Spectroscopic Properties of Lumiflavin: A Quantum Chemical Study

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1 **ABSTRACT**

2 In this work, the electronic structure and spectroscopic properties of lumiflavin are calculated
3 using various quantum chemical methods. The excitation energies for ten singlet and triplet
4 states as well as the analysis of the electron density difference are assessed using various
5 wave function based methods and density functionals. The relative order of singlet and triplet
6 excited states is corrected and established on the basis of the coupled cluster method CC2.
7 We find that at least seven singlet excited states are required to assign all peaks in the UV/Vis
8 spectrum. In addition, we have studied the solvatochromic effect on the excitation energies
9 and found differential effects except for the first bright excited state. Multiple vibrational
10 frequencies including IR, Raman and Resonance Raman are simulated and compared to their
11 experimental counterparts. We have assigned peaks, assessed the effect of anharmonicity
12 and confirmed the previous assignments in case of the most intense transitions. Finally, we
13 have studied the NMR shieldings and established the effect of the solvent polarity. The
14 present study provides data for lumiflavin in the gas phase and in implicit solvent model that
15 can be used as a reference for the protein embedded flavin simulations and assignment of
16 experimental spectra.

1 INTRODUCTION

2 Flavins belong to a group of natural pigments responsible for the yellow color of various
3 plants (*flavus* means yellow in latin). In 1937, Paul Karrer received the Nobel Prize in
4 Chemistry for his characterization and synthesis of vitamin B₂ (riboflavin) (1). The yellow
5 color of flavin in its oxidized form, as discussed in the present work, relies on the absorption
6 with a maximum at 447 nm. A second electronic transition is in UV region and centered at
7 λ_{max} of 360 nm (2).

8 The simplest flavin compound is the lumiflavin as shown in the oxidized and reduced forms
9 in Scheme 1A and B. Substitution of a methyl group with an aliphatic side chain of 2,3,4,5-
10 tetrahydroxypentyl leads to riboflavin (Scheme 1C). In nature, enzymatic phosphorylation
11 of riboflavin produces flavin mononucleotide (FMN), and further adenylation transforms
12 FMN to flavin adenine dinucleotide (FAD) (3). These substituents do not have a significant
13 effect on the absorption wavelength because they are not part of the conjugated system (2,
14 4-6), thus the present study is focused on the non-substituted lumiflavin.

15 The oxidized form of lumiflavin can be reduced twice. In the first reduction step, combined
16 with a proton uptake, the neutral radical, also called semiquinone, is produced.
17 Spectroscopically, the flavin radical can be distinguished from the oxidized form since it has
18 absorption bands at 620, 580 and 500 nm, depending on the chromophore environment (5,
19 7, 8). Further reduction and protonation of the second nitrogen of the isoalloxazine ring leads
20 to the form which has a major absorption at 250 nm and shoulders at 400 and 280 nm (9).
21 Hence, flavins have the potential for transfer of electrons and protons, making them versatile
22 for substrate modification and photochemically triggered redox reactions.

1 This abundance in reactivity makes flavins widespread functional cofactors in proteins. One
2 class of flavoproteins is **cryptochrome** (10) closely related to **DNA photolyases** which
3 conduct repair of the ultraviolet damaged DNA (11, 12). Both cryptochromes and DNA
4 photolyases contain FAD as a cofactor and a conserved “tryptophan-triad” next to it. The
5 latter forms an electron transfer chain when FAD is electronically excited and abstracts
6 electrons to form an anionic radical ($\text{FAD}^{\bullet-}$) (13). Also, BLUF domains, found in bacteria
7 contains FAD (BLUF = “blue light using FAD”) (14-16). They are involved in blue-light
8 sensing. **Phototropin** is an FMN-containing protein controlling phototropism, i.e, the
9 orientation of plants towards light sources (17). The photosensory domain of phototropin
10 includes two similar light-oxygen-voltage (LOV) domains, called LOV1 and LOV2. Each
11 LOV domain contains an FMN chromophore within the protein binding pocket, bound
12 through a network of hydrogen bonds and hydrophobic interactions (18). Obviously, the rich
13 photo- and redox chemistry of flavins opens plenty of possibilities for biotechnological
14 applications.

15 *< Scheme 1 >*

16 In order to interpret the experimental measurements and gain insight into the photochemistry
17 of flavins the use of the modern computational methods, in particular quantum chemical
18 calculations, has proved to be vital. For example, previous theoretical studies by Zheng *et*
19 *al.* (19) and Li *et al.* (20) have shown that the oxidized flavin adopts a planar structure
20 whereas the reduced-form is bent. The calculation of vertical excitation energies has been
21 helpful to assign the peaks in the absorption spectrum (21-26). Also the calculation of the
22 vibrational frequencies has been instrumental in the assignment of the infrared spectrum (27,
23 28).

1 In the present study we reexamine the quantum chemical calculations of various
2 spectroscopic properties. In the case of the vertical excitation energies of lumiflavin a few
3 lower-lying electronic states are reported often with contradicting energetic order or focus
4 only on the bright excited states. Note that during the photochemical reaction states which
5 are dark initially might be populated as demonstrated recently by Burghardt and coworkers
6 (29). Further a non-radiative decay from a singlet to a triplet state can occur via an
7 intersystem crossing (30). The energetic order of the latter might be affected in TD-DFT
8 calculations due to the triplet instability (31, 32). Therefore, we would like to evaluate the
9 energetic order of singlet and triplet excited states. Here, we report the results of calculations
10 of 10 low-lying singlet and triplet excited states of lumiflavin using RI-ADC(2) and RI-CC2
11 as well as range separated hybrid CAM-B3LYP methods with cc-pVTZ basis set. This work
12 is focused on the analysis of the orbital character, charge-transfer, and excitation energies in
13 both gas phase and solution.

14 Another focus of this work is the assignment of vibrational spectra. Previous studies have
15 put an emphasis on the assignment of the carbonyl stretching modes. A large deviation
16 between the experiment and quantum chemical calculations was described by Wolf et al for
17 these particular type of vibrations (27, 28). The discrepancy was rationalized on the basis of
18 environmental effects. However, in the present study we will evaluate the influence of
19 anharmonicity. In addition, Raman and NMR spectra of the isolated and implicitly solvated
20 lumiflavin are computed. The overall objective here is to review previous computational
21 studies, revise some of the shortcoming and therefore create a reference for the gas phase
22 that can be used in future studies where flavin is embedded within an environment.

23 MATERIALS AND METHODS

Computational Section

Geometry optimization

The geometry optimization for lumiflavin was carried out using the B3LYP functional (33) in conjunction with the 6-31G* basis set. The chemical structure of the above mentioned flavin chromophore is shown in Figure 1A. All calculations were performed without a symmetry constraint. For DFT calculations the D3 Grimme's dispersion correction was used (34). The B3LYP equilibrium geometry was used as our starting point for all calculations. The geometry optimization was carried out using in Q-Chem 4.4 program package (35).

