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Isotopic effects in the electronic spectra of tryptophan

Yu. A. Zolotarev[1], **Yu. A. Borisov**[2], **A. K. Dadayan**[1], and **N. F. Myasoedov**[1]

[1] Institute of Molecular Genetics, Russian Academy of Sciences, Moscow, Russia
[2] A. N. Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, Moscow, Russia

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Summary. No influence of isotopic substitution in deuterium-substituted tryptophan on the florescence excitation spectrum has previously been found out. Here, the isotopic effects of electronic excitation of deuterium-substituted tryptophan were experimentally and theoretically analyzed for first time. It was shown a short-wave shift of the UV-absorption maximum at 220 nm corresponding to the 360 cal/mol and short-wave shift for fluorescence spectrum corresponding to the 210 cal/mol. To account for this effect, the quantum chemical calculations of the geometric and electron structure, frequencies of normal vibrations and transition energies have been performed. The isotopic effects originate from the zero-point energies of ground and excited states. It was found that isotopic shifts depend on the position of isotope in the molecule and kind of transition. So, it can be utilized in the analysis of proteins structure and complexation.

Keywords: Fluorescence – Phosphorescence – Isotopic effects – Electronic transitions – Tryptophan

Abbreviations: RCIS, restricted configuration interaction singles method of *ab initio* calculations; B3LYP, hybrid density functional method of *ab initio* calculations; ZPC, zero-point energy corrections; HPLC, high performance liquid chromatography; UV, ultra-violet

Introduction

The substitution of atoms in a compound by their isotopes gives rise to isotope effects. Investigation of thermodynamic and kinetic isotopic effects is an important tool in studying the mechanisms of chemical reactions. Considerable isotope effects in vibrational spectra are well known. Changes in the characteristic frequencies of isotopomer molecules strongly depend on the relative change in the weight of the isotope atoms. Conversely, the electronic properties of free atoms of isotopes are only slightly different even for the lightest of them. The isotope shift of the ground state level for hydrogen, deuterium, and tritium does not exceed 0.03% (Radtsig and Smirnov, 1986).

Fluorescence and phosphorescence spectroscopies of tryptophan residues are widely used for analysis structure and complexation in proteins (Rettig et al., 1999). Tryptophan is especially useful, because its fluorescence is so sensitive to environment. Unfortunately, its complex photophysics have made structural analysis based on tryptophan fluorescence challenging. Deuterium isotope effects on fluorescence quantum yield for tryptophan was analyzed (McLaughlin and Barkley, 1997). In tryptophan, the ammonium group clearly enhances the solvent isotope effect on the fluorescence quantum yield and lifetime. Isotopic shifts in the fluorescence and phosphorescence spectra of deuterium-substituted tryptophan have not hitherto been observed. To prepare tryptophan uniformly labeled with tritium, the reaction of high-temperature solid-phase catalytic isotope exchange was used (Zolotarev et al., 1995). Isotopic shift in UV-absorbance spectra of tritium-substituted tryptophan has been observed for first time (Zolotarev et al., 2005). In this paper, we have experimentally and theoretically analyzed isotopic shifts in the electronic spectra of tryptophan during hydrogen substitution for deuterium.

Materials and methods

Calculation methods

The calculations of tryptophane and its isotope-substituted isomers were performed by the Restricted Hartree-Fock method (RHF) (Weeny and Sutclife, 1969), the Restricted Becke-Lee-Young-Parr modification (RB3LYP) of the density functional theory (Becke, 1993) and the method of configuration interaction (RCIS) (Foresman et al., 1992). The Dunning-Hay D95 atomic basis set was used for the optimization of the geometry and for the calculation of frequencies of normal vibrations. 6-31G* basis set was used for the calculation of one-electron energies (Dunning and

Hay, 1976). Excited states were calculated using the configuration interaction method RCIS and the D95 basis set. Zero point energies corrections were calculated by a combined application of all three methods (RB3LYP, RHF, RCIS). RHF method was used for definition ZPC of the ground state, and RCIS for definition of the excited states of a molecule Trp. All calculations were performed with the GAUSSIAN-98 software running on CRAY J-90 supercomputers (National Energetic Research Supercomputer Center, Oakland, California, USA).

