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Spectroscopy and Photophysics of Iso- and Alloxazines: Experimental and Theoretical Study

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We present a systematic study of the effect of methyl substitution on iso- and alloxazines in acetonitrile solutions. Substitution patterns have profound effects on both spectral and photophysical properties, with fluorescence quantum yields varying by more than an order of magnitude. TD-DFT calculation were used for the first time to correlate electronic structure changes with the substitution patterns, with good agreement between calculated and theoretical band positions and oscillator strengths. Both $n - \pi^*$ and $\pi - \pi^*$ states in these compounds are predicted, with the oscillator strengths indicating that only the $\pi - \pi^*$ states should be observable in the absorption spectra. Substitution patterns are shown to be responsible for energy order inversion between these states.

KEY WORDS: TD-DFT calculations; alloxazines; iso-aloxazines; prediction; absorption spectra; T-T absorption spectra.

INTRODUCTION

Iso- and alloxazines are closely related compounds, representing two classes of nitrogen heterocycles with active centres at N(10), N(5), N(3) and N(1), and at both carbonyl oxygens at C(2) and C(4). Alloxazine, All, (benzo[*g*]pteridine-2,4(1*H*,3*H*)-dione) and lumichrome, Lch, (7,8-dimethylalloxazine=7,8-dimethyl-benzo[*g*]-pteridine-2,4(1*H*,3*H*)-dione) are representative of alloxazines, a class of nitrogen heterocycles related to lumazine and flavins. Isoalloxazines (isoalloxazine: 10-substituted 2,3,4,10-tetrahydro-benzo[*g*]pteridine-2,4-dione) and especially flavins, possess the yellow chromophore characteristic of flavoproteins—enzymes occurring widely in animals and plants. The term "Flavins" refers to the 10-substituted 7,8-dimethyl-2,3,4,10-tetrahydro-benzo[*g*]-pteridine-2,4-diones, among which are the natural coenzymes: riboflavin, FMN and FAD. Lumiflavin, Lfl, (7,8,10-trimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione), is another representative of flavins. Iso- and alloxazines are closely related compounds, yet the spectroscopic and photophysical properties of these two groups are quite different. In particular, isoalloxazines exhibit intense fluorescence and relatively long fluorescence lifetimes. This property has been widely used for their characterisation and determination. It is generally accepted that isoalloxazines have fluorescence quantum yields one order of magnitude larger and correspondingly longer fluorescence lifetimes than alloxazines. The photochemistry of isoalloxazines has been the subject of intense research, see for example [1–4]. Since the amount of data on ground- and excited-state properties of flavins is overwhelming, we shall only refer to the proceedings of a symposium, titled "Flavins and Flavoproteins" [5], which illustrate both the wealth of the information available and the progress that has been made in photochemistry, structure and functionality of flavins.

In contrast to flavins, alloxazines have received relatively little attention, but some activity in experimental studies [6–11] and theoretical calculations [4,12,13]

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should be noted. Recently, the interest in alloxazines has become more intense due to realization of possible involvement of alloxazines in a wide variety of biological systems [2,14]. For example, it has been shown that lumichrome may be used to inhibit flavin reductase in living *Escherichia coli* cells [15]. Said *et al.* [16] reported that the mechanism of riboflavin uptake by human-derived liver cells Hep G2, colonic epithelial NCM460 cells, and Caco-2 human intestinal epithelial cells, is inhibited by lumichrome. A further point of interest is the possibility of using alloxazine nucleosides as fluorescent probes, as they have been predicted to have hydrogen–bonding characteristics similar to those of thymidine [17]. Most of the work on the photochemistry of alloxazines has been performed on lumichrome and its 1- and 3-methyl and 1,3-dimethyl derivatives [6,7,10,11,18–25]. From the previous experimental studies, which have usually been limited to steady-state spectral measurements, the effect of the position of the methyl substituent on the properties of alloxazines has been elucidated [7,26]. However, those observations were only poorly reproduced theoretically by the semiempirical methods used [4,12,13]. Spectral and photochemical properties of a variety of methyl-substituted isoalloxazine derivatives have also been investigated [3,27], once more, semiempirical methods had been used for interpretation, with limited success.