Vertical excitation energies

The vertical excitation energies (VETs) of the ten low-lying singlet and triplet states were assessed using various wave function based methods as well as time dependent density functional theory (TD-DFT). For all methods, the cc-pVTZ basis set and wherever required cc-pVTZ-RI auxiliary basis set were used.

Among the wave function methods we have employed ADC(2) and CC2. The second order algebraic-diagrammatic construction (ADC(2)) scheme (36) has favorable computational cost and good quality description of excited states. It has also the advantage of being size-consistent and having the eigenvalue equation formulated as a Hermitian matrix that is suitable for derivation of properties (37). The second-order approximate coupled cluster singles and doubles (CC2) method (38) is also proved to be efficient for an accurate description of excited states. The accuracy of CC2 was assessed previously in the benchmark studies by Thiel and coworkers (39) using 28 organic molecules. Recently, also an assessment of the ADC(2) method was made by Dreuw and coworkers (40) using the same set of molecules. The singlet excitation energies of CC2 method were found to have an

accuracy of 0.29 ± 0.28 eV (mean value $\pm$ standard deviation) for 103 vertical excitation energies compared to the theoretical best estimates, while ADC(2) produced 0.22 ± 0.38 eV for 104 vertical excitation energies. Similarly, for triplets a comparison of 63 vertical excitation energies showed a deviation of 0.17 ± 0.13 eV and 0.12 ± 0.16 eV for CC2 and ADC(2) methods, respectively. The two wave function based methods were used in the “resolution of the identity” (RI) formulation (41), which is an order of magnitude more efficient in comparison to the conventional algorithms. The calculations for RI-ADC(2) and RI-CC2 were performed in Turbomole 7.0 (42).

Time-dependent density functional theory TD-DFT (43) method is employed for calculation of electronically singlet and triplet excitation energies. Two functionals were involved: B3LYP and the long-range corrected version: coulomb attenuating method (CAM)-B3LYP (33, 44). The hybrid functional B3LYP is known to give accurate $n-\pi^*$ and $\pi-\pi^*$ excitation energies, with an expected error range of ca. 0.20 – 0.25 eV from the experimental values (43). A benchmark study of TD-DFT by Thiel and coworkers (45) revealed a deviation of -0.07 ± 0.33 eV for singlet and -0.45 ± 0.49 eV for triplet vertical excitation energies, with reference to the theoretical best estimates reported in (39). Notably, these statistics are based on consideration of 104 singlet and 63 triplet excitation energies, which further infers that the hybrid TD-B3LYP describes the excitation energies more accurately compared to other functionals like BP86 and BHLYP (45). Further, TD-CAM-B3LYP method can correctly describe excited states with charge transfer (CT) character (46). Another important issue is the “triplet instability problem” in the ground state wave function, as described by Tozer and co-workers (31). In their work, they have shown that the use of hybrid and range-separated functionals within the “full” TD-DFT scheme does not produce accurate singlet-triplet energy gaps. Instead, the use of Tamm-Dancoff approximation (TDA) was reported to show

1 a dramatic improvement of the singlet-triplet gaps (31, 32). Hence, in order to have more
2 accurate results, we have used TDA for the calculation of singlet-triplet gaps. The TD-DFT
3 vertical excitation energies were also computed with the Conductor-like Polarizable
4 Continuum Model (CPCM) (47) to estimate the solvatochromic shifts. The programs ORCA
5 4.0.1.2 (48) and Gaussian 09 revision D01 (49) are used for TD-DFT calculations.

6 **IR and Resonance Raman spectra**

7 One of the most valuable tool in the characterization of the molecular structure is the
8 vibrational spectroscopy, which has two main directions: Infrared and Raman. The main
9 difference between the two types lies in the selection rules of the transitions. The vibrational
10 modes which change the dipole moment of the molecule are active in the IR, whereas the
11 modes which change the polarizabilities are Raman active. In contrast to the conventional
12 Raman spectroscopy, which is based on the direct absorption of the electromagnetic radiation
13 in the IR region, the resonance Raman (RR) method is based on the vibronic transitions
14 (which occur between different electronic and vibrational states) that can lead to a significant
15 enhancement of the Raman bands (50). From the computational perspective the vibrational
16 modes correspond to energy levels that are obtained from the solution of the nuclear wave
17 function equations. Hence, the fundamentals for the IR and Raman signals are computed in
18 the same way but the calculation of the intensities is different. The IR intensities require
19 mixed second derivatives of the energy with respect to geometric displacement and an
20 external electric field, while the Raman intensities require mixed third derivatives to account
21 for a change in the molecular polarizability along the geometric motion.

22 The gas phase IR intensities were computed using the B3LYP functional and the 6-311G*
23 basis set in Gaussian 09 program package. The obtained frequencies were scaled by a factor
24 of 0.966 as suggested in the Computational Chemistry Comparison and Benchmark Database

(51). In addition, we have tested the effect of the anharmonicity by using the Generalized second-order Vibrational Perturbation Theory (GVPT2) (52).

The Raman spectrum was calculated using the ORCA program at the B3LYP/6-311G* level of theory. For the RR spectrum the gradients were obtained by the TD-B3LYP method. From previous benchmarking studies (53), B3LYP functional is found to be the most efficient for determination of the ground state properties (vibrational frequencies and normal modes) as well as for the resonance Raman (RR) intensities. The method employed in this work is based on *Independent mode, displaced harmonic oscillator* (IMDHO) as described by Petrenko and Neese (54). The solvation effect was included in the calculation of the RR spectrum, using CPCM. The ORCA modules “orca_mapspc” and “orca_asa” were used for extracting the polarizabilities and simulating the RR spectrum, respectively (48). For the visualization, Lorentzian function with Full width at half maximum (FWHM) of 10 cm⁻¹ is used for the peak broadening.

NMR spectrum

The chemical shielding values were calculated using GIAO approach in Gaussian 09. These calculations were performed at B3LYP level using the 6-311G* basis set. The polarity effects of water and cyclohexane were taken into account using CPCM.

Experimental Section

For measuring the UV-vis absorption spectrum, lumiflavin was dissolved in chloroform and the spectrum was recorded from 250-600 nm by UV-2450 spectrometer (Shimadzu). The data acquisition is performed with a step interval of 0.5 nm.

The infrared absorption spectrum was measured in this work. For this purpose lumiflavin powder was first mixed with KBr powder (wL/wK = 1:50) and grinded until they became

homogeneous. Subsequently, the mixture was subjected to high pressure for two minutes to form a thin transparent pellet. The pellet was then measured by Fourier transform IR spectrometer (Thermo Nicolet) from 4,000-400 cm^{-1} at a spectral resolution of 1 cm^{-1} . The data processing was done using Omnic32 software.