Determination of the spectral properties of tryptophan

The spectra of [^{2}H]Trp and [^{1}H]Trp were read with reference to the chromatographic buffer as well as those of [^{2}H]Trp with reference to [^{1}H]Trp in the 200–320 nm range on U-2800 UV-Vis spectrophotometer (Hitachi) and fluorescent spectrophotometer Safire (Tecan). The [^{2}H]Trp and [^{1}H]Trp samples studied were purified by HPLC. A correlation between the absorbance for calculated differential spectrum and shift of maximum for these spectrums are shown (Fig. 3). The value absorbance for experimental differential spectrum was measured for [^{2}H]Trp with reference to the same concentration of [^{1}H]Trp. The isotopic shift in the Trp spectrum was performed using value of absorbance at 226 nm for the experimental differential spectrum and correlation between value of the shift and the absorbance for calculated differential spectrum on Fig. 3.

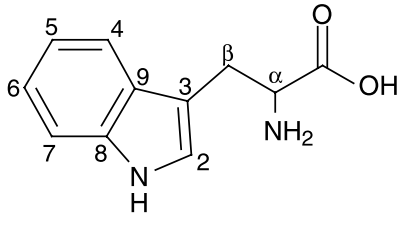
HPLC analysis and purification

HPLC of [^{2}H]Trp and [^{1}H]Trp was done using an Ultrasphere ODS 5 C_{18} 4.6 × 250 mm column (Altex) and 20 mM KH_2PO_4 in 20% methanol as chromatographic buffer. 1 µg each amino acid was analyzed using UV Beckman 165 variable-wavelength detector. The same method was used for purification initial amino acids.

Results and discussion

Isotopic shifts in the electronic spectra were analyzed for L-tryptophan (indole D_5) ([^{2}H]Trp) (Cambridge Isotope Laboratories, Inc) and for L-tryptophan ([^{1}H]Trp) (Sigma). The structure and numeration in Trp are shown in Scheme 1. In [^{2}H]Trp, all its 5 H_C atoms were substituted for deuterium in the molecule's indole part.

The isotopic effects for the electronic transition in tryptophan were studied by UV spectroscopy in the range 200–320 nm and by fluorescence spectroscopy. The UV spectra of [^{2}H]Trp and [^{1}H]Trp with respect to the chromatographic buffer are similar in appearance (Fig. 1). If UV-spectra of [^{2}H]Trp and [^{1}H]Trp are read with reference to the chromatographic buffer, the isotopic shifts are small to be correctly analyzed. To elucidate the differences between these spectra, we obtained the difference UV spectrum of [^{2}H]Trp with respect to [^{1}H]Trp. The values of small shifts UV spectrum may be calculated from absorbance of differential spectrum. The difference spectrum shows a maximum at 212 nm and a minimum at 226 nm, which correspond to the isotopic shift of the band at 220 nm in the UV-absorption spectrum of tryptophan.



