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Synthesis, spectral-fluorescence properties and TD-DFT calculations of 4-canotryptophan and its derivatives

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Tryptophan-based fluorescent amino acids are promising alternatives to native tryptophan (Trp) for biological fluorescence studies. This work reports the synthesis and structure characterization of 4-cyanotryptophan (4-CN-Trp) based on the modified Mannich reaction. The optical spectra of 4-CN-Trp measured in solvents of different natures revealed the essential red-shifted absorption and emission in aqueous solutions compared to unsubstituted Trp. Moreover, the high fluorescence quantum yield of 4-CN-Trp makes it a promising replacement for native Trp for the study of folding and denaturation of proteins containing several Trp residues. In addition, the TD-DFT calculations were utilized for computer-aided design of dicyano-substituted Trp, suggesting that 4,6- and 4,7-diCN-Trp are promising for protein studies due to their red-shifted fluorescence.

Keywords: organic synthesis, heterocycles, non-natural amino acid, fluorescent probe, DFT.

Синтез, спектрально-флуоресцентні властивості та TD-DFT розрахунки 4-ціанотриптофану та його похідних. *Р.Г. Шипов, О.В. Буравов, Е.С. Гладков, Л.В. Чепелєва, О.В. Кириченко.*

Флуоресцентні амінокислоти на основі триптофану є багатообіцяючою альтернативою нативному триптофану (Trp) для досліджень біологічної флуоресценції. У цій роботі повідомляється про синтез і спектральні властивості 4-ціанотриптофану (4-CN-Trp) на основі модифікованої реакції Манніха. Оптичні спектри 4-CN-Trp, виміряні в розчинниках різної природи, виявили суттєве червоне зміщення поглинання та випромінювання у водному розчині порівняно з незаміщеним Trp. Високий квантовий вихід флуоресценції 4-CN-Trp робить його перспективною заміною нативного Trp для дослідження згортання та денатурації білків, що містять декілька залишків Trp. Крім того, розрахунки методом TD-DFT були використані для дизайну диціано-заміщених Trp та показали, що 4,6- та 4,7-diCN-Trp є перспективними для досліджень білків завдяки їхній флуоресценції з червоним зсувом.

1. Introduction

The study of the tertiary structure and dynamics of complex protein macromolecules using fluorescence spectroscopy methods requires the inclusion of one or more external fluorophores in the molecule, which should not significantly affect any native behavior of the studied system [1-3]. It requires developing diverse fluorescent probes that differ in chemical,

physical, and photophysical properties. Among such compounds, fluorescent amino acids based on tryptophan (Trp) are promising alternative [4-5]. Numerous Trp derivatives have become a fluorescent template for developing new red fluorescent amino acids [6].

The one-reactor biocatalytic synthesis method [7], which demonstrated the synthesis of D-tryptophan from indoles in good yield and high enantiomeric yield (91% to >99%), is a convenient and widespread method for the synthesis of tryptophan derivatives. This method was applied to the synthesis on a preparative scale of a wide range of D-tryptophan derivatives containing electron-donating or accepting substituents in all positions of the benzene part of the indole. Enantioselective synthesis of tryptophan derivatives by the tandem Friedel–Crafts conjugate addition/asymmetric protonation reaction between 2-substituted indoles and methyl 2-acetamidoacrylate has been reported [7]. The reaction was catalyzed by (*R*)-3,3′-dibromo-BINOL in the presence of stoichiometric $SnCl_4$ as the catalyst. The convergent nature of this transformation has allowed the production of a series of unnatural tryptophan derivatives for use in a broad array of synthetic and biological applications [7].

In addition, a rapid synthesis of Fmoc and Boc protected analogues of L-tryptophan with a disubstituted ring A was proposed, starting from the corresponding 2,4- or 2,3-disubstituted phenylhydrazines and optically active N,N-diprotected g-aldehydes of L-glutamic acid [9]. The main stage of this synthesis is the Fischer indole synthesis reaction. The method is convenient for the synthesis of optically active tryptophans, starting from a simple and common chiral precursor. These building blocks are widely used in peptide and combinatorial chemistry [9]. In addition, a scalable synthesis of the Fmoc-protected blue fluorescent amino acid, *L*-4-cyanotryptophan using enantioselective phase transfer-catalyzed alkylation has been reported [8]. Some preliminary spectroscopic studies of cyanotryptophans [9-11] and Trp-containing oligopeptides [12-15] have already been reported. However, in-depth understanding of their photophysics is still required.