RESULTS AND DISCUSSION

Optimized geometry and IR spectrum

The optimized geometry of the oxidized form of lumiflavin along with the bond lengths is given in Figure 1A. The numbering of atoms is used consistently throughout the manuscript. The vibrational frequencies for a bond stretching modes are related to the mass of the atoms involved and to the strength of the corresponding chemical bond. The characteristic IR intensities of the ground state lumiflavin were calculated in vacuum and a scaling factor of 0.966 was applied according to the level of theory (Table 1). To the best of our knowledge, there is no experimental gas phase IR spectrum of lumiflavin. Hence, we have measured a spectrum in the KBr disc and compared the observed peaks with the calculated frequencies. The solid environment present in the crystalline KBr pellet provides sharp peaks, but the tight packing in the solid could lead to interactions with lumiflavin and in addition the thickness of the pellet could affect the absorbance of the sample. Therefore, we cannot expect a direct correspondence with the calculated frequencies of the isolated lumiflavin even after the scaling of the frequencies. The assignments of the peaks in Figure 1B are made using a reference study based on isotope labeling (55). In the following discussion we focus mainly on the most intense bands of the spectrum.

< **Figure 1** >

Two amide I vibrations ($\text{C}_4=\text{O}_4$ and $\text{C}_2=\text{O}_2$ stretching modes coupled with $\text{N}_3\text{-H}$ bending) in the

experimental solid-state spectra, are observed at 1708 and 1663 cm^{-1} . Similar values were obtained for the flavin in the protein environment: for FMN in LOV2 domain at 1714 and 1678 cm^{-1} (56) and in BLUF domain at 1710 and 1641 cm^{-1} (57, 58). Further, for the oxidized lumiflavin in LOV1 domain (C57S mutant) these vibrations were assigned to 1693 and 1681 cm^{-1} (59). The calculated vibrational modes of the $\text{C}_4=\text{O}_4$ and $\text{C}_2=\text{O}_2$ stretching appear at 1738 and 1727 cm^{-1} , respectively. Although a scaling factor of 0.966 has been applied the deviation, especially for the $\text{C}_2=\text{O}_2$ mode, is fairly large. However, it is not surprising that one global scaling factor is not sufficient to have a close agreement with the experiment. Mroginiski *et al.* have reported the use of 11 scaling factors for the linear tetrapyrrole chromophore in phytochromes in order to achieve an agreement with the experiment of ca 10 cm^{-1} (60). In the specific case of the two $\text{C}=\text{O}$ stretching modes in flavins, Domratcheva and coworkers (27) have first proposed hydrogen bonding to be responsible for the discrepancy between the experiment and the quantum chemical calculation. In a subsequent study the same group has attributed this discrepancy to the bulk solvent effect (28). By adding an implicit solvent to the calculation the agreement with the experiment in DMSO has improved for the two $\text{C}=\text{O}$ stretching modes. Nevertheless, the measured $\text{C}=\text{O}$ stretching frequencies in DMSO are in good agreement with our experimental results obtained in the solid state: 1711 cm^{-1} and 1676 cm^{-1} . Hence, this agreement might question the interpretation of the solvent. The deviation could be also due to the lack of anharmonicity in the calculated frequencies. In order to test this hypothesis we accounted for the missing anharmonic correction by the GVPT2 method. The resulting frequencies are listed in Table S1 of the Supporting Information. The obtained anharmonic frequencies are found to be in a better agreement with the experimental counterparts for the $\text{C}_4=\text{O}_4$ and $\text{C}_2=\text{O}_2$ vibrations (1709 and 1695 cm^{-1} , respectively). However, the GVPT2 method has downshifted all frequencies which were previously in good agreement with experiment.

Thus, our findings points to a possible environmental effect is the major reason for the difference observed in the C=O stretching mode frequency as reported previously by Domratcheva and coworkers (28). However, the environmental effect is not specific to the polar solvent DMSO.

The contributions from the skeletal stretching of ring I coupled with ring II and III in the solid-state spectrum were found at 1621 and 1461 cm^{-1} . These vibrations appear at 1615 and 1421 cm^{-1} in the computed spectrum. Further intense bands in the measured spectrum are found at 1583, 1552 and 1169 cm^{-1} , which also correspond to ring II stretching vibrations (55). The gas phase calculation yields bands at 1570 and 1535 cm^{-1} that correspond to the ring II stretching with main contribution from N₁₀-C_{9a} and C_{4a}-N₅ stretching. These ring II stretching modes were also described in the experimental IR spectra of flavin from plant cryptochrome (CRY1) at 1578 and 1543 cm^{-1} (61). Also, in earlier reports (62, 59), similar vibrations, originating from ring II of the isoalloxazine were assigned to 1550 and 1584 cm^{-1} . The calculated vibrational band at 1151 cm^{-1} corresponds to the C_{4a}-C₄ and N₅-C_{5a} stretching coupled with N₃-H bending. A low intensity N₃-H bending mode measured at 1413 cm^{-1} was computed at 1375 cm^{-1} . The large deviation can be explained by the involvement of the N₃-H group in hydrogen bonding within the protein binding pocket (63, 64). For example, in FMN bound to LOV domain, the N₃-H bending mode appears at 1443 cm^{-1} according to the reported DFT calculations (65). We also noted a delocalized skeletal vibration accompanied with N₁₀-C_{9a} and C_{4a}-N₅ stretching in our calculated spectrum at 1284 cm^{-1} . These vibrations were also referred to ring II stretching modes, which correspond to the peak at 1301 cm^{-1} in the experimental solid-state IR spectrum of lumiflavin. However, according to our calculations this also involve ring III. The amide III vibration (C₂-N₃ antisymmetric stretching mode) coupled with N₃-H bending mode was found experimentally at 1238 cm^{-1} , which in the gas phase occurs at 1169 cm^{-1} . A similar antisymmetric stretching is assigned in FMN bound to LOV2 domain to 1395 cm^{-1} , which also involves C₄-N₃ vibration

1 along with stretching vibrations from C₂-N₃ and bending vibrations from N₃-H.(56) Frequencies
2 lower than 1000 cm⁻¹ are mostly referred to the skeletal deformation vibrations (55, 63). For
3 example, a calculated peak at 800 cm⁻¹, corresponds to a stretching vibration of rings I and II. In
4 this frequency region the vibrations are mostly delocalized over the fused multiple rings (66).

5 < **Table 1** >

6
7 **Raman and resonance Raman (RR) spectra**

8 The computed and measured Raman as well as RR spectra of the oxidized lumiflavin are
9 shown in Figure 2. The experimental Raman spectrum of lumiflavin was measured in powder
10 (67), since we cannot account for this environment we have chosen to calculate the Raman
11 spectrum *in vacuo* at the B3LYP/6-311G* level of theory (Figure 2A and B). However, we
12 have selected the experimental RR spectrum obtained for lumiflavin in the riboflavin binding
13 protein (RBP) in water solution that was excited at 488 nm (68). Therefore, we decided to
14 compute the spectrum in the implicit water model (CPCM) at the B3LYP/6-311G* level of
15 theory (Figure 2C and D). Hence, not only the intensities but also the frequencies will be
16 different between the computed Raman and RR spectra.