Scheme 1. Structure and numeration of trytophan

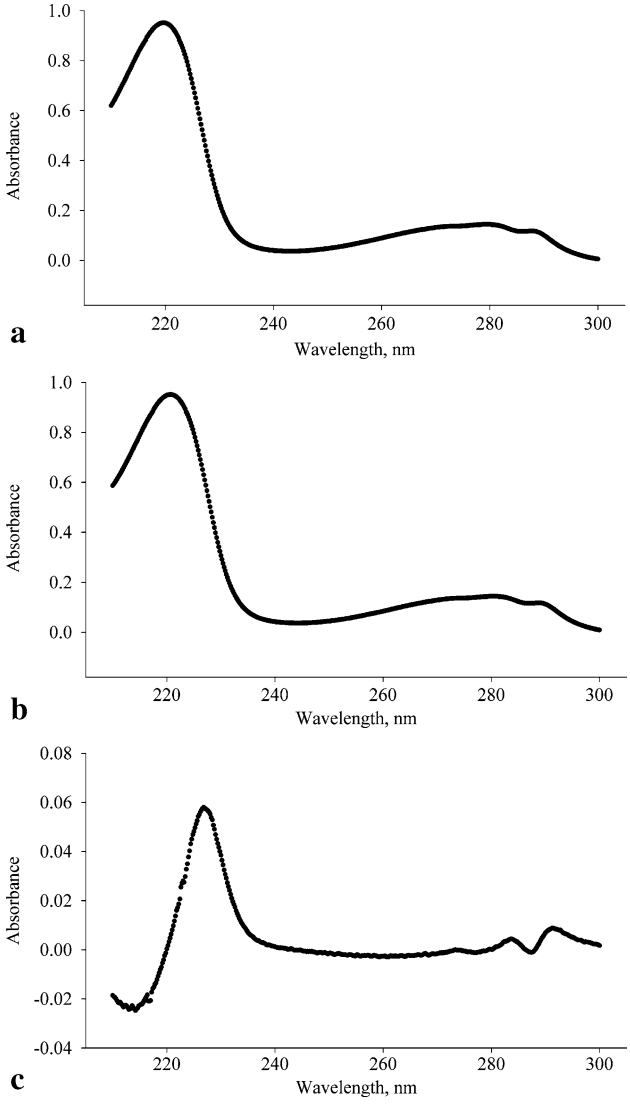


Fig. 1. a UV-absorption spectrum of [^{1}H]Trp, **b** UV-absorption spectrum of [^{2}H]Trp, **c** The differential UV-absorption spectrum for [^{1}H]Trp with regard to [^{2}H]Trp

There are two maxima in the UV-absorption spectrum of tryptophan at 280 nm and 220 nm corresponding to electronic transitions I_1 and I_3. Figure 1 displays a part of the UV-absorption spectrum (Fig. 2a.1), the fluorescence spectrum (Fig. 2b.3), the differential UV-absorption spectrum (Fig. 2a.2) and the differential fluorescence spectrum (Fig. 2b.4) of [^{2}H]Trp. In the part of the differential

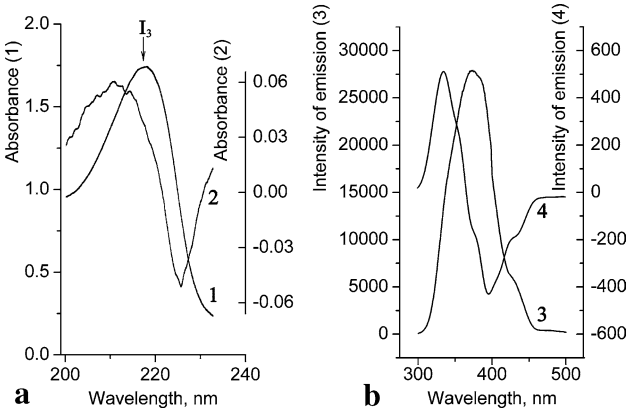


Fig. 2. The experimental analysis of isotopic effects for electron transitions of tryptophan. The part of UV-absorption (**a1**) and fluorescence (**b3**) spectrum of [^{2}H]Trp. The differential UV-absorption (**a2**) and fluorescence (**b4**) spectrum for [^{2}H]Trp with regard to [^{1}H]Trp

UV spectrum corresponding to electronic transition I_3 (Fig. 2a.2), there is a maximum at 212 nm and a minimum at 226 nm. The lowest value is 0.058 A. Starting from this minimum value at 226 nm in the differential spectrum, the short-wave isotopic shift of the band at 220 nm in [^{2}H]Trp was calculated. It was equal to 0.62 nm or 360 cal/mol from a correlation between the absorbance for calculated differential spectra and shift of bands for these spectra.