In this study, we present the synthesis and spectral characterization of 4-cyanotryptophan (4-CN-Trp) and its derivatives. The introduction of the cyano group leads to a significant red shift in the absorption and emission spectra of 4-CN-Trp compared to the typical spectral range of protein bioluminescence. Moreover, these spectral data were used to calibrate our TD-DFT calculations, which allowed us to subsequently propose other red-shifted probes based on dicyano-substituted unnatural amino acids.

2. Experimental

Materials and Methods

^1H and ^{13}C NMR spectra were recorded on a Bruker 170 Avance 500 spectrometer (at 500 MHz for protons and 126 MHz for Carbon-13) and a Varian Unity Plus 400 spectrometer (at 400 MHz for protons and 101 MHz for Carbon-13 in DMSO-d_6). The signals are given in the δ scale. Mass spectra were recorded on an Agilent 1100 high-performance liquid chromatograph (HPLC) equipped with a diode matrix and an Agilent LC/MSD SL mass-selective detector, a SUPELCO Ascentis Express C18 chromatographic column 2.7 μm × 4.6 mm × 15 cm. Control throughout the reaction and the individuality of the obtained substances was carried out by the TLC method on silica gel-coated Polychrom SI F254 plates with a fluorescent detector in the hexane-THF 3:1 system, the developer was an ultraviolet lamp. The elemental analysis was realized on a EuroVector EA-3000 instrument. The melting points of all synthesized compounds were determined using a Hanon Instruments MP450 open capillary tube automatic melting point apparatus.

The starting 1H-indole-4-carbonitrile was commercially available and it was used without additionally purification.

Spectroscopic measurements

Electronic absorption spectra were measured by an Agilent Cary 3500 UV-Vis Multicell Spectrophotometer. Fluorescence spectra were acquired using a Hitachi 850 steady-state fluorescence spectrometer equipped with double-grating excitation and emission monochromators. The fluorescence measurements were made in a 10×10 mm cuvette maintained at 20 °C.

DFT Calculations

The structure of tryptophan and its derivatives was first optimized by the density functional theory (DFT) approach at the B3LYP/cc-pVDZ theory level using the PCM model for the water solution. UV-vis absorption spectra of tryptophan derivatives were calculated using the time-dependent DFT (TD-DFT) with

the hybrid B3LYP functional [16] and the cc-pVDZ basis set [17]. The PCM model was used to mimic the water solution. Up to ten vertical transitions $S_0 \rightarrow S_n$ were calculated. The excited-state transitions with non-zero oscillator strength were approximated by the Gaussian function to take into account the spectral band broadening in solution. So, the simulated TD-DFT absorption spectra were represented by a superposition of a sum of these single Gaussian bands. All DFT calculations were performed with the Gaussian 16 software package [18].

Synthesis: 3-((Dimethylamino)methyl)-1H-indole-4-carbonitrile (1)

13.9 ml (0.11 mol) of dimethylamine (40% solution in water) and 10.5 ml (0.11 mol) of 37% formaldehyde solution were added to 50 mL of THF at a temperature of -4 °C. 13.1 mL (0.23 mol) of acetic acid was added to the solution and the mixture was left to stir while cooling for 30 minutes. The solution of 14.2 g (0.1 mol) of 1H-indole-4-carbonitrile in 100 mL of acetic acid was added dropsy to the mixture. The reaction mixture was stirred at room temperature for 24 h. After the end of the reaction (TLC control), the reaction mixture was neutralized with a Na_2CO_3 solution until a precipitate formed. The precipitate was filtered and washed with acetonitrile and dried.

Yield: 16.3 g (80%), pale beige solid, mp 174-175 °C.

The ^{1}H NMR spectrum, δ, ppm: 11.6 (bs, NH), 7.65 (d, 1H), 7.49 (s, 1H), 7.46 (d, 1H), 7.20 (t, 1H), 3.62 (s, 2H, CH_2), 2.16 (s, 6H, CH_3).