17 < **Figure 2** >

18 The frequencies of the calculated Raman spectrum of lumiflavin are in qualitative agreement
19 with the experimental results in the region 1700-1100 cm⁻¹, given that the latter is measured
20 in powder (67). The band at 1080 cm⁻¹ in the experimental spectrum corresponds to
21 stretching vibration of C₇-C_{7a} and C₈-C_{8a} along with skeletal deformation of rings I, II, and
22 III, which was found at 1094 cm⁻¹ in the calculated counterpart. The experimental peaks
23 within the fingerprint region of 1300-1100 cm⁻¹ correspond to a combination of the bending

1 of C₆-H, C₉-H, and N₃-H, which in the calculated spectrum were found at 1238, 1209, 1190,
 2 and 1163 cm⁻¹. The intense peaks in Figure 2B at 1347 and 1360 cm⁻¹ correspond to
 3 vibrations of the fused ring system, dominated by the N₁₀-C₁₁ and N₃-C₄ stretchings. The
 4 corresponding computed values are 1345 and 1368 cm⁻¹, respectively. Peaks in the
 5 experimental spectrum at 1415 and 1465 cm⁻¹ are due to the ring II and III vibrations, C_{7a}
 6 and C_{8a} methyl deformation, as well as C₉-H bending, which are shown at 1402 and 1469 cm⁻¹
 7 in Figure 2A. The largest discrepancy is found for the three intense peaks at 1574, 1588
 8 and 1624 cm⁻¹ in the calculated spectrum. Their relative intensity is much higher than in the
 9 experimental spectrum. They are best assigned to the peaks measured at 1552, 1583, and
 10 1623 cm⁻¹. The first mode is a skeletal stretching of ring II and III with contributions from
 11 N₁-C_{10a} and the C₁₁-methyl group, the second is associated with the ring III stretching, C_{7a}
 12 and C_{8a} methyl groups and the third one is stretching between N₁-C_{10a}-C_{4a}-N₅, coupled with
 13 C₈-C₉. The stretching mode of C_{4a}-N₅ is also found at 1580 and 1585 cm⁻¹ in case of FMN
 14 in LOV1 domain and in water (62).

15 As mentioned in the Methodology section, the intensity in the RR spectrum is greatly
 16 enhanced for those modes that are active in the excited state. We will focus on the most
 17 intense peaks in the following discussion because we have described the assignment of the
 18 Raman peaks already above. The calculated frequencies in the Raman and RR spectra should
 19 be identical and only the relative intensities are altered. However, we have used an implicit
 20 water solvent in the RR calculation because the experimental spectrum was also recorded in
 21 water solution of RBP. One cannot expect a quantitative agreement with the experiment since
 22 we don't have the specific protein interaction, nevertheless, the qualitative trend can be
 23 observed. We noted the reduced intensity of the two Raman peaks at 1345 and 1368 cm⁻¹,
 24 while the two peaks at 1217 and 1240 cm⁻¹ have increased the intensity in the RR spectrum.

1 These two intense peaks originate from ring I vibrations: the one at 1217 cm^{-1} due to $\text{C}_2\text{-N}_3$
2 and $\text{C}_4\text{-C}_{4a}$ stretching and the other one at 1240 cm^{-1} due to the bending of $\text{C}_2\text{-N}_3\text{-C}_4$. They
3 agree well with the experimental counterpart, where the most intense peak is found at 1243
4 cm^{-1} . Further intense RR peaks are found at 1541 , 1604 , and 1663 cm^{-1} with two of them
5 corresponding to the intense peaks at 1574 and 1588 cm^{-1} in the Raman spectrum and are
6 described above. Due to the lack of the baseline correction, their assignment is difficult. In
7 the measured spectrum the first peak is likely associated to signal at 1553 cm^{-1} and is also
8 found at 1553 cm^{-1} in a RR spectrum of FAD in water (69), while the second is located at
9 1585 cm^{-1} . The intense peak at 1663 cm^{-1} corresponds to a less prominent peak at 1670 cm^{-1}
10 in the Raman and to 1630 cm^{-1} in the experimental RR spectrum. It is associated with the
11 stretching of $\text{C}_{10a}\text{-C}_{4a}$, $\text{C}_9\text{-C}_{9a}$, $\text{C}_{5a}\text{-C}_6$ and $\text{C}_6\text{-C}_7$ from ring II and III.

12 In the extended low-energy region of the RR spectrum ($400\text{-}1100\text{ cm}^{-1}$), that is not covered
13 in the Raman spectrum, the most intense peak is found at 523 cm^{-1} . It corresponds to 527
14 cm^{-1} in the experimental RR spectrum (Figure 2D) and is attributed to the stretching of ring
15 I and II as well as the C_{7a} and C_{8a} methyl groups (55, 66). Other peaks at 604 and 640 cm^{-1}
16 that agree well with the experimental spectra (Figure 2D) arise from the bending of the $\text{C}_2\text{-}$
17 $\text{N}_3\text{-C}_4$ and $\text{N}_5\text{-C}_{5a}\text{-C}_6$ angles, respectively. The computed RR peak at 748 cm^{-1} corresponds
18 to the vibration of ring III, which is shifted in the experimental counterpart by 7 cm^{-1} . Further
19 peaks at 793 and 839 cm^{-1} , correspond to the displacement of $\text{C}_6\text{-C}_{5a}$ along with stretching
20 between $\text{C}_2\text{-N}_3$ shifted by -3 cm^{-1} and $\text{C}_{4a}\text{-N}_5\text{-C}_{5a}$ shifted by 4 cm^{-1} , respectively. The
21 vibrations at N_{10} were assigned to the intense signal at 997 cm^{-1} in the experimental spectrum,
22 which was found at 997 cm^{-1} in the calculated one.

24 **Assessment of vertical excitation energies of lumiflavin**

1 A comprehensive understanding of the electronic structure associated with the absorption
2 peaks of the lumiflavin spectrum is the main target of numerous computational studies (21,
3 24, 70, 22, 26, 25, 71-73). The nature of the excited states and their energetic order is also
4 important for the study of the photochemical reaction initiated at the Frank Condon point.
5 Therefore, the aim of this work is to assess the excited states in lumiflavin using *ab initio*
6 quantum chemical methods ADC(2) and CC2 and compare the results to previous studies.
7 We have calculated the 10 lowest singlet and triplet excited states which can become relevant
8 in the investigation of the excited state reactivity including singlet-triplet transitions.