This paper describes steady-state and time–resolved studies of the ground and excited singlet states of iso- and alloxazines in acetonitrile. A study of the electronic structure of alloxazines by means of time-dependent density functional theory [28] (TD-DFT) is also reported. To the best of our knowledge, there are no published TD-DFT predictions of electronic spectra for alloxazines, while the available theoretical predictions for similar compounds have been obtained using only semiempirical methods [4,12,13,26]. The structures and abbreviations of the compounds discussed are presented in Fig. 1. The aim of the present paper is to characterise and reconcile the diverse photophysical and spectroscopic properties of alloxazines in acetonitrile solutions and to compare them to those of isoalloxazines. To obtain further information on the methyl group effect on spectroscopy and photophysics of iso- and alloxazines, we studied their derivatives, from mono- to tetramethyl substituted, having the methyl group at different positions in the molecule. The present investigation was undertaken with the aim to give a systematic insight into the photophysics of iso- and alloxazines in solution, and to provide its theoretical interpretation based on TD-DFT methods.

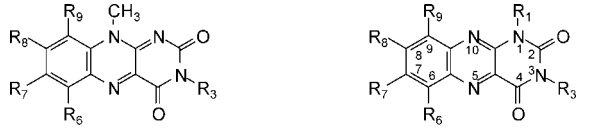

Fig. 1. Structures of lumiflavin and lumichrome and their atom numbering.

EXPERIMENTAL

Alloxazine, lumichrome, lumiflavin and the solvent acetonitrile, all from Aldrich, were used as received. The alloxazine derivatives, 1-methyllumichrome and 3-methyllumiflavin, were available from previous work [7].

Three different systems were used to measure the fluorescence decays. However, some of the measurements were performed using all three available instruments as a check of the reliability of the measurements. Time–resolved fluorescence was recorded using a time–correlated single–photon counting system, which has been described in detail in [29], and using time–correlated single–photon-counting method on an IBH model 5000U fluorescence lifetime spectrometer. Some of the measurements were conducted with a model C–700 fluorometer from Photon Technology International. The system utilises a nanosecond flash lamp as an excitation source and a stroboscopic detection system [30]. Steady–state fluorescence spectra were obtained with a Jobin Yvon–Spex Fluorolog 3–11 spectrofluorometer, and UV–visible absorption spectra on a Varian Cary 5E spectrophotometer.

RESULTS AND DISCUSSION

A summary of photophysical parameters of alloxazine and its methyl derivatives and of isoalloxazines in their lowest excited singlet states is given in the Table I. The results presented allow us to selectively observe the effect of various methyl substitutions. Iso- and alloxazines exhibit absorption spectra with a few major bands in the UV-visible region, see Fig. 2. Molecules of an isoalloxazine structure, 3-methyllumiflavin and its methyl derivatives, and lumiflavin itself, have spectroscopic and photophysical properties very different from those of the alloxazines. As a typical example of isoalloxazines, the absorption and fluorescence spectra of 3-methyllumiflavin (3MLfl) and the fluorescence decay kinetics in acetonitrile are shown in Fig. 2 and can be contrasted with the data presented for 1,3-dimethyllumichrome. In absorption 3-methyllumiflavin shows two characteristic

Table I. Spectroscopic and Photophysical Data for the Singlet States of Iso and Alloxazines in Acetonitrile[a]