The fluorescence spectrum of [^{2}H]Trp (Fig. 2b.3) reached its maximum at 369 nm. A minimum in the differential fluorescence spectrum (Fig. 2b.4) at 398 nm and a maximum at 336 nm were observed. The isotopic shift of the band at 369 nm of fluorescence spectrum was calculated using correlation between value of the shifts and intensity of emission at 336 nm for calculated differential fluorescence tryptophan spectra. The short-wave shift was equal to 1.0 nm or 210 cal/mol from this correlation.

An isotopic shift of the band at 220 nm in [^{2}H]Trp was also observed during HPLC using 20 mM KH_2PO_4 in 20% methanol as eluent. UV absorption was detected at two wavelengths: one was fixed at 280 nm, and the second wavelength X was varied from 205 to 230 nm. UV detection at two wavelengths was instrumental in revealing the ratio of the absorbances A_X/A_{280} for these amino acids. It is worth mentioning that the absorbance ratios A_X/A_{280} remain constant during elution of the whole chromatographic peak as for [^{1}H]Trp or for [^{2}H]Trp, indicating that the studied amino acids are free from impurities. It was shown that this ratio coincides at 220 nm for [^{1}H]Trp and [^{2}H]Trp. However, it was shown that this A_X/A_{280} ratios for L-[^{1}H]Trp and L-[^{2}H]Trp may be different in 205–230 nm region. The ratio of A_{210}/A_{280} absorption values was 3.5% larger and the ratio of A_{226}/A_{280} is 6.3% smaller for [^{2}H]Trp relative to [^{1}H]Trp. It should be mentioned that the largest positive deviation is observed for 210 nm and the largest negative deviation, for 226 nm, i.e., at the wavelengths corresponding to the highest rate of change in the molar extinction coefficient for the electronic transition with a maximum at 220 nm. This data correlate with isotopic short-wave shift of the band in UV-absorption spectrum. In this way, the occurrence of isotopic effect during electronic transitions has been experimentally shown for tryptophan. Numerical simulation was utilized for further investigation of the origin of the isotopic shift of the absorption maximum in UV absorption and for singlet–triplet excitation.

Previously, the semiempirical AM1 method and *ab initio* Hartree-Fock calculations with the 3-21G basis set were used to study the conformations, the charges of the first two excited states, and electron transfer in tryptophan (Smolyar and Wong, 1999; Tong and Li, 2002). Here, geometric parameters of the tryptophan molecule were calculated using complete geometry optimization by the hybrid density functional method B3LYP with Dunning-Hay's two-exponential basis set D95 (Becke,

Table 1. The comparison calculated and experimental (Tong and Li, 2002) bond lengths and bond angles of tryptophan in the gas phase

Bond lengths, A			Bond angles, degree		
Position	Calculated	Experimental	Position	Calculated	Experimental
N1–C2	1.397	1.371	C2–C3–Cβ	125.42	125.8
C2–C3	1.389	1.350	C9–C3–Cβ	128.10	127.5
C3–C9	1.457	1.435	N1–C2–C3	109.92	110.5
C4–C9	1.418	1.393	C8–N1–C2	109.01	108.7
C4–C5	1.401	1.379	C4–C9–C3	133.81	134.2
C6–C7	1.402	1.368	C7–C8–N1	130.11	130.8
C7–C8	1.408	1.390	C5–C4–C9	118.73	118.7
N1–C8	1.397	1.370	C6–C7–C8	117.33	117.7

1993). The calculated geometric parameters of the tryptophan molecule agree well with the experimental data based on the X-ray structural analysis of L,D-tryptophan (Smolyar and Wong, 1999) (Table 1).