Diethyl 2-acetamido-2-((4-cyano-1H-indol-3-yl)methyl)malonate (2)

0.84 g (0.021 mol) sodium hydroxide was carefully added to 120 mL boiling toluene. Then, a mixture of 13.9 g (0.07 mol) 3-((dimethylamino)methyl)-1H-indole-4-carbonitrile (1) and 16.0 g (0.74 mol) diethyl 2-acetamidomalonate was incorporated into the reaction mixture. The reaction mixture was vigorously stirred at 120°C for 24 h under Ar atmosphere. Upon completion of the reaction (TLC control), the reaction mixture was cooled to 0°C and mixed until crystallization occurred. The resulting precipitate was filtered and washed with hexane and dried under vacuum.

Yield: 23.4 g (89%), pale yellow solid, mp 179-181 °C.

The ^{1}H NMR spectrum, δ, ppm: 11.0 (bs, 1H, NH), 7.92 (s, 1H, NH), 7.62 (d, J = 6.5 Hz, 1H), 7.61 (d, J = 7.0 Hz, 1H), 7.45 (d, J = 6.5 Hz, 1H), 7.31 (t, J = 7.5 Hz, 1H), 7.18 (s, 1H), 4.1 (q, 4H, CH_2), 3.30 (s, 2H, CH_2), 1.95 (s, 3H, CH_3), 1.3 (t, 6H, CH_3)

The ^{13}C NMR spectrum, δ, ppm: 169.0, 168.2, 167.7, 135.1, 125.9, 123.0, 120.9, 117.1, 115.4, 110.8, 104.5, 75.2, 61.3, 30.1, 23.3, 14.1.

The mass spectrum, m/z (I_{rel}, %): 372.2 $[M+H]^+$ (100).

2-Acetamido-2-((4-cyano-1H-indol-3-yl)methyl)malonic acid (3)

20 g (0.054 mol) of diethyl 2-acetamido-2-((4-cyano-1H-indol-3-yl)methyl)malonate (2) was dissolved in 120 mL of a mixture of methanol and water (1:1). Then, 9.1 g (0.162 mol) of potassium hydroxide was added to the reaction mixture. The reaction mixture was stirred at room temperature for 24 hours. After the end of the reaction (TLC control), the reaction mixture was brought to pH = 1 with a hydrochloric acid solution, extracted with ethyl acetate. The extract was evaporated from the solvent under vacuum.

Yield: 16.1 g (95%), pale white solid, mp 110 °C (with decomposition).

The ^{1}H NMR spectrum (400 MHz, DMSO-d_6), δ, ppm: 12.72 (s, 2H, 2 x COOH), 11.2 (bs, 1H, NH), 7.92 (s, 1H, NH), 7.72 (d, J = 6.5 Hz, 1H), 7.52-7.48 (d, J = 7.0 Hz, 2H), 7.20 (t, J = 6.5 Hz, 1H), 3.30 (s, 2H, CH_2), 1.95 (s, 3H, CH_3).

The ^{13}C NMR spectrum (100 MHz, DMSO-d_6), δ, ppm: 176.0, 169.0, 168.2, 135.1, 125.9, 123.0, 120.9, 117.1, 115.4, 110.8, 79.6, 29.5, 23.3.

The mass spectrum, m/z (I_{rel}, %): 316.1 $[M+H]^+$ (100).

2-Acetamido-3-(4-cyano-1H-indol-3-yl)propanoic acid (4)

15.0 g (0.048 mol) of 2-acetamido-2-((4-cyano-1H-indol-3-yl)methyl)malonic acid (3) was suspended in 150 mL of distilled water and heated until the solution boils. The reaction mixture was stirred at 100°C for 24 h. After the end of the reaction (TLC control), the reaction mixture was acidified with a hydrochloric acid solution to pH = 1. The precipitate formed was filtered and washed with acetonitrile and dried.

Yield: 11.6 g (88%), pale white solid, mp 176-177 °C.

The ^{1}H NMR spectrum, δ, ppm: 12.89 (s, H, COOH), 11.2 (bs, 1H, NH), 8.22 (s, 1H, NH), 7.72 (d, J = 6.5 Hz, 1H), 7.52-7.48 (d, J = 7.0 Hz, 2H), 7.20 (t, J = 6.5 Hz, 1H), 4.22 (s, 1H, CH), 3.46-3.20 (dd, 2H, CH_2), 1.9 (s, 3H, CH_3).