No.	Compound	λ_2 (nm)	λ_1 (nm)	λ_F (nm)	ϕ_F	τ_F (ns)	k_r ($10^8 s^{-1}$)	Σk_{nr} ($10^8 s^{-1}$)
1	Alloxazine	320	372	432	0.009	0.35	0.26	28
2	6-Methylalloxazine[b]	333	380	452	0.017	0.9	0.19	11
3	7-Methylalloxazine[b]	320	382	443	0.025	0.6	0.42	16
4	8-Methylalloxazine[b]	335	370	426	0.017	0.4	0.41	25
5	9-Methylalloxazine[b]	333	379	449	0.018	0.7	0.29	16
6	6,7-Dimethylalloxazine	332	388	469	0.041	2.41	0.17	4.0
7	6,8-Dimethylalloxazine	350		461	0.020	1.24	0.16	7.9
8	6,9-Dimethylalloxazine	343	388	491	0.080	8.88	0.09	1.0
9	7,8-Dimethylalloxazine, (Lumichrome)[b]	334	380	437	0.028	0.64	0.43	15
10	1,7,8-Trimethylalloxazine	334	379	437	0.027	0.63	0.43	15
11	3,7,8-Trimethylalloxazine	335	379	436	0.026	0.64	0.41	15
12	1,3,7,8-Tetramethylalloxazine	335	375	437	0.028	0.64	0.43	15
13	7,9-Dimethylalloxazine	330	388	463	0.043	1.65	0.26	5.8
14	8,9-Dimethylalloxazine	351	375	461	0.019	1.14	0.16	6.1
15	7,8,10-Trimethylisoalloxazine, (Lumiflavin)	342	443	533	0.16	7.7	0.21	1.1
16	3,7,8,10-Tetramethylisoalloxazine (3-Methyllumiflavin)	342	444	531	0.17	7.1	0.24	1.2
17	3,6,10-Trimethylisoalloxazine[c]	344	440	493	0.14	1.8	0.77	4.7
18	3,7,10-Trimethylisoalloxazine[c]	330	446	532	0.43	9.4	0.46	0.61
19	3,8,10-Trimethylisoalloxazine[c]	340	435	518	0.28	4.9	0.57	1.47
20	3,9,10-Trimethylisoalloxazine[c]	344	439	532	0.07	1.8	0.39	5.17
21	3,10-Dimethylisoalloxazine[c]	328	437	529	0.38	7.0	0.54	0.89

[a] λ_1, λ_2 are the positions of the two lowest-energy bands in the absorption spectra, λ_F the fluorescence emission maximum, ϕ_F the fluorescence quantum yield, τ_F the fluorescence lifetime, k_r the radiative rate constant and Σk_{nr} the sum of nonradiative rate constants.
[b] From Ref. [11].
[c] From Ref. [3].

bands at 342 nm (ca. 29 200 cm^{-1}) and 444 nm (ca. 22 500 cm^{-1}); the corresponding absorption and emission spectra of lumiflavin in acetonitrile are practically identical to those of 3-methyllumiflavin. However, for 3,10-dimethylisoalloxazines substituted with methyl group at positions 6, 7, 8 or 9 significant spectral and photophysical effects as a function of substitution pattern have been observed, see Table I, and interpreted in term of different bonds orders between the C-atoms of the methyl group and of the aromatic ring [3,27]. Addition of a methyl group at C(6) or C(9) results in a decrease of the quantum yield of fluorescence and a shortening of the fluorescence lifetime, whereas a methyl group at C(7) has the opposite effect.