The electronic transition energies were theoretically analyzed by two independent methods: the restricted configuration interaction singles (RCIS) method and the B3LYP/6-31G* method based on Koopmans' theorem (Weeny and Sutclife, 1969). Figure 3a shows the electronic diagram of the tryptophan molecule's single-electron levels, which was plotted using calculations based on Koopman's theorem (B3LYP/6-31G*). There are three twice-occupied energy levels ε(52), ε(53), ε(54) of the π-pattern. Vacant orbitals ε(55), ε(56), ε(57), ε(58) are also shown. The level of ε(56) is of the σ-character, the remaining levels are of the π-character. The numeration of the atomic levels corresponds to 108 electrons in Trp. The third transition (I3) from the level ε(54) to the vacant

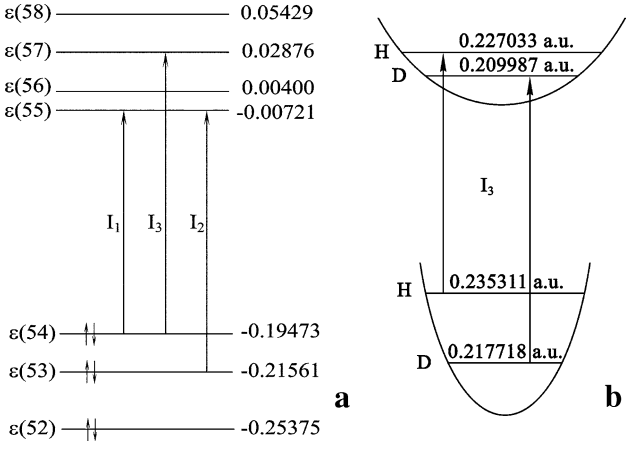


Fig. 3. The theoretical analysis of isotopic effects for electronic transitions of tryptophan. **a** The diagram of one-electron levels for the tryptophan. **b** The electronic transition energy in X-H compound. Zero-point oscillation energies for [^{1}H]Trp-H and [^{2}H]Trp-D of the ground and excited states

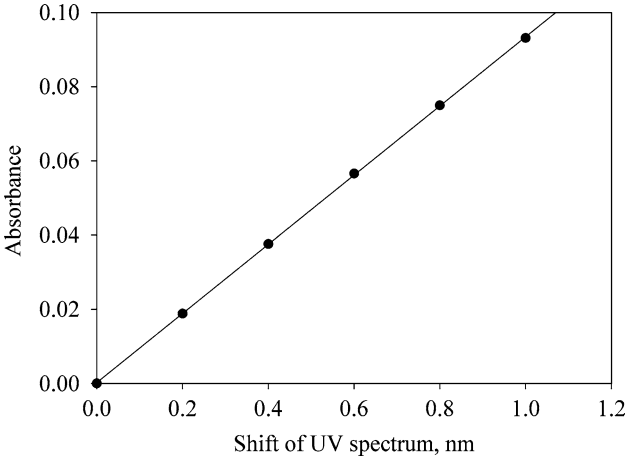


Fig. 4. The absorbance at 226 nm for the calculated differential spectrum from shift values in the Trp spectrum

level ε(57) is 5.336 eV. This transition corresponds to wavelengths of 232 nm.

The electronic transitions in tryptophan were also calculated by the RCIS method, which consists in constructing a multireference wave function with inclusion of only single excitations. According to these calculations, the electron transfers from ε(54) to ε(55) and from ε(53) to ε(57) account, respectively, for more than 60 and 30% of the I_1 energy (5.652 eV). The I_3 transition energy was 6.105 eV (202 nm) according to the configuration interaction method RCIS. The contribution of the electronic transition I_3 from the ε(54) occupied level to the ε(57) vacant level is the largest one (up to 80%). The oscillator strengths for transitions I_1 and I_3 are 0.1748 and 0.7084, respectively. The higher probability of the I_3 transition is consistent with the experiment (Fig. 1.).