The ^{13}C NMR spectrum, δ, ppm: 174.7, 170.7, 168.2, 135.1, 125.9, 123.0, 120.9, 117.1, 115.4, 104.8, 60.0, 27.7, 23.3.

The mass spectrum, *m/z* (I_{rel}, %): 272.3 $[M+H]^+$ (100).

Found, %: C 61.95; H 4.82; N 15.53. $C_{14}H_{13}N_3O_3$. Calculated, %: C 61.99; H 4.83; N 15.49.

Methyl 2-amino-3-(4-cyano-1H-indol-3-yl)propanoate (5)

94 mL of TMSCl (0.738 mol) was added dropwise to 300 mL of MeOH at –4ºC. After 30 minutes of stirring, 20.0 g (0.0738 mol) of 2-acetamido-3-(4-cyano-1H-indol-3-yl)propanoic acid (4) was added to the mixture. The reaction mixture was stirred at RT for 48 h. The reaction mixture was evaporated from the solvent under vacuum. 100 mL of water was added and the product was extracted with ethyl acetate and evaporated under vacuum.

Yield: 4.96 g (22%), pink solid, mp 146-147 ºC.

The ^{1}H NMR spectrum, δ, ppm: 12.5 (s, H, COOH), 12.0 (bs, 1H, NH), 8.7-8.5 (s, 3H, NH_3), 7.8 (d, *J* = 6.5 Hz, 1H), 7.6 (d, *J* = 7.0 Hz, 1H), 7.51 (d, *J* = 7.0 Hz, 1H), 7.20 (t, *J* = 6.5 Hz, 1H), 4.22 (s, 1H, CH), 3.64 (s, 3H, OCH_3), 3.58-3.41 (dd, J = 16 Hz, 2H, CH_2).

The ^{13}C NMR spectrum, δ, ppm: 174.7, 168.2, 138.1, 128.9, 126.0, 123.9, 117.1, 115.4, 100.8, 55,3, 51.9, 27.7, 23.3.

The mass spectrum, *m/z* (I_{rel}, %): 244.0 $[M+H]^+$ (100).

Found, %: C 64.23; H 5.35; N 17.28. $C_{13}H_{13}N_3O_2$. Calculated, %: C 64.19; H 5.39; N 17.27.

2-Amino-3-(4-cyano-1H-indol-3-yl)propanoic acid hydrochloride (6)

4.0 g (0.0136 mol) of methyl 2-amino-3-(4-cyano-1H-indol-3-yl)propanoate (5) was added to a solution of 1.52 g (0.0272 mol) of potassium hydroxide in 50 mL of water. The reaction mixture was stirred at RT for 24 h. The reaction mixture was acidified to pH = 1 with a solution of HCl, and the solvent was evaporated under vacuum. The resulting precipitate was washed with absolute methanol and the resulting solution was evaporated from the solvent under vacuum.

Yield: 3.07 g (85%), orange solid, mp 242-243 ºC (with decomp.)

The ^{1}H NMR spectrum, δ, ppm: 12.5 (bs, H, COOH), 11.5 (bs, 1H, NH), 8.22 (s, 3H, NH_3), 7.72 (d, *J* = 6.5 Hz, 1H), 7.52-7.48 (d, *J* = 7.0 Hz, 2H), 7.20 (t, *J* = 6.5 Hz, 1H), 4.22 (s, 1H, CH), 3.58-3.30 (dd, 2H, CH_2).

The ^{13}C NMR spectrum, δ, ppm: 174.7, 168.2, 138.1, 128.9, 126.0, 123.9, 117.1, 115.4, 100.8, 52.2, 27.7, 23.3.

The mass spectrum, *m/z* (I_{rel}, %): 230.1 $[M+H]^+$ (100).

Found, %: C 62.83; H 4.87; N 18.30. $C_{12}H_{11}N_3O_2$. Calculated, %: C 62.87; H 4.84; N 18.33.

