, a number of photophysical characteristics of the conjugates, namely: relative fluorescence quantum yield, molar extinction coefficient, relative fluorescence lifetime, radiative and non-radiative rate constants were calulated. 3D fluorescence spectra of the compounds in DMSO – BS mixture (1:1) were obtained. Absorption and emission titration of the compounds was carried out. The results of the titration allowed us to confirm additionally a stoichiometric composition of the conjugates. Quantum chemical analysis of structural and spectral properties of the compounds in a ground state was carried out. The results of quantum chemical calculations gave an insight to a nature of the BODIPY – amino acid interactions in the conjugates. Besides, the synthesized amino acids – BODIPY conjugates are found to be highly soluble in polar solvents. Thus, a range of unique properties of the objects of the present research enables us to use them for visualization of bioactive macromolecules as well as biochemical processes in living cells.

Key words: BODIPY, amino acids, conjugation, markers, fluorescence

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INTRODUCTION

One of the encouraging areas of modern science is creating fluorescent markers for compounds of biological origin. These objects, labeled with fluorescent molecules, find their application in a huge amount of life science researches [1-3]. In recent years, a class of boron-dipyrromethene (BODIPY) dyes has drawn scientific community attention due to the range of its special features: excellent quantum yield, high molar extinction coefficient, sharp absorption and emission peaks, and great ability to structural modification [4, 5]. All of these cause extensive use of BODIPY derivatives in biomolecule labeling [6].

Synthesis of conjugates based on BODIPY and some biomolecules is relatively new promising direction in chemistry. In this regard, one of the most interesting objects of research are amino acids – BODIPY conjugates. This type of bioconjugates can be applied for visualization of various biochemical processes, such as protein interactions with other biomolecules, protein localization and dynamics, enzyme activity, etc. [7-9].

There is currently not a great number of scientific papers devoted to a development of amino acids – BODIPY conjugates. The only works on this subject are [10-12]. Guzow et al. [10] synthesized an alanine-BODIPY conjugate which can be utilized in peptide synthesis. Mendive-Tapia et al. [11] obtained a tryptophan-BODIPY conjugate and incorporated it into several peptides for *ex vivo* imaging of human tissues. Our scientific group [12] earlier synthesized lysine-, methionine-, and tryptophan-BODIPY conjugates which can be used in bioimaging.

It is noteworthy that conjugates based on BODIPY and other classes of small biomolecules are extensively studied. For example, there are bioconjugates of BODIPY derivatives and nucleotides [13, 14], lipids [15-17], hormones [18, 19], vitamins [20], etc.

The present work describes synthesis and characterization of two novel amino acids – BODIPY conjugates with histidine and tyrosine residues bound to fluorophore via amino group. Spectral properties of the obtained compounds were investigated. Advantageous photophysical characteristics and high solubility in polar solvents make these conjugates promising fluorescent markers for various biological objects.

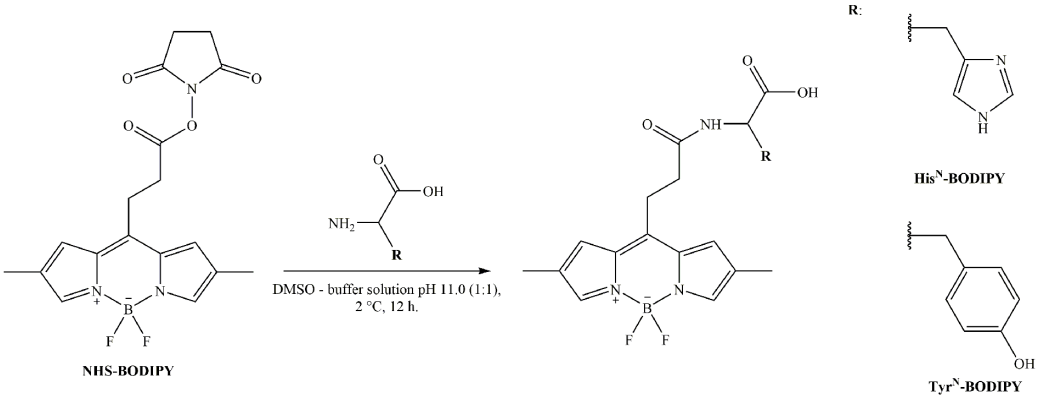
EXPERIMENTAL

Materials

The reagents and solvents were obtained from commercial sources (Sigma-Aldrich, Fisher Chemical, Lab-Scan, Reakhim, Khimmed) and used without further purification.