The singlet–triplet transition energy was calculated at the Hartree-Fock level of theory within the limits of the Franck-Condon approximation, which implies that the electronic transition does not entail changes in the geometry of the excited molecule. Then, the energy of this

Table 2. Zero-point energy corrections levels (ZPC) for ground and electronic excited states of [^{1}H]Trp and [^{2}H]Trp. Isotopic shift for electronic transitions (I_i) from ground (S_0) to singlet excited states (S_1, S_2, S_3) and for singlet–triplet transitions (I_T) between lowest triplet and ground state

States	ZPC, a.u.		Isotopic effect for ZPC, kcal/mol	Isotopic shift for transitions I_i, cal/mol	Transitions I_i
	[^{1}H]Trp	[^{2}H]Trp			
S_0	0.235311	0.217718	11.0398		–
S_1	0.227712	0.210505	10.7976	242.22	I_1
S_2	0.233622	0.216445	10.7787	261.04	I_2
S_3	0.227033	0.209987	10.6965	343.25	I_3
T_1	0.227492	0.210701	10.5365	503.26	I_T

transition can be determined as the difference between the total energies of the triplet and ground singlet states of the tryptophan molecule. The energy of the singlet–triplet transition calculated by means of Hartree-Fock method was 2.837 eV. The experimental value was 2.695 eV (Tong and Li, 2002).

For calculating the isotope shifts of the I_1, I_2, and I_3 transitions, zero-point energy corrections (ZPC) were determined for ground and the electronically excited singlet states of the tryptophan molecules (Table 2, Fig. 3b). Numerical values of ZPC are energy distances from the bottom potential curves to the first vibration level. ZPC for ground states of [^{2}H]Trp is 11.0398 kcal/mol lower than of [^{1}H]Trp and ZPC for the third excited state of [^{2}H]Trp is 10.6965 kcal/mol lower than of [^{1}H]Trp, respectively. So, the calculated isotopic shift of the I_3 transition energy in [^{2}H]Trp is 343 cal/mol. There is good agreement between experimental (360 cal/mol) and calculated isotopic shift of the band at 220 nm with transition energy I_3.

Isotopic shift for singlet–triplet transitions between lowest triplet and ground singlet states was calculated from value of ZPC for this states for [^{1}H]Trp and [^{2}H]Trp. The calculated isotopic shift for singlet–triplet excitation is 503 cal/mol in phosphorescence spectrum of tryptophan. It was found that calculated isotopic shift for electronic transitions in Trp depends on the positions of deuterium label (Table 3). The highest isotopic effect for singlet–triplet transitions corresponds to [2-^{2}H]Trp, just as the highest isotopic effect for singlet–singlet transitions I_3 corresponds to [1-^{2}H]Trp. The substitution of 3 H-C atoms in aliphatic part of Trp has only weak isotopic effect as for singlet–singlet transitions I_1 and I_2, as for singlet–triplet transition.

We think that measurements of isotopic shift of electronic transitions in aromatic, heteroaromatic, metalorganic compounds could be utilized as a new informative method of investigation chemical bonds and complexation. The origin of this phenomenon is attributed to the difference in the zero-point energy of the ground and excited states of the isotope-substituted molecule.

Table 3. Calculated isotopic shifts for electronic transitions from ground to singlet (I_1, I_2, I_3) and triplet (I_T) excited states in Trp with different positions of deuterium label

Deuterium substituted tryptophan	Isotopic shift for transitions I_i, cal/mol			
	I_1	I_2	I_3	I_T
[1-^{2}H]Trp	14	11	169	85
[2-^{2}H]Trp	58	42	118	365
[4-^{2}H]Trp	56	50	73	100
[5-^{2}H]Trp	29	56	60	90
[6-^{2}H]Trp	52	60	13	107
[7-^{2}H]Trp	48	56	80	85
[α,β,β-^{2}H]Trp	4	2	45	14

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Authors' address: Dr. Yurii A. Zolotarev, Institute of Molecular Genetics, Russian Academy of Sciences, Kurchatov sq. 2, 123182 Moscow, Russia,
E-mail: zolya@img.ras.ru