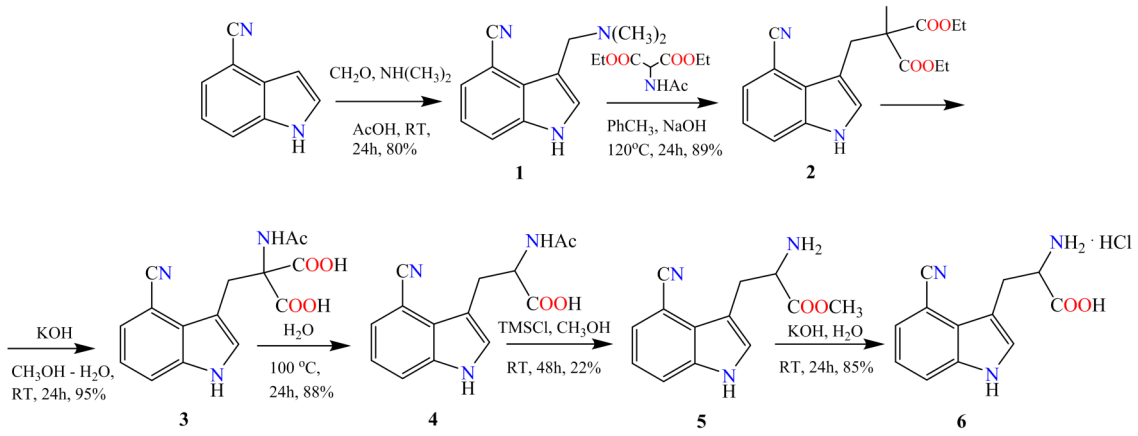
3. Results and discussion

Synthesis

Cyanotryptophan derivatives were synthesized from 1H-indole-4-carbonitrile via 3-((dimethylamino)methyl)-1H-indole-4-carbonitrile (*1*) (4-cyanogramine), which allows us to obtain the target compounds with an good yield [19]. In order to obtain new fluorescent analogs of tryptophan, we synthesized its derivative –2-amino-3-(4-cyano-1H-indol-3-yl)propionic acid (4-cyanotryptophan, 4-CN-Trp) (*5*) and some derivatives containing protective groups – 2-acetamido-3-(4-cyano-1H-indol-3-yl)propanoic acid (*4*) and methyl 2-amino-3-(4-cyano-1H-indol-3-yl)propanoate (*6*) (Scheme 1).

The 3-((dimethylamino)methyl)-1H-indole -4-carbonitrile (*1*) (4-cyanogramine) was synthesized from 1H-indole-4-carbonitrile by the modified Mannich reaction with dimethylamine and formaldehyde. Diethyl 2-acetamido-2-((4-cyano-1H-indol-3-yl)methyl)malonate (*2*) was synthesized by heating from diethyl 2-acetamidomalonate in toluene in the presence of sodium hydroxide. 2-Acetamido-2-((4-cyano-1H-indol-3-yl)methyl)malonic acid (*3*) was obtained by hydrolysis of ester (*2*) and subsequent decarboxylation led to the acetyl-protected amino acid - 2-acetamido -3-(4-cyano-1H-indol-3-yl)propanoic acid (*4*). After acidic hydrolysis and esterification (HCl in CH_3OH) of the acetyl derivative (4) at RT, methyl 2-amino-3-(4-cyano-1H-indol-3-yl)propanoate (*5*) was obtained. The unprotected 2-amino-3-(4-cyano-1H-indol-3-yl)propanoic acid (*6*) was synthesized by hydrolyze with KOH in water at RT. The proposed synthesis scheme and corresponding mild hydrolyses conditions made it possible to keep the cyano-group unchanged and obtain the target product with a satisfactory yield (Scheme 1).