Instruments

^{1}H and ^{11}B nuclear magnetic resonance (NMR) spectra were obtained by means of a Bruker Avance III 500 NMR spectrometer. Infrared (IR) spectra were recorded on a Bruker VERTEX 80v FTIR spectrometer. Matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectra were registered by a Shimadzu AXIMA Confidence MALDI TOF-TOF mass spectrometer. Absorbance and fluorescence spectroscopy experiments were performed on a Solar SM 2203 spectrofluorometer and an Agilent Cary Eclipse fluorescence spectrophotometer.



Scheme 1. Scheme of amino acids – BODIPY conjugates synthesis
Схема 1. Схема синтеза конъюгатов BODIPY с аминокислотами

Synthesis of amino acids – BODIPY conjugates

The succinimidyl ester of 3-(4,4-difluoro-2,6-dimethyl-4-bora-3a,4a-diaza-s-indacene-8-yl)-propionic acid (NHS-BODIPY) (1 mg, 2.57 μmol, 1 equiv.) synthesized following the procedure [12] was dissolved in 10 ml of dimethyl sulfoxide (DMSO). An amino acid (2.57 μmol, 1 equiv.) was dissolved in 10 ml of $NaHCO_3$ – NaOH buffer solution (BS) [21] with pH of 11.0. At this pH, more than 90% of amino acid species in aqueous solutions are anionic ones, which are perfect for binding to the amine-reactive NHS-BODIPY. The mixture of the NHS-BODIPY in DMSO and an amino acid in BS was stirred in an ice bath for 12 h. After the end of the reaction, the mixture was washed with dichloromethane (DCM) to eliminate the unreacted NHS-BODIPY. The solvents were removed under reduced pressure to give the pure products His^N-BODIPY and Tyr^N-BODIPY (Scheme 1).

N^6-(3-(4,4-Difluoro-2,6-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-8-yl)propanoyl)-L-hystidine His^N-BODIPY: Light brown solid (1.1 mg, 97%). ^{1}H NMR (500.17 MHz, D_2O – H_2O mixture (1:9)): δ (ppm) 8.97 (dd, 1H, J = 7.6, 6.6 Hz, >$NH_{amino\ acid}$), 8.45 (dd, 1H, J = 7.6, 2.1 Hz, –CH=$_{amino\ acid}$), 8.30 (s, 1H, –NH–$_{amide}$), 7.34-7.31 (m, 1H, –CH=$_{amino\ acid}$), 4.47 (dt, 1H, J = 13.0, 7.0 Hz, >CH–$_{amino\ acid}$), 3.82 (td, 2H, J = 7.1, 1.6 Hz, –CH_2–), 3.47 (t, 2H, J = 7.1 Hz, –CH_2–), 3.10-2.96 (m, 2H, –CH_2–$_{amino\ acid}$), 2.58 (d, 3H, J = 1.0 Hz, –CH_3), 2.39 (t, 3H, J = 0.6 Hz, –CH_3). ^{11}B NMR (160.48 MHz, D_2O – H_2O mixture (1:9)): δ (ppm) 2.3 (br, 1B, –BF_2–). IR (KBr): ν (cm^{-1}) 3414 (w, br, N–H_{amide}), 2974-2794 (w, br, C–H), 1690 (w, C=O_{amide}), 1621 (w, C–N_{amide}, N–H_{amide}), 1440 (s, sh, C–N_{amide}), 1057 (w, B–F). MS (MALDI TOF, α-cyano-4-hydroxycinnamic acid (CHCA) matrix): *m*/*z* calculated for $C_{20}H_{21}BF_2KN_5O_3$ [M–H+K] 467.13, found 466.89.