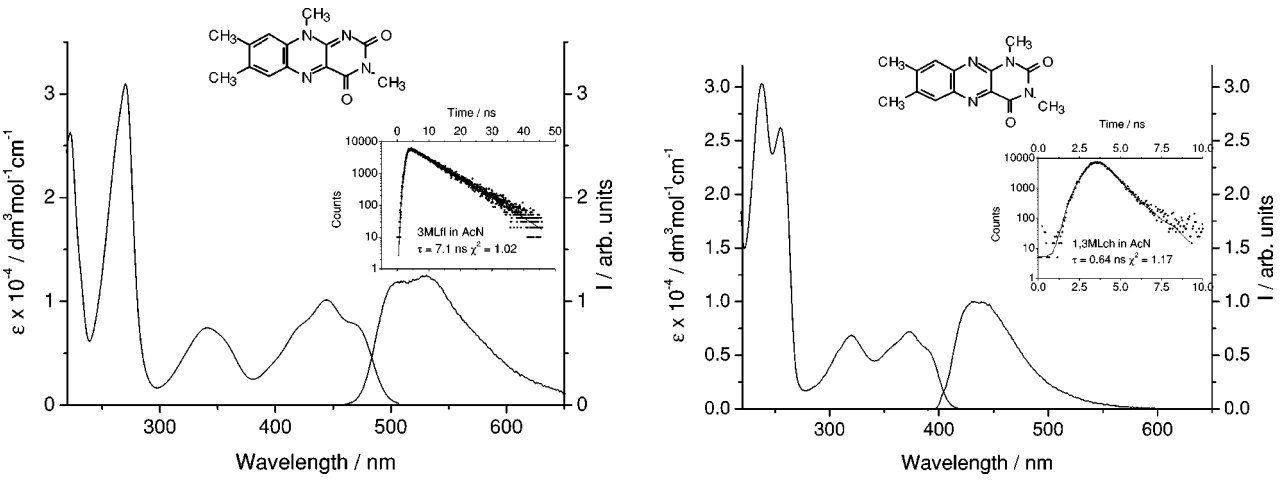

Fig. 2. Absorption and fluorescence spectra of 3-methyllumiflavin, 3Mfl, (left panel) and 1,3-dimethyllumichrome, 1,3MLch, (right panel) in acetonitrile, together with their fluorescence decay kinetics.

It is well-known that the methyl group in the N(10) position of isoalloxazines yields very closely structurally related iso- and alloxazines, possessing divergent spectral and photophysical properties. In particular, isoalloxazines exhibit intense fluorescence, and the maximum of fluorescence of isoalloxazines is strongly red-shifted if compared to alloxazines. For example, the maximum of the lumiflavin fluorescence is red-shifted by about 100 nm relative to that of lumichrome, giving a broad, unresolved band with a maximum at 533 nm (ca. 18 800 cm^{-1}). Alloxazines exhibit absorption spectra differing from those of isoalloxazines in that there is a hypsochromic shift of both long-wavelength maxima from about 440 and 340 nm to about 380 and 330 nm. The lowest-energy band positions for all the compounds are listed in Table I. Typical fluorescence emission spectra of iso- and alloxazines excited at 355 nm are presented in Fig. 2. Fluorescence emission spectra of iso- and alloxazines in acetonitrile show a single band peaking at about 440 nm and 380 nm respectively, the exact position depending on the location and number of substituents.

Theoretical calculations and polarised luminescence data indicate that all the UV–visible absorption and emission bands of iso- and alloxazines are assignable to the electric dipole allowed $\pi \rightarrow \pi^*$ transitions [12,13]. However, it is very desirable to learn more about the effect of n, π^* states in elucidating the spectroscopy and photophysics of iso- and alloxazines. According to relatively old theoretical studies, the energy of the n, π^* singlet state is very close to the energy of the lowest excited singlet π, π^* state in iso- and alloxazines [12,13]. The exact position and energy of the n, π^* state was not fully determined; however it was generally believed that the weak fluorescence emission of alloxazines relatively to isoalloxazines may reflect a close spacing of n, π^* and π, π^* excited singlet states, with the lowest-energy state being of n, π^* character. Isoalloxazines with their lowest excited singlet state of π, π^* character and with lower excited singlet state energies have fluorescence quantum yields one order of magnitude higher and correspondingly longer fluorescence lifetimes, than the alloxazines. As we will show later, the energy of the lowest excited π, π^* singlet state depends on the position of the methyl substituent, which can result in energy order inversion between n, π^* and π, π^* excited singlet states.

For all the compounds examined, the absorption and the corrected fluorescence excitation spectra agree well with one another. The fluorescence decays are modelled well by single-exponential functions in all cases, as shown by the usual statistical goodness–of–fit criteria. The fluorescence lifetimes and quantum yields in acetonitrile of all studied compounds are given in Table I. The radiative and non-radiative decay constants for the lowest excited singlet states can be calculated based on the fluorescence lifetimes and quantum yields as

$$k_r = \frac{\phi_F}{\tau_F} \text{ and } \sum k_{nr} = \frac{1 - \phi_F}{\tau_F}.$$