9 A summary of vertical excitation energies of lumiflavin is presented in Table 2. In the
10 analysis of the singlet excited states we consider a bright state to have an oscillator strength
11 (*f*) greater than 0.1. Molecular orbitals (MOs) that are involved in the low-lying electronic
12 transitions are shown in Figure 3. The first bright state (S_1) obtained with CC2 and ADC(2)
13 is in the visible spectral range (ca 400 nm), whereas other bright states fall into the near-UV
14 spectral region. In general, the vertical excitation energies computed with ADC(2) are
15 typically lower by ca 0.10 eV for both singlet and triplet transitions, compared to that of
16 CC2. In ADC(2), four bright states are found among the 10 lowest singlet states: 2.94 eV
17 (S_1), 4.06 eV (S_4), 4.79 eV (S_7), and 4.91 eV (S_9). However, CC2 calculation reveals three
18 bright states located at 3.06 eV (S_1), 4.13 eV (S_4), and 4.97 eV (S_9). This is because S_7 has
19 an oscillator strength of 0.088 at the CC2, which is close to the arbitrary selected cutoff of
20 0.1 for the bright states. Therefore the only change in the relative order of the first 10 singlet
21 states is found to be S_7 at CC2 level of theory, which corresponds to S_9 at ADC(2) level of
22 theory. We attribute this swap in the position to the close proximity of the states S_7 , S_8 and
23 S_9 , which are found within 0.04 eV at CC2 level and 0.12 eV at the ADC(2) level, which are
24 nearly degenerate. However, the nature of singlet excited states for CC2 and ADC(2) is in

1 agreement with the DFT/MRCI excitation energies computed by Salzmann *et al.* (23) for 7
2 states. However, the DFT/MRCI results are slightly different from Neiss *et al.* (21) which
3 might be explained by the use of a smaller basis set (SV(P) basis set in contrast to TZVP in
4 the former case). Another high level method is XMCQDPT2, however, the published results
5 include only two π - π^* states (25). While the DFT/MRCI energies are slightly lower than
6 CC2, the XMCQDPT2 energies are slightly higher.

7 The experimental results shows that the first peak of lumiflavin in water appears at 2.79 eV
8 and in the protein environment at 2.77 eV (74, 71). A comparison of this peak with the first
9 low-lying bright state of CC2 in gas phase yields a blue-shift of 0.27 eV. The second
10 experimental peak is found at 3.38 and 3.77 eV in water and protein, respectively, which in
11 gas phase is blue shifted by 0.75 and 0.36 eV. The assignment of the third experimental peak
12 with reference to the gas phase results is complicated because several states with non-
13 negligible oscillator strength are found in a close proximity. In case of S_6 and S_7 states, the
14 oscillator strength is 0.016 and 0.088, respectively. However, the S_9 state is found at 4.97 eV
15 that is within 0.04 eV of S_7 and has a nearly tenfold increased oscillator strength ($f=0.776$).
16 Hence, we expect the brightest peak to overlap with S_7 and impede the assignment of the
17 experimental spectrum. Due to the high oscillator strength of S_9 and the high intensity of the
18 third peak (Figure 5) we have given the values in the same row of Table 2.

19 < **Table 2** >

20 < **Figure 3** >

1 For a detailed characterization of the excited states, we have analyzed the electron density
2 differences (EDD) between the S_n and S_0 . The EDDs were calculated for the four excited
3 states with the highest oscillator strength from CC2 calculations (Figure 4). The ground and
4 excited state densities are delocalized over the entire molecule, except for the S_0 - S_4
5 transition, which possess a charge-transfer from ring III to rings I and II. The S_1 - S_0 EDD is
6 more pronounced on the rings I and II, which shows that the electron density is transferred
7 from N_1 to N_5 upon excitation. These results are in agreement with the previous reports (72).

8 *< Figure 4 >*

9 In comparison to the results of the wave function methods, the previously reported TD-DFT
10 results by Neiss *et al.* (21), Vdovin *et al.* (24) and Zenichowski *et al.* (75), as well as our
11 results (see Supporting Information Table S2), suggest not only the different ordering of the
12 singlet but also triplet excited states. The TD-DFT calculations using the B3LYP functional
13 result in a bright, first singlet state with π - π^* character. However, the following three states
14 are dark n - π^* states in calculations with a double-zeta basis set. Only the fifth excited state
15 corresponds to the bright π - π^* transition. If a triple-zeta basis set is employed there are only
16 two of these dark states between the two bright states in agreement with ADC(2), CC2 and
17 DFT/MRCI. Also the long-range corrected functional CAM-B3LYP can recover the correct
18 order among the first five excited states. This has been shown for double-zeta basis set by
19 List *et al.* (73) as well as triple-zeta basis set in Table 2. The fact that CAM-B3LYP is
20 adjusting the energetic order indicates a partial charge transfer character of the S_4 state which
21 is not correctly described using B3LYP. The nature of the charge transfer is evident from
22 difference density plots for the bright states in Figure 4, as discussed above. A detailed
23 inspection of the excited states shows that despite the correct order of bright states the
24 electronic structure for two pairs of states is interchanged at the CAM-B3LYP level (S_2 and

S₃, S₇ and S₈). The states at 3.55 eV (S₁), 4.37 eV (S₄), and 5.42 (S₉) are found to be bright which is consistent with the CC2 results. The highest computed excited state S₁₀ involves different orbitals compared to CC2 and therefore is omitted in from Table 2. The range-separation in CAM-B3LYP correctly describes the order of the states, however, the excitation energies are overestimated because the γ -parameter (controlling the short and long range separation) is not universal (76).

In the case of the triplet excited states B3LYP yields four states below the first singlet. For TD-CAM-B3LYP there are three triplet states and for DFT/MRCI two triplet states below S₁ (Table 2). Both, ADC(2) and CC2, methods indicate that only one triplet state is below the lowest singlet excited state, which is in-line with CIS(D), a simple configuration interaction method (Table S2). Hence, the problem with triplet states could be attributed to the documented triplet instability in TD-DFT (31, 32). The S₁-T₁ energy gap is 0.54 eV and 0.50 eV for ADC(2) and CC2, respectively. In summary, we conclude that the use of TD-DFT should be avoided in particular for studies of the lumiflavin photochemistry involving singlet-triplet transitions.