N-(3-(4,4-Difluoro-2,6-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-8-yl)propanoyl)-L-tyrosine Tyr^N-BODIPY: Light brown solid (1.2 mg, 99%). ^{1}H NMR (500.17 MHz, D_2O – H_2O mixture (1:9)): δ (ppm) 8.31 (s, 1H, J = 0.9 Hz, –NH–$_{amide}$), 7.84 (s, 1H, –$OH_{amino\ acid}$), 7.05-6.99 (m, 2H, –CH=$_{amino\ acid}$), 6.71-6.64 (m, 2H, –CH=$_{amino\ acid}$), 4.42 (dt, 1H, J = 12.0, 7.0 Hz, >CH–$_{amino\ acid}$), 3.86 (td, 2H, J = 7.1, 2.1 Hz, –CH_2–), 3.53-3.38 (m, 2H, –CH_2–), 3.09-2.97 (m, 2H, –CH_2–$_{amino\ acid}$), 2.58 (t, 3H, J = 1.0 Hz, –CH_3), 2.31 (s, 3H, –CH_3). ^{11}B NMR (160.48 MHz, D_2O – H_2O mixture (1:9)): δ (ppm) 2.7 (br, 1B, –BF_2–). IR (KBr): ν (cm^{-1}) 3470 (m, br, N–H_{amide}), 2956-2808 (w, br, C–H), 1692 (m, C=O_{amide}), 1624 (w, C–N_{amide}, N–H_{amide}), 1461 (s, sh, C–N_{amide}), 1060 (w, B–F), 652 (m, N–H_{amide}), 610 (m, N–H_{amide}). MS (MALDI TOF, 2,5-dihydroxybenzoic acid (DHB) matrix): *m*/*z* calculated for $C_{23}H_{22}BF_2Li_2N_3O_4$ [M–2H+2Li] 467.20, found 466.91.

Computational details

The quantum chemical calculations of the investigated molecules were carried out on the basis of the methodology described in [12].

Determination of photophysical characteristics

The fluorescence quantum yields Φ of the fluorophores under study were estimated by comparison with Rhodamine 6G as a standard of the known quantum yield (Φ = 94 % in ethanol [22]) using the equation (1) [23].

$$\Phi_x = \Phi_{st} \frac{S_x}{S_{st}} \frac{A_{st}}{A_x} \frac{n_x^2}{n_{st}^2} \quad (1)$$

where Φ is the fluorescence quantum yield, S is the integrated area under the emission spectrum, A is the absorbance at the excitation wavelength, n is the refractive index of the solvent. The subscripts x and st refer to unknown and reference solutions, respectively.

The actual fluorescence lifetimes τ were computed by the PhotochemCAD program [24-26] which is based on the Strickler – Berg approach [27].

The radiative k_r and non-radiative k_{nr} rate constants were calculated using the equations (2) and (3) [23], respectively.

$$k_r = \frac{\Phi}{\tau} \quad (2)$$

$$k_{nr} = \frac{1-\Phi}{\tau} \quad (3)$$

RESULTS AND DISCUSSION

Absorption and emission spectra of amino acids – BODIPY conjugates

It was obtained absorption and emission spectra along with a number of photophysical characteristics of the amino acids – BODIPY conjugates His^N-BODIPY and Tyr^N-BODIPY in DMSO – BS mixture (1:1) (Fig. 1, Table).

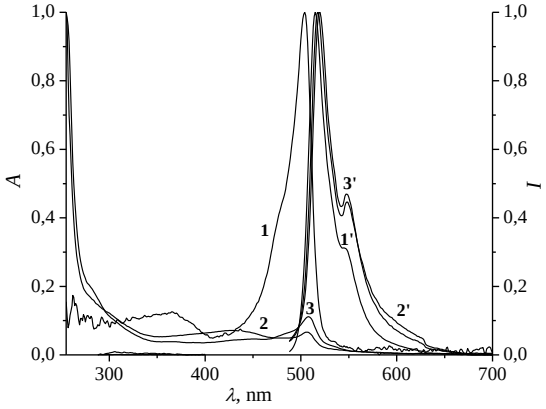


Fig. 1. Normalized absorption and emission spectra of NHS-BODIPY [12] (1 and 1', respectively), His^N-BODIPY (2 and 2', respectively), and Tyr^N-BODIPY (3 and 3', respectively) in DMSO – BS mixture (1:1)

Рис. 1. Нормализованные спектры поглощения и флуоресценции NHS-BODIPY [12] (1 и 1', соответственно), His^N-BODIPY (2 и 2', соответственно) и Tyr^N-BODIPY (3 и 3', соответственно) в смеси диметилсульфоксид (ДМСО) – буферный раствор (БР) (1:1)

Table

Photophysical characteristics of NHS-BODIPY [12], His^N-BODIPY, and Tyr^N-BODIPY in DMSO – BS mixture (1:1)