The structure of all intermediates 1-3 and the target compound 4-6 was established by ^{1}H-, ^{13}C NMR spectroscopy and mass spec-



Scheme 1. Synthesis of 2-amino-3-(4-cyano-1H-indol-3-yl)propanoic acid (6) and its derivatives

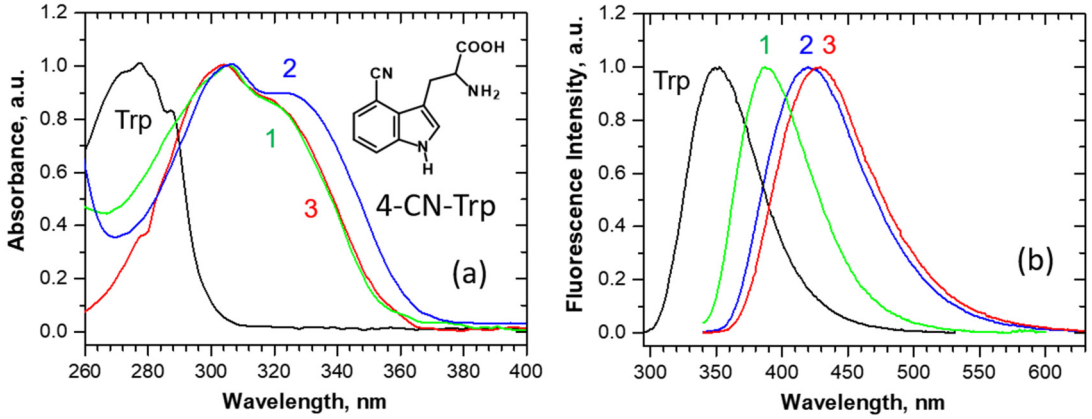


Fig. 1. Comparison of electronic absorption and fluorescence spectra of tryptophan (Trp) in phosphate buffer pH 6.86 and 4-CN-Trp in solvents of the different nature: *1* – 1,4-dioxane, *2* – DMSO, *3* – phosphate buffer pH 6.86. The fluorescence spectra were measured after excitation at 290 nm

trometry. All the spectra are well with those for previously described compounds [8].

Spectral Fluorescent Properties

Indole and Trp fluorescence is known to be environment sensitive, so that their emission properties depend strongly on hydrogen bonding with solvent molecules [5, 20-21]. Therefore, using optical spectroscopy methods, we investigated the electronic effect of the cyano group in the position 4 of an indole chromophore on the emission properties of 4-CN-Trp and their dependence on the solvent nature and polarity. Fig. 1 presents the electronic absorption and fluorescence spectra of 4-CN-Trp in solvents of different natures (1,4-dioxane, DMSO, phosphate buffer). In a phosphate buffer pH 6.86, a bathochromic shift of the absorption spectrum maximum of 4-CN-Trp relative to native Trp from 275 nm to 305 nm is observed, which is accompanied by a significant broadening of the spectral band (Fig. 1a). Thus, 4-CN-Trp has substantial technological advantages in the biological fluorescence study of proteins that contain more than one Trp fluorophore, since it provides selective excitation by visible light.

Fig. 1b shows a bathochromic shift in the fluorescence spectra of 4-CN-Trp relative to that of native Trp: in phosphate buffer (pH 6.86), Trp has a fluorescence maximum at 345 nm, while 4-CN-Trp emits at 430 nm. The fluorescence of 4-CN-Trp is sensitive to the polarity of the solvent. When passing from an aqueous solution to nonpolar 1,4-dioxane, the maximum of the fluorescence spectrum is blue-shifted to 386 nm. Moreover, in the water environment, 4-CN-Trp fluorescence is characterized by a large fluorescence quantum yield (FQY) of 0.68 (Table 1) compared to that of 0.15 for unsubstituted Trp [14]. Thus, the fluorescent 4-CN-Trp probe is a promising replacement for native Trp to study the processes of folding and denaturation of proteins containing several tryptophan residues.

Table 1. Spectral properties of 4-CN-Trp in different solvents

Solvent	$\lambda_{abs\ maximum}$, nm	$\lambda_{fluor\ maximum}$, nm	FQY
1,4-dioxane	303	386	0.21
DMSO	307	420	0.27
phosphate buffer pH 6.86	305	430	0.68