Here, k_r is the radiative decay rate constant of the excited species and Σk_{nr} is the sum of all first order and pseudo–first order rate constants for its non–radiative decay. The sum, Σk_{nr}, may include contributions from the pseudo-first order concentration quenching and oxygen quenching of the excited species. The values of k_r and Σk_{nr} are also tabulated in Table I. For all examined compounds of alloxazine type the data show that the decay of the singlet state is dominated by the rates of the non–radiative processes, these being one or two orders of magnitude higher than those of the radiative processes. Flavines, in contrast, exhibit longer fluorescence lifetimes and the rates of radiative and non-radiative processes of a similar order of magnitude. For example, the fluorescence lifetime of lumiflavine has been determined as 7.6 ns [11], due mainly to a remarkable reduction in the rate of non-radiative processes (by more than an order of magnitude) relative to lumichrome.

Taking into account the structure, their spectral and photophysical properties, the compounds studied may be grouped into four classes. The first class includes alloxazine and its monomethyl derivatives. The effect of introducing a methyl group into the benzene ring of the alloxazine becomes apparent when the spectral and photophysical characteristics of this class of compounds are compared to those of alloxazine itself. In particular, a methyl substituent in the benzene ring increases the fluorescence lifetime and quantum yield, although the effect on the lifetime is marginal in the case of 8-methyl substitution, due mainly to a decrease in non-radiative deactivation rates. Monomethyl substitution induces changes in the spectral properties as compared to the parent molecule. These changes include a distinct shift in the positions of maxima and in the band shapes in the absorption spectra. Alloxazine itself exhibits an absorption spectrum with two well-separated bands at longer wavelengths. A methyl substitution at positions 6 or 9 causes a very similar batochromic shift of these two absorption bands. The fluorescence maximum is similarly shifted as compared to alloxazine. The strongest effect is observed on the fluorescence lifetimes, 6MAll and 7MAll having the longest lifetimes within the first class of compounds. Methyl substitution at position 7 causes better separation of the two absorption bands, leaving the second band unaltered and bathochromically shifting the first one. Methyl substitution

at position 8 merges both absorption bands due to shifts in opposite directions, making them difficult to distinguish.

The second class, representing dimethylsubstituted alloxazines, includes compounds with two methyl groups in the benzene ring (namely, 6,7-, 6,8-, 6,9-, 7,8-, 7,9-, and 8,9-dimethylalloxazines). Inspection of data presented in Table I reveals that in each case the spectral data reflect a sum of individual effects of the methyl substituents in particular positions observed for monomethyl-derivatives. Less straightforward is the interpretation of the changes in photophysical parameters. The data of Table I lead to an observation that the second methyl group in the benzene ring of alloxazine lengthens the fluorescence lifetime, increases the fluorescence quantum yield and reduces the nonradiative rate constant. Significant substituent effects on the photophysics can be observed, for example in 6,9-dimethylalloxazine compared to 7,8-dimethylalloxazine. The fluorescence lifetime, but not the quantum yield, makes the 6,9-dimethylalloxazine a very special case. The similar but less spectacular changes can be also noted for 6,7-dimethylalloxazine. To our best knowledge, the fluorescence lifetimes of dimethylsubstituted alloxazines are among the longest ever reported for alloxazines. In some cases the fluorescence lifetimes are even longer than the fluorescence lifetimes of some isoalloxazines, also reported in Table I.

7,8-dimethylalloxazine, known widely as lumichrome, appears to be a special case in which opposite effect of methyl groups located at positions 7 and 8 are observed. Thus, we grouped lumichrome and its 1- and 3-methyl and 1,3-dimethyl derivatives into a separate third class. Especially interesting is that all characteristics of the excited singlet state, ϕ_F, τ_F, k_r, and k_{nr}, have very similar values for the lumichrome and its 1- and 3-methyl and 1,3-dimethyl derivatives. Introduction of a methyl group at position N(1) and/or N(3) practically does not influence the spectral and photophysical properties of the studied molecules in their ground and excited singlet states.