Solvatochromic effects on vertical excitation energies

In the previous section we have shown that the first two bright states from ADC(2), CC2, and CAM-B3LYP calculations involve $\pi_H \rightarrow \pi^*_L$ and $\pi_{H-1} \rightarrow \pi^*_L$ transitions, and their positions are found in qualitative agreement with the experimental peaks (Table 2). However, the gas phase results are blue-shifted with respect to the absorption peaks of lumiflavin measured in water and within the protein embedded system (73). Our recorded spectrum in chloroform is shown in Figure 5 that is consistent to the previous experimental studies by Sikorski *et al.* and Sikorska *et al.* that have reported the first two band maxima for lumiflavin in water at $\lambda_1=445$ nm (2.79 eV) and $\lambda_2=367$ nm (3.38 eV) (77, 78). In this section, we want

1 to address to the solvent effect on the few low-lying excited states that are needed to
2 understand the experimental spectrum (79, 80). In order to analyze the solvation effect in a
3 systematic way we calculated the VETs using the Conductor-like Polarizable Continuum
4 Model (CPCM) (47), with water, chloroform, and cyclohexane solvents. Unfortunately, the
5 CPCM implicit solvent model is neither available for CC2 or ADC(2) methods in
6 Turbomole. Thus, we have decided to use the CAM-B3LYP method to study the effect of
7 solvation (Table 3). Comparison of the low-lying bright states in the gas-phase with those
8 computed in water reveal a red-shift from 0.14 to 0.28 eV. This solvatochromic shift
9 improves the gas phase results in comparison to the experimental peak maxima (Table 2 and
10 3). If we assume the shift is independent of the electronic structure method then the solvent
11 model will also improve the CC2 excitation energies. In case of cyclohexane the shift of the
12 bright state energies with respect to the gas phase is marginal (ca. 0.1 eV), due to the low
13 dielectric constant of this non-polar solvent.

14 Calculations in other solvents revealed different solvatochromic effect on the bright states.
15 The series of the solvents includes cyclohexane, chloroform and water. The polarity of the
16 solvent is increasing from cyclohexane having the lowest value that makes it the closest to
17 gas phase to water with highest polarity. The S_1 energy is nearly unaffected but the oscillator
18 strength is decreasing when going from the non-polar cyclohexane to the polar water. In this
19 series of solvent the S_4 shows a decrease in excitation energy and an increase in oscillator
20 strength. The largest change in the excitation energy is found for the S_6 , where a blue shift
21 of ca. 0.5 eV from cyclohexane to water is observed. In comparison to the experiment, the
22 computed values (chloroform) are systematically blue shifted by ca 0.65 eV. This might be
23 due to simple nature of the implicit solvent model, which for example cannot account for
24 hydrogen bonding. To illustrate, List and coworkers have performed a simulation of the

absorption spectrum of lumiflavin with polarizable embedding technique to account for the solvent effects. The obtained absorption maxima were blue shifted by ca. 0.4 eV in water (73, 74) and the order of states was slightly different: the dark S_2 state is destabilized and shifted above S_4 . The consideration of low energy, low frequency modes is another important factor as demonstrated by Marazzi *et al.* (81). According to the reference above, inclusion of the vibrational effect will provide a red-shift. We have observed that CAM-B3LYP excitation energies in PCM solvent overestimate the measured absorption maxima by 0.64 eV for S_1 . Hence the vibrational effect will reduce the difference between the computed and measured spectra. However, in this study we have mainly focused on the relative position of the bands.

Note that the low-energy band consists of three peaks, which yet correspond to the transitions to the different vibrational levels of the same electronically excited state (Figure 5). This was previously proven by Klaumünzer *et al.*, by simulating a vibronic spectrum of riboflavin (82). Also, it can be seen from our computed transitions: the energy gap between first two bright states is 0.74 eV (CAM-B3LYP, chloroform), which is significantly higher than the gap between different shoulders of the first absorption band (ca. 0.15 eV).

< **Table 3** >

< **Figure 5** >

NMR chemical shielding analysis

The NMR chemical shielding values are given in Table 4. The experimental data for the carbon and nitrogen atoms was obtained from previous studies of the oxidized form of tetraacetylriboflavin (83, 84). The conversion of the measured chemical shift values to

chemical shieldings is described in ref (84). Since chloroform can only donate a hydrogen bond but not accept it, Walsh and Miller have corrected the values for N₁ and N₅ by -12.5 ppm (84). Further, it is also important to note that the experimental shifts were measured in chloroform. Hence, in the present work we have investigated the solvent effect on the shielding values by choosing three different solvents with increasing polarity. The three solvents are cyclohexane, chloroform and water consistent with the excitation energy calculations.

The calculated NMR shielding values are sensitive to the choice of basis set. A large shift in these values is observed when going from double zeta (6-31G*) to triple zeta (6-311G*) basis set (Table 4 and Table S3 in the SI). The aim of our study is not to reach a quantitative agreement but rather compare the relative carbon and nitrogen shieldings which agree well with the experimental trends for both basis sets. Among all carbon atoms the highest deshielded values belong to C_{7a} and C_{8a}. The downfield shift is attributed to the fact that the electron density of these methyl groups at ring III shifts towards rings I and II which contain electronegative atoms (85). The introduction of implicit solvent in the calculation doesn't change the values of these carbon atoms. A significant effect of the increased polarity when going from the gas phase to the water environment, is found in the upfield shift of C₂, C₄, C₇, and C₈ by 4.92, 4.7, 5.23, and 6.57 ppm, respectively. In the non-polar solvent, the maximum upfield value is found for C₈ with 2.35 ppm.

< Table 4 >

Among the nitrogen shielding values only the one of N₅ is negative. A negative value indicates an electron rich atom, which in case of N₅ is due to the lone pair and the strong shielding effect of the fused ring current. Also N₅ is further away from the electron withdrawing carbonyl group compared to N₁ that also has a lone pair. The effect of polarity

(water) is responsible for the highest deshielding noticed for N₁ with a downfield shift of 17.81 ppm, while the other three nitrogens are not affected. This change is less pronounced in the cyclohexane solution, namely by 6.79 ppm. This indicates a high sensitivity of N₁ to the environment. In contrast, the shielding at N₁₀ is upshifted. The change in water is by 13.44 ppm and in cyclohexane by 4.86 ppm. In summary, based on the assignment of the NMR shieldings we confirm the experimental findings that identified N₅ as the most favorable nucleophilic reaction center (85, 86). However, we find the shielding of N₁ to be the most sensitive to the polarization of the solvent environment.

CONCLUSIONS

This work provides a detailed investigation of the various spectroscopic properties of lumiflavin in the gas phase and in solution to give a reference for further photochemical studies. Ten low-lying singlet and triplet states were calculated by various levels of theory. The nature of all excited states was analyzed in terms of the character of the involved orbitals and electron density difference for the bright states. Furthermore, the IR, Raman, as well as resonance Raman spectra were simulated in order to assign the experimentally observed vibrational spectra. In addition to the above, the GIAO NMR shielding of nitrogen and carbon atoms were computed and compared to the experiment.