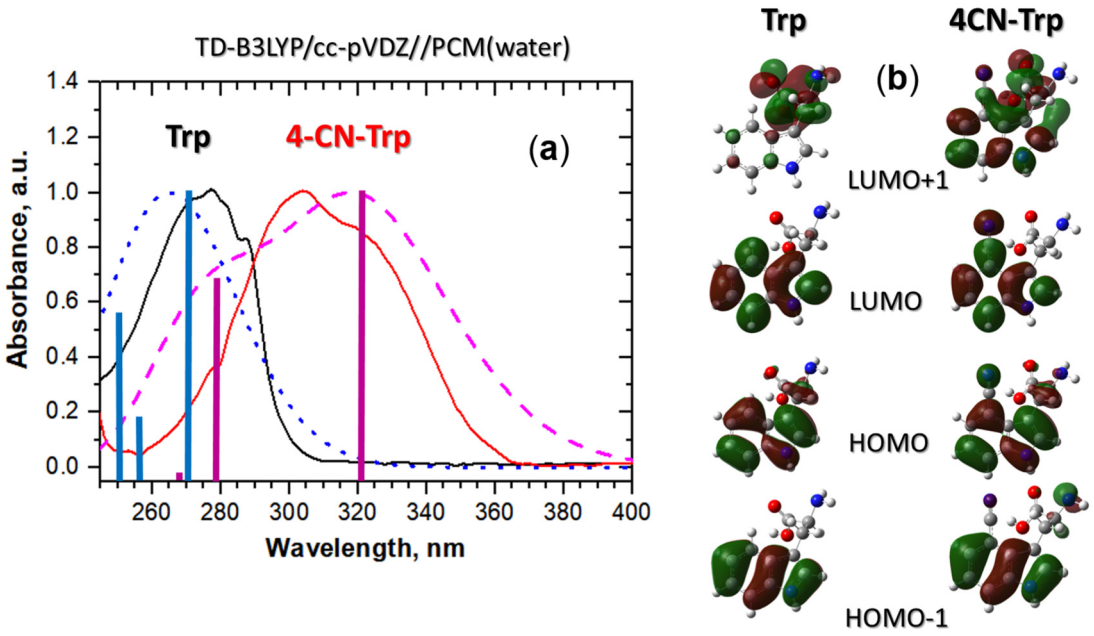


Fig. 2. (a) Experimental (solid lines) and calculated (dotted lines) absorption spectra for Trp and 4-CN-Trp in water. The calculated vertical $S_0 \rightarrow S_n$ transitions are plotted by color-coded bars. (b) Frontier molecular orbitals of Trp and 4-CN-Trp calculated by the TD-B3LYP/cc-pVDZ(PCM-water) method.

TD-DFT Calculations

To better understand the nature of electronic excited states and the origin of the spectral shift in 4-CN-Trp, we calculated its spectrum using the time-dependent density functional theory (TD-DFT) method. The TD-DFT calculations have demonstrated good performance in evaluating the electronically excited states of indoles and tryptophans [22-25].

Fig. 2 provides a detailed comparison of the experimental absorption spectra of Trp and 4-CN-Trp in phosphate buffer pH 6.86 with their theoretical spectra estimated by the TD-B3LYP/cc-pVDZ method using the PMC-water solvation model. As shown in Fig. 2, this spectral comparison is crucial, since it reveals the red-spectral shifts of 4-CN-Trp versus Trp, which are significant in our study.

Table 2 summarizes the TD-DFT-estimated excited-state properties of Trp and 4-CN-Trp. The analysis of the natural orbitals and the excited-state configurations allowed us to assign the lowest excited-state transition $S_0 \rightarrow S_1$ in Trp to the $L_a(\pi\pi^*)$ state, which agrees with the assignments in indole and its derivatives [24-26]. In 4-CN-Trp, the TD-DFT calculations indicate the same $L_a(\pi\pi^*)$ nature of the lowest S_1 state, which is also in good agreement with its high FQY (Table 1). Thus, the good performance of our TD-DFT calculations in reproducing the main spectral features of the studied tryptophans makes it possible to guide the theoretical design of other promising red-shifted probes based on dicyano-substituted Trp.

Table 2 and Fig. 3 summarize our computer-aided design of dicyano-substituted Trp and show a further red shift of the absorption band maxima for 4,6- and 4,7-diCN-Trp in the aqueous solution. The high oscillator strength of the lowest excited-state singlet state transition of 4,6- and 4,7-diCN-Trp promises their bright fluorescence with a shift to the red region of the spectrum.