The fourth class of compounds in our studies is represented by lumiflavin, a compound existing in the isoalloxazinic form, and its derivatives. The spectroscopic and photophysical properties of isoalloxazines are very different from those of alloxazines. For example, the maximum of lumiflavin fluorescence is shifted to longer wavelengths by about 100 nm relative to that of alloxazines, giving a broad, structureless band with a maximum at about 530 nm. It is well known that isoalloxazines exhibit longer fluorescence lifetimes and a similar order of magnitude for the rates of both radiative and non-radiative processes, which are similar to those of the radiative processes in alloxazines. For example, the fluorescence lifetime of lumiflavin has been determined as 7.6 ns [11], due mainly to a remarkable reduction in the rate of non-radiative processes (by more than an order of magnitude) relative to lumichrome. Isoalloxazines also exhibit intense fluorescence, which has been widely used for their characterisation and determination. Methyl substituents at the positions 6 and 9 in the benzene moiety in the isoalloxazines have an opposite effect to those in alloxazines. As Visser and Muller reported [3], both methyl groups at position of 6 and 9 diminish the fluorescence quantum yield and shorten the lifetime considerably, if compared to 3,10-dimethylisoalloxazine. Methyl substitution at position C(7) lengthens the lifetime, increases the quantum yield and shifts the fluorescence maximum to longer wavelength, whereas a methyl group at C(8) has the opposite effect.

Electronic structure of alloxazines and of some isoalloxazines has been studied by means of time-dependent density-functional theory (TD-DFT) [28]. Recently, similar TD-DFT calculations have been performed for singlet and triplet absorption spectra of lumiflavin [31–34] by others, and by us for lumiflavin and lumichromes [35] and demonstrated some very encouraging improvements as compared to previous semi-empirical and TD-DFT calculations [4,36], in that they succeeded in reproducing the correct order of the observed singlet excited states and oscillator strengths of the respective transitions. Moreover, to the best of our knowledge, there are no published TD-DFT calculations of the electronic spectra for neither alloxazines nor most of the isoalloxazines examined in this study. The available theoretical predictions for similar compounds were obtained using semiempirical methods only [4,12]. In this work, the TD-DFT calculations were performed using the B3LYP hybrid method [37] in conjunction with a modest 6-31G* split-valence polarized basis set [38]. Excitation energies and transition intensities were calculated for the optimized ground-state geometries. Oscillator strengths were calculated in the dipole length representation. Calculations were performed using the Gaussian 98 package of TD-DFT programs [39]. The TD-DFT results are presented in Figs. 3 and 4.

It is desirable to compare the results of these TD-DFT calculations to gas-phase spectra of the corresponding compounds. However, to the best of our knowledge, there are no published experimental gas-phase spectra for the compounds examined in our studies. Thus we turn to spectra recorded in solvents, but even here we are limited by the limited solubility of the compounds in nonpolar solvents. As a result, we chose acetonitrile as a solvent for comparison of theoretical predictions and experimental observations, of course bearing in mind the possible effects of the environment on the position and shape of the corresponding bands. Lumiflavin shows two characteristic