***Таблица*. Фотофизические характеристики NHS-BODIPY [12], His^N-BODIPY и Tyr^N-BODIPY в смеси ДМСО – БР (1:1)**

	NHS-BODIPY [12]	His^N-BODIPY	Tyr^N-BODIPY
Φ, %	92	33	29
ε, l/mol·cm	64700	7100	3400
τ, ns	3.88	3.71	10.60
k_r, ns^{-1}	0.237	0.089	0.027
k_{nr}, ns^{-1}	0.021	0.181	0.066

The absorption spectra of the amino acids – BODIPY conjugates give three absorption bands: 291, 426, and 506 nm for the His^N-BODIPY and 282, 450, and 508 nm for the Tyr^N-BODIPY. The emission spectra give one band with the maximum absorption wavelength of 518 nm in the case of the His^N-BODIPY and two bands with the maxima absorption wavelengths of 306 and 519 nm in the case of the Tyr^N-BODIPY. The His^N-BODIPY and the Tyr^N-BODIPY exhibit relatively small Stokes shifts of 12 and 11 nm, respectively. The BODIPY characteristic absorption and emission bands maxima of the conjugates are 2-4 nm bathochromically shifted compared with the spectra of the NHS-BODIPY.

It is observed a significant three times decrease of the fluorescence quantum yields of the His^N-BODIPY and the Tyr^N-BODIPY in contrast to that of the NHS-BODIPY. This fact can be explained by several reasons [23]. Firstly, the decrease of the fluorescence quantum yields of the conjugates is connected with an increase of the molecular size resulting in an increase of an internal conversions probability. Secondly, this is due to the photoinduced electron transfer (PeT) process occurring (see section *Quantum chemical calculations of BODIPY precursor and amino acids – BODIPY conjugates*).

During the work, it was discovered that the amino acids – BODIPY conjugates have high solubility in a number of polar solvents, including water. Unfortunately, spectral properties of the bioconjugates in water have not been investigated within the scope of the present study but this research is the aim of our further work.

3D Fluorescence spectra of BODIPY precursor and amino acids – BODIPY conjugates

It was obtained 3D fluorescence spectra of the BODIPY precursor NHS-BODIPY and the amino acids – BODIPY conjugates His^N-BODIPY and Tyr^N-BODIPY in DMSO – BS mixture (1:1) (Fig. 2).