The vertical excitation energies were computed using the accurate *ab initio* methods CC2 and ADC(2). Both of them yield the same order of the singlet excited states as previously reported DFT/MRCI, nevertheless, the ordering of the triplet states is qualitatively different. The results of CC2 and ADC(2) were also compared to the results of the earlier TD-DFT studies. It was found that the most popular B3LYP functional cannot predict the correct ordering of singlet and triplet states. However, the inclusion of range-separation (CAM-

B3LYP) provides a balanced description of the singlet excited states, yet, the order of the triplets is incorrect. Thus, on the basis of the current and previous studies, we make several conclusions: (1) Despite the identification of the two lowest bright states of lumiflavin there are two dark $n\text{-}\pi^*$ states in-between which can cross in the course of the photoreaction and thereby affect the reaction; (2) TD-DFT methods provide reasonable excitation energies for the bright states, however, the relative ordering of the states and singlet to triplet transitions might not be correctly described. (3) As mentioned above, CAM-B3LYP is free of the limitations of B3LYP for the singlet states. Further, our study of the solvatochromic effect, revealed no dependence of the first bright excited state on the solvent polarity, whereas remaining bright states undergo a significant change in both excitation energies and oscillator strength. Despite the fact that deviation from the experimental values is significant (0.64 eV), it is systematic.

The comparison of the simulated and the measured IR spectrum showed good agreement for the majority of the frequencies. However, two modes, namely $\text{C}_4=\text{O}_4$ and $\text{C}_2=\text{O}_2$ show a significant deviation by ca. 30 and 64 cm^{-1} respectively. The account for anharmonicity reduces one frequency more than the other, i.e. difference of 1 and 32 cm^{-1} . However, the agreement of the most of the other frequencies deteriorates. Hence, we conclude that the lack of anharmonicity is not the major source of the large deviation in the carbonyl stretchings because this correction shifts most of the vibrational frequencies. Further calculations included the conventional and resonance Raman spectra where we found good agreement to the corresponding experiments in solid state (Raman) and in protein (RR). In particular, the enhancement of the Raman intensity due to the resonance condition was qualitatively reproduced. However, the identification of the intensities in the region 1200 to 1700 cm^{-1} in the experimental RR spectrum was hindered by the lack of the baseline correction.

1 Finally, we studied the GIAO NMR shielding values of the carbon and nitrogen atoms in
2 lumiflavin. We have identified which shielding values are sensitive to solvent polarity and
3 managed to observe trends that help to assign experimental spectra. The values of carbon
4 atoms C₂, C₄, C₇, and C₈ changed the most while for the nitrogens it was N₁ and N₁₀.
5 The importance of this work lies in the identification of the spectroscopic values which are
6 difficult to observe (e.g. dark electronic states) or to assign unambiguously (e.g.
7 solvatochromic effects). Overall we believe that this computational work will serve as a
8 reference for embedded calculations and will also support the assignment of experimental
9 spectra of flavins.

10

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SUPPLEMENTARY MATERIALS

Supporting Information containing **Table S1:** Experimental vibrational frequencies compared with calculated anharmonic frequencies; **Table S2:** Vertical excitation energies computed with RI-CIS(D) and TD-DFT B3LYP; **Table S3:** Vertical excitation energies computed with TDA TD-DFT B3LYP with CPCM solvent; **Table S4:** GIAO NMR shielding values computed at B3LYP/6-31G* level, can be found at DOI: 10.1562/2006-xxxxxx.s1.

1 FIGURE & TABLE CAPTIONS

Scheme I: Chemical structure of the oxidized form of lumiflavin (A), the reduced form of lumiflavin (B) and riboflavin (C).

Figure 1: (A) A B3LYP/6-31G equilibrium geometry of lumiflavin. The bond lengths are given in Å. (B) Experimental IR spectrum of lumiflavin measured in KBr disc.*

Figure 2: (A) Calculated Raman spectrum of lumiflavin (B3LYP/6-311G* level of theory). (B) Experimental Raman spectrum of lumiflavin measured in powder, adopted from ref (67) with permission Copyright © 1999 American Chemical Society. (C) Calculated RR spectrum (B3LYP/6-311G* level of theory). (D) Experimental RR spectra of lumiflavin bound to RBP in water, excited at 488 nm. The figure is adopted from ref (68) with permission Copyright © 1980 Oxford University Press.) Lorentzian function with FWHM of 10 cm^{-1} was used to broaden the peaks in (A) and (C).

Figure 3: Molecular orbitals of lumiflavin involved in lowest singlet and triplet transitions.

The orbitals are computed based on the CC2/cc-pVTZ calculation.

Figure 4: Electron density difference for bright states of lumiflavin represented with blue (positive) and red colors (negative) electron density between (S_n and S_0). The EDD obtained are based on CC2 calculations.

Figure 5: Measured absorption spectrum of lumiflavin in chloroform (black line). Computed excitation energies (CAM-B3LYP/cc-pVTZ, CPCM, Chloroform) with oscillator strength for singlet transition (A' symmetry) are shown as blue sticks. The transitions with A'' symmetry are in order of magnitude less intense and marked as red arrow.

Table 1: Comparison of experimental and calculated vibrational frequencies of lumiflavin.
The level of theory is B3LYP/6-311G*.

Expt. Freq. (cm ⁻¹)	Calculated Freq. (cm ⁻¹)	Mode type	Characteristic frequencies in protein environment
1708	1738	C ₄ =O ₄	- 1714 in LOV2 domain ⁽⁵⁶⁾ - 1693 in LOV1 (C57S mutant) domain ⁽⁵⁹⁾ - 1710 in BLUF domain ⁽⁵⁷⁾
1663	1727	C ₂ =O ₂	- 1678 in LOV2 domain ⁽⁵⁶⁾ - 1681 in LOV1 (C57S mutant) domain ⁽⁵⁹⁾ - 1641 in BLUF domain ⁽⁵⁸⁾
1621	1615	Ring III: C _{5a} -C _{9a} , C ₇ -C ₈ , C ₈ -C ₉	- 1637 in LOV2 domain ^(56, 62)
1583	1570	Ring II stretching: N ₁₀ -C _{10a} , C _{4a} -N ₅ , C _{4a} -C _{10a} , C _{10a} -N ₁	- 1584 in LOV1 (C57S mutant) domain ⁽⁵⁹⁾ - 1578 in plant cryptochrome (CRY1) ⁽⁶¹⁾
1552	1535	Ring II stretching: C _{4a} -N ₅ , C _{10a} -N ₁	- 1550 in LOV1(C57S mutant) domain ⁽⁵⁹⁾ - 1543 in plant cryptochrome (CRY1) ⁽⁶¹⁾
1461	1421	C _{10a} -N ₁ , C _{4a} -N ₅ , C ₈ -C ₉ , C ₉ -C _{9a} , C ₈ -m	
1413	1375	N ₃ -H bending (weaker in expt.)	- 1443 in LOV domain ^(55, 65)
1346	1323	C ₄ -N ₃ , N ₅ -C _{5a} , C _{4a} -C _{10a} , C _{10a} -N ₁₀	- 1348 in LOV2 domain ⁽⁵⁶⁾
1301	1284	C _{4a} -N ₅ , N ₅ -C _{5a} , C _{4a} -C _{10a} , C ₈ -C ₉ , N ₁₀ -C _{9a}	
1238	1169	C ₂ -N ₃ , N ₃ -H bending, C ₄ -N ₃	- 1395 FMN binding LOV2 domain ⁽⁵⁶⁾
1169	1151	C _{4a} -C ₄ , N ₅ -C _{5a} , N ₃ -H bending	

Table 2: Vertical excitation energies for singlets and triplets computed with CC2, ADC(2), and CAM-B3LYP using cc-pVTZ basis set in comparison with previous quantum chemical calculations. The amplitude of the most significant electronic transition is based on CC2 calculations.