4. Summary and perspectives

Biological fluorescence studies require developing modified fluorescent amino acids with red-shifted fluorescence. Here, we synthesized

Table 2. The first three excited-state vertical electronic transitions, their oscillator strength, electronic configuration and assignment calculated by TD-B3LYP/cc-pVDZ(PCM-water)

Electronic Transition	Vertical Excitation Energy, nm	oscillator strength, *f*	Electronic configuration (HOMO=H, LUMO=L)
Trp			
$S_0 \rightarrow S_1$	270.9	0.0798	$L_a(\pi\pi^*)$ H→L (0.67516) H-1→L+2 (0.12723)
$S_0 \rightarrow S_2$	256.7	0.0101	$L_b(\pi\pi^*)$ H-1→L (-0.42005) H→L+1 (0.49174)
$S_0 \rightarrow S_3$	251.1	0.0352	H-1→L (0.37468) H→L+1 (0.50347) H→L+2 (-0.31577)
4-CN-Trp			
$S_0 \rightarrow S_1$	322.0	0.1184	$L_a(\pi\pi^*)$ H→L (0.69095)
$S_0 \rightarrow S_2$	277.3	0.0792	$L_b(\pi\pi^*)$ H-1→L (0.63955) H→L+1 (0.21670)
$S_0 \rightarrow S_3$	268.9	0.0006	H-2→L (0.70276)
4,6-diCN-Trp			
$S_0 \rightarrow S_1$	333.2	0.1281	$L_a(\pi\pi^*)$ H→L (0.68414)
$S_0 \rightarrow S_2$	293.4	0.0033	H-2→L (0.49485) H→L+1 (-0.46229)
$S_0 \rightarrow S_3$	283.3	0.0036	H-1→L (0.67469) H-2→L (-0.19655)
4,7-diCN-Trp			
$S_0 \rightarrow S_1$	366.1	0.1114	$L_a(\pi\pi^*)$ H→L (0.69102)
$S_0 \rightarrow S_2$	307.6	0.0192	H-1→L (0.70331)
$S_0 \rightarrow S_3$	301.8	0.1167	H-2→L (0.66555) H→L+1 (-0.21050)
5,7-diCN-Trp			
$S_0 \rightarrow S_1$	325.7	0.0598	$L_a(\pi\pi^*)$ H→L (0.69102)
$S_0 \rightarrow S_2$	293.3	0.0185	H→L+1 (-0.43448) H-1→L (0.46822)
$S_0 \rightarrow S_3$	274.9	0.0024	H-1→L (0.39977) H-2→L (0.57733)

and characterized unnatural 4-cyanotryptophan (4-CN-Trp) amino acid. The synthesis of 4-CN-Trp was carried out under mild conditions using a modified Mannich reaction that avoids hydrolysis of the cyano-group. We found that the essential feature of 4-CN-Trp is that its absorption and emission spectra are red-shifted compared to unsubstituted Trp. The introduction of the 4-CN group increased the fluorescence quantum yield, making 4-CN-Trp a promising alternative for native Trp for studying proteins containing multiple Trp residues. Finally, our computer-aided design of dicyano-substituted Trp using TD-DFT excited-state

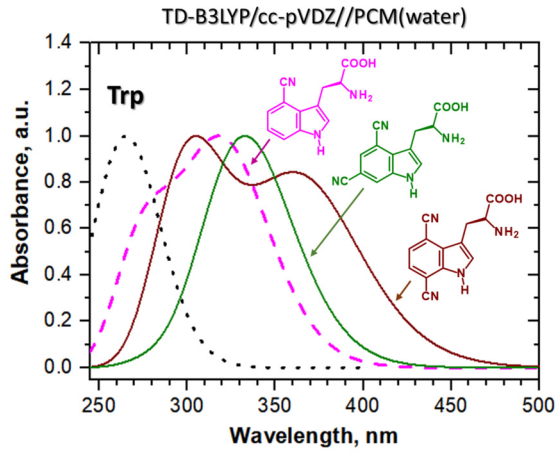


Fig. 3. Normalized absorption spectra of Trp, 4-CN-Trp and dicyano-substituted Trp estimated by the TD-B3LYP/cc-pVDZ//PCM(water) calculations

calculations suggested that the two derivatives 4,6- and 4,7-diCN-Trp are promising probes with red-shifted fluorescence for protein studies.

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