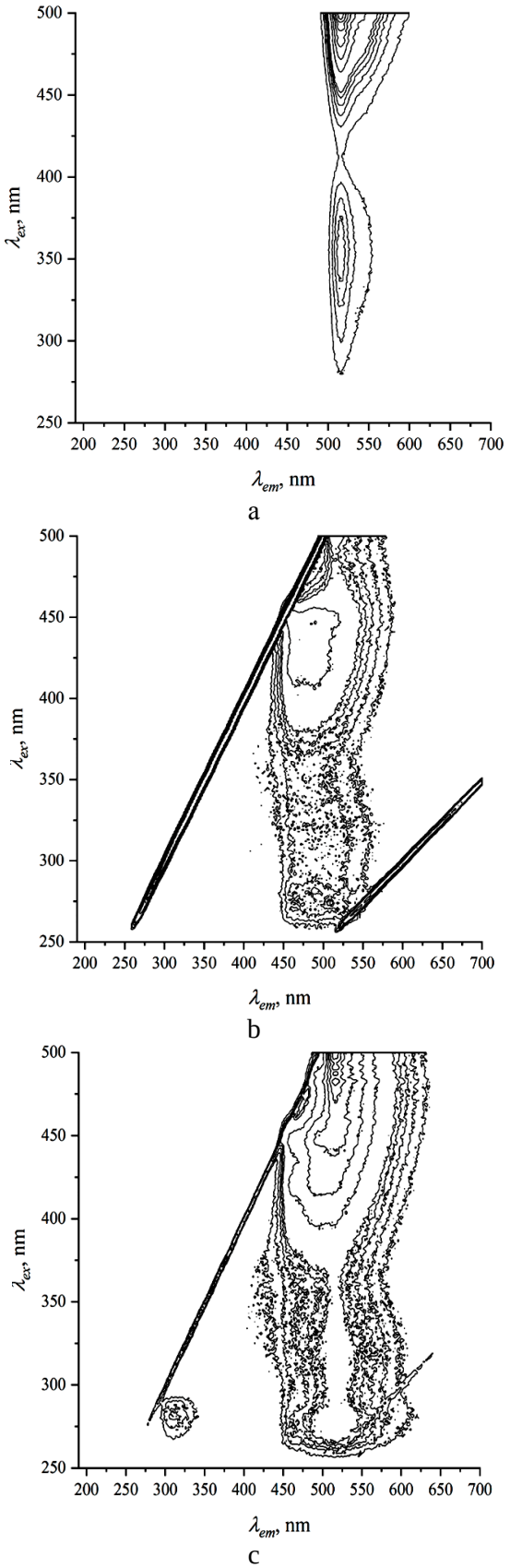


Fig. 2. 3D Fluorescence spectra of NHS-BODIPY (a), His^N-BODIPY (b), and Tyr^N-BODIPY (c) in DMSO – BS mixture (1:1)

Рис. 2. 3D Спектры флуоресценции NHS-BODIPY (a), His^N-BODIPY (b) и Tyr^N-BODIPY (c) в смеси ДМСО – БР (1:1)

3D Fluorescence spectra, obtained by measuring fluorescence emission via scanning both emission and excitation wavelengths, are a valuable tool for analyzing fluorophores. These spectra are consider to be much more useful to discriminate among similar fluorescent dyes than individual numerical data [28].

The 3D fluorescence spectrum of the NHS-BODIPY gives two characteristic excitation / emission peaks: $\lambda_{ex}/\lambda_{em}$ = 350/515 and 500/515 nm. The fluorescence intensity is about ten times higher at λ_{ex} = 500 nm than 350 nm. The peaks mentioned above are due to S_0–S_2 and S_0–S_1 electron transitions, respectively.

The 3D fluorescence spectrum of the His^N-BODIPY gives two characteristic excitation / emission peaks: $\lambda_{ex}/\lambda_{em}$ = 280/500 and 488/500 nm. The fluorescence intensity is about three times higher at λ_{ex} = 488 nm than 280 nm. The peaks mentioned above are due to S_0–S_2 and S_0–S_1 electron transitions, respectively. The 3D fluorescence spectrum of the Tyr^N-BODIPY gives three characteristic excitation / emission peaks: two peaks with $\lambda_{ex}/\lambda_{em}$ = 280/519 and 500/519 nm and one new peak with $\lambda_{ex}/\lambda_{em}$ = 280/306 nm. The fluorescence intensity at 519 nm is about five times higher at λ_{ex} = 500 nm than 280 nm. The first two peaks are due to S_0–S_2 and S_0–S_1 electron transitions, respectively, while the third peak is due to S_0–S_3 electron transition (see section *Quantum chemical calculations of BODIPY precursor and amino acids – BODIPY conjugates*) and is connected to tyrosine residue fluorescence [29].

Thus, the results of 3D fluorescence spectroscopy are in good agreement with the results of absorbance and 2D fluorescence spectroscopy as well as quantum chemical calculations (see sections *Absorption and emission spectra of amino acids – BODIPY conjugates* and *Quantum chemical calculations of BODIPY precursor and amino acids – BODIPY conjugates*).

Absorption and emission titration of amino acids – BODIPY conjugates

It was carried out absorbance and fluorescence titration of the NHS-BODIPY dissolved in DMSO by an amino acid dissolved in BS. Fig. 3 shows the results of fluorescence titration of the NHS-BODIPY by the His as an example. The trends mentioned below are similar for the Tyr and the other types of titration and that is why are not described here.

During the fluorescence titration, it is observed a weak 1 nm bathochromic shift of the emission bands maxima with increasing His concentration. An initial addition of the amino acid causes a strong fluorescence quenching due to an orientation interaction between the BODIPY and the His [12]. At equal concentration of the components, there is the inflection point on the titration curve (Fig. 3(*b*)) that indicates the formation of the His-BODIPY conjugate of stoichiometric composition.