			CC2	ADC(2)	CAM-B3LYP	DFT MRCI/TZVP ^a	SAC-CI/D95V(d) ^b	XMCQDPT2/cc-pVDZ ^c	CAM-B3LYP/cc-pVDZ ^d	Expt ^{e,f}
S ₀ →	Electronic Structure		ΔE (f)	ΔE (f)	ΔE (f)	ΔE	ΔE (f)	ΔE (f)	ΔE (f)	ΔE
S ₁	(0.93) A'	π _H → π* _L	3.06 (0.243)	2.94 (0.247)	3.55 (0.365)	2.94	2.46 (0.091)	3.09 (0.317)	3.31 (0.310)	2.79 ^e 2.77 ^f
S ₂	(0.78) A''	n _{O2} → π* _L	3.50 (0.001)	3.38 (0.000)	3.97 (0.000)	3.21	3.18 (0.002)		3.91 (0.000)	
S ₃	(0.75) A''	n _{N2} → π* _L	3.59 (0.000)	3.51 (0.000)	3.68 (0.001)	3.35	3.59 (0.000)		3.61 (0.000)	
S ₄	(0.91) A'	π _{H-1} → π* _L	4.13 (0.158)	4.06 (0.205)	4.37 (0.159)	3.84	3.84 (0.145)	4.23 (0.287)	4.22 (0.140)	3.38 ^e 3.77 ^f
S ₅	(0.90) A''	n _{O1} → π* _L	4.39 (0.000)	4.17 (0.000)	4.77 (0.000)	3.93				
S ₆	(0.71) A'	π _{H-2} → π* _L	4.55 (0.016)	4.41 (0.018)	4.93 (0.033)		4.55 (0.213)			
S ₇	(0.58) A'	π _H → π* _{L+2}	4.93 (0.088)	4.91 (0.273)	5.32 (0.000)		4.56 (0.076)			
S ₈	(0.87) A''	n _{N5} → π* _L	4.96 (0.000)	4.91 (0.000)	5.12 (0.009)					
S ₉	(0.81) A'	π _H → π* _{L+1}	4.97 (0.776)	4.79 (0.550)	5.42 (1.067)	4.77				4.59 ^e 4.85 ^f
S ₁₀	(0.64) A'	π _H → π* _{L+2}	5.18 (0.055)	5.16 (0.030)		4.91				
T ₁	(0.95) A'	π _H → π* _L	2.52	2.44	2.34	2.24				
T ₂	(0.67) A''	n _{N2} → π* _L	3.18	3.30	3.10	2.90				
T ₃	(0.92) A'	π _{H-1} → π* _L	3.35	3.29	3.17	3.05				
T ₄	(0.70) A''	n _{O2} → π* _L	3.48	3.13	3.66	3.21				
T ₅	(0.85) A'	π _H → π* _{L+1}	4.04	3.96	3.86					
T ₆	(0.72) A'	π _{H-2} → π* _L	4.21	4.09	4.09					
T ₇	(0.84) A''	n _{O1} → π* _L	4.26	4.04	4.45					

T ₈	(0.60) A'	$\pi_H \rightarrow \pi^*_{L+2}$	4.45	4.39	4.24
T ₉	(0.82) A''	$n_{N5} \rightarrow \pi^*_L$	4.68	4.59	4.72
T ₁₀	(0.57) A'	$\pi_{H-1} \rightarrow \pi^*_{L+2}$	4.98	4.93	4.74

1 ^a Ref (72)

2 ^b Ref (26)

3 ^c Ref (25)

4 ^d Ref (73)

5 ^e measured in water, Ref (74)

6 ^f absorption of FMN in LOV1 domain, Ref (71)

7

8 **Table 3:** Vertical excitation energies of lumiflavin computed with TDA TD-CAM-B3LYP

9 with CPCM solvent model and measured experimentally. The excited states are ordered

10 based on the CC2 results in Table 2.

		TDA TD-DFT CAM-B3LYP/ cc-pVTZ, CPCM				
		Cyclohexane	Chloroform	Water	Exp. Chloroform	
S ₀ →	Symmetry	ΔE (f)			ΔE	
S ₁	A'	π _H → π* _L	3.43 (0.485)	3.41 (0.479)	3.41 (0.440)	2.77
S ₂	A''	n _{O2} → π* _L	4.15 (0.000)	3.79 (0.002)	4.37 (0.000)	
S ₃	A''	n _{N2} → π* _L	3.75 (0.002)	4.27 (0.000)	3.84 (0.002)	
S ₄	A'	π _{H-1} → π* _L	4.23 (0.255)	4.15 (0.282)	4.09 (0.294)	3.59
S ₅	A''	n _{OI} → π* _L	4.96 (0.000)	5.08 (0.000)	5.37 (0.000)	
S ₆	A'	π _{H-2} → π* _L	4.99 (0.145)	5.02 (0.264)	5.47 (0.611)	
S ₇	A'	π _H → π* _{L+1}	5.30 (0.831)	5.32 (0.498)	5.04 (0.321)	4.58
S ₈	A''	n _{N5} → π* _L	5.21 (0.000)	5.29 (0.000)	5.16 (0.000)	

11

12 **Table 4:** GIAO NMR shielding (in ppm) of carbon and nitrogen of lumiflavin molecule,

13 calculated at the B3LYP/6-311G* level of theory.

	Gas phase	Solvent (cyclohexane)	Solvent (chloroform)	Solvent (water)	Experiment (chloroform) ^a
C ₂	29.27	27.89	26.99	24.35	29.3
C ₄	24.13	22.57	21.65	19.43	25.2
C _{4a}	42.48	43.21	43.73	45.16	49.4
C _{5a}	44.06	44.02	43.95	43.17	50.4
C ₆	43.36	43.87	44.29	44.84	52.4
C ₇	45.13	43.24	41.99	39.9	48.4
C _{7a}	166.94	167.01	166.93	166.89	165.6
C ₈	33.27	30.92	29.31	26.70	37.5
C _{8a}	159.82	159.84	159.79	159.45	163.6
C ₉	66.97	66.03	65.32	64.51	69.5
C _{9a}	47.46	47.31	47.17	47.02	53.8
C _{10a}	30.72	30.21	29.74	29.26	35.9
N ₁	18.94	25.73	29.64	36.75	32.0
N ₃	77.30	77.18	77.23	77.89	85.0
N ₅	-126.13	-123.23	-120.99	-121.62	-113.9
N ₁₀	95.40	90.54	86.5	81.96	92.7

1 ^aRef (84)

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