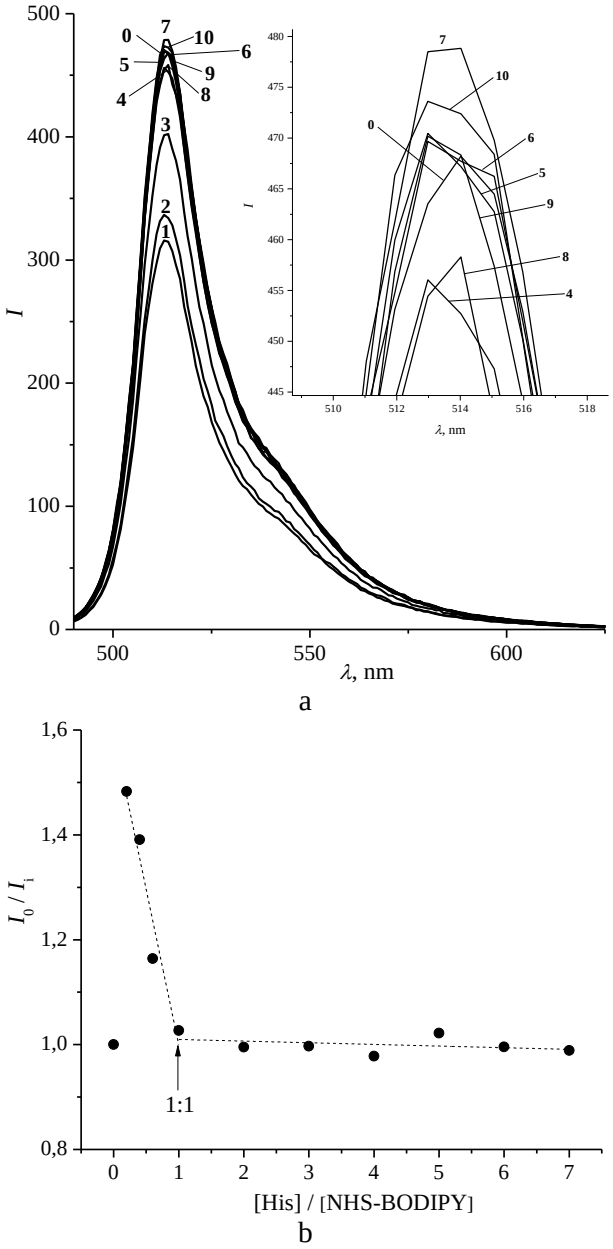


Fig. 3. Emission spectra of NHS-BODIPY in DMSO with increasing His concentration in BS ([His] / [NHS-BODIPY]: 0 – 0.0; 1 – 0.2; 2 – 0.4; 3 – 0.6; 4 – 1.0; 5 – 2.0; 6 – 3.0; 7 – 4.0; 8 – 5.0; 9 – 6.0; 10 – 7.0) (a) and dependence of a ratio of NHS-BODIPY fluorescence intensity at maximum emission wavelength without (I0) and with (Ii) an addition of His on a molar ratio of components ([His] / [NHS-BODIPY]) (b)

Рис. 3. Спектры флуоресценции NHS-BODIPY в ДМСО при увеличении концентрации His в БР ([His] / [NHS-BODIPY]: 0 – 0,0; 1 – 0,2; 2 – 0,4; 3 – 0,6; 4 – 1,0; 5 – 2,0; 6 – 3,0; 7 – 4,0; 8 – 5,0; 9 – 6,0; 10 – 7,0) (a) и зависимость отношения интенсивности флуоресценции NHS-BODIPY на длине волны максимума испускания без (I0) и с (Ii) добавлением His от молярного соотношения компонентов ([His] / [NHS-BODIPY]) (b)

Quantum chemical calculations of BODIPY precursor and amino acids – BODIPY conjugates

It was carried out the quantum chemical analysis of structural and spectral properties of the BODIPY precursor NHS-BODIPY and the amino acids – BODIPY conjugates His^N-BODIPY and Tyr^N-BODIPY in a ground state for interpreting a nature of spectral properties of the fluorophores.

TDDFT analysis results are presented in Fig. 4.

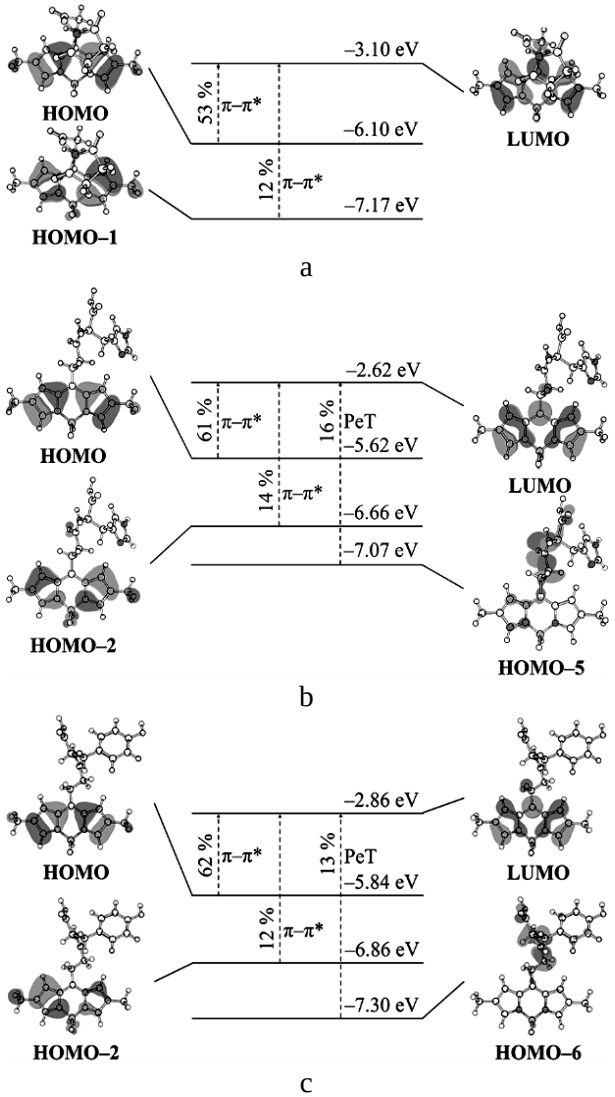


Fig. 4. Frontier molecular orbitals (FMOs) distributions of NHS-BODIPY (a), His^N-BODIPY (b), and Tyr^N-BODIPY (c) (B3LYP-D3(PCM)/def2-TZVP)

Рис. 4. Распределение граничных молекулярных орбиталей NHS-BODIPY (a), His^N-BODIPY (b) и Tyr^N-BODIPY (c) (B3LYP-D3(PCM)/def2-TZVP)

There are three bands of different intensities in the TDDFT spectra of the amino acids – BODIPY conjugates His^N-BODIPY and Tyr^N-BODIPY, as opposed to the BODIPY precursor NHS-BODIPY with two bands. The experimental results exhibit exactly the same trends (see section *Absorption and emission spectra of amino acids – BODIPY conjugates*). All the spectral bands are due to S_0–S_n electron transitions.

In the case of the NHS-BODIPY, S_0–S_1 band has a maximum at 413 nm, while S_0–S_2 band – at 305 nm. Both transitions are caused by intramolecular π–π* energy transfer in a BODIPY moiety.

In the case of the His^N-BODIPY and the Tyr^N-BODIPY, S_0–S_1 band has a maximum in the range of 413-416 nm, S_0–S_2 band – in the range of 307-310 nm, and S_0–S_3 band – at 279 nm. The dominating contribution to spectral properties of the conjugates is made by the highest occupied molecular orbital (HOMO) – lowest unoccupied molecular orbital (LUMO) transition with the oscillator strength of 61-62%. Both S_0–S_1 and S_0–S_2 transitions are caused by intramolecular π–π* energy transfer in a BODIPY moiety of a conjugate. HOMO-5 – LUMO transition in the His^N-BODIPY molecule and HOMO-6 – LUMO transition in the Tyr^N-BODIPY molecule are caused by PeT from an electron donating amino acid moiety of a conjugate to an electron withdrawing BODIPY one (PeT mechanism).

CONCLUSIONS

The two novel fluorescent amino acids – BODIPY conjugates His^N-BODIPY and Tyr^N-BODIPY were obtained through a direct one-step synthesis and thoroughly characterized using ^1H and ^{11}B NMR-spectroscopy, IR-spectroscopy, MALDI-TOF-spectrometry. Spectral properties of the amino acids – BODIPY conjugates His^N-BODIPY and Tyr^N-BODIPY were investigated by absorbance spectroscopy, 2D and 3D fluorescence spectroscopy, computer modeling. The synthesized amino acids – BODIPY conjugates His^N-BODIPY and Tyr^N-BODIPY are found to be highly soluble in polar solvents. This valuable feature allows us to expect an increase in their bioavailability and affinity for peptides and proteins and hence their potential use as fluorescent markers for various biological objects.

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