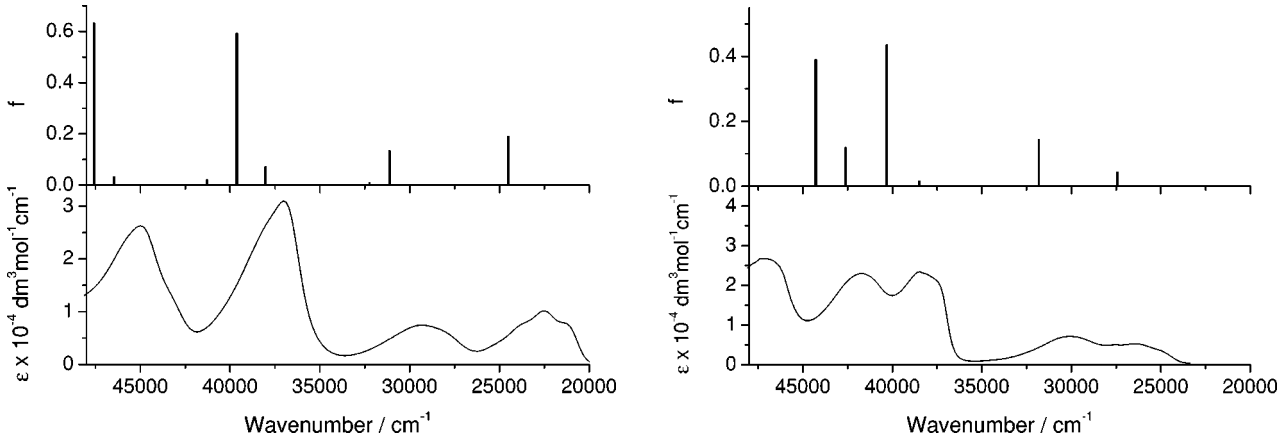

Fig. 3. Absorption spectra of 3-methyllumiflavin (left panel) and 9-methylalloxazine (right panel) in acetonitrile solutions. Calculated (B3LYP /6-31G*) transition energies and oscillator strengths (f) are indicated by solid vertical lines.

absorption bands at about 342 nm (ca. 29 300 cm^{-1}) and 443 nm (ca. 22 600 cm^{-1}). For both lumiflavins, the two calculated lowest-energy transitions presented in Fig. 3 are of the $\pi - \pi^*$ character, and appear at approximately 321 nm (31 100 cm^{-1}) and 408 nm (ca. 24 500 cm^{-1}), the computed oscillator strengths confirming that only the $\pi - \pi^*$ transitions should be observable. For both lumiflavins, the lowest excited singlet state has the $\pi - \pi^*$ character. However, the $\pi - \pi^*$ transitions are accompanied by two closely located $n - \pi^*$ transitions at 323 nm (31 000 cm^{-1}) and 402 nm (24 900 cm^{-1}) of low oscillator strengths.

Theoretical results for alloxazines, in contrast to lumiflavins, predict that the lowest excited singlet state is of the $n - \pi^*$ character – the exceptions being 6,7MAll, 6,9MAll, 7,9MAll, and 8,9MAll. Significant substituent effects on the energy of the lowest excited $n - \pi^*$ singlet state can be observed, see Fig. 4, for example in 6,9-dimethylalloxazine compared to 7,8-dimethylalloxazine. Once again, the 6,9-dimethylalloxazine is a very special case, in which methyl groups in positions 6 and 9 cause order inversion between the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions if compared to other alloxazines. The results of calculations shows that the energies of the $\pi \rightarrow \pi^*$ transitions vary with the positions and number of methyl groups in the benzene ring. In contrast, the energies of $n \rightarrow \pi^*$ transitions are little influenced by the position and number of the methyl groups. The two calculated lowest-energy transitions in alloxazines presented in the Fig. 3 are of the $\pi - \pi^*$ character, located at approximately 314 nm (31 700 cm^{-1}) and 365 nm (ca. 27 400 cm^{-1}), and accompanied by two closely-located $n - \pi^*$ transitions at 313 nm (31 900 cm^{-1}) and 365 nm (27 400 cm^{-1}) of low oscillator strengths. Hence the observed transitions are also of the $\pi - \pi^*$ character. The difference between the predicted and observed transition energies in acetonitrile is about 1500 cm^{-1}.

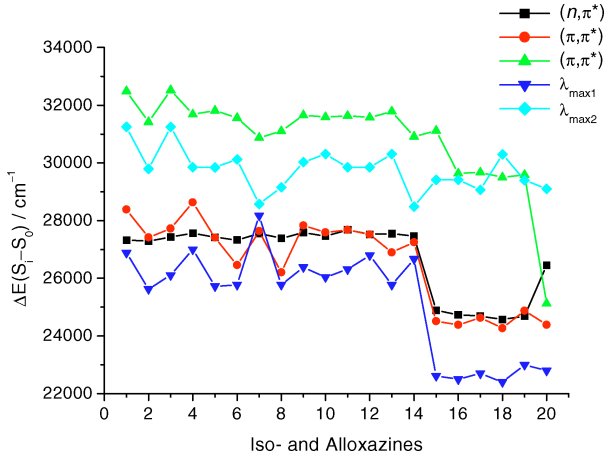

Fig. 4. Predicted and measured lowest singlet–singlet transitions of iso- and alloxazines. Numbering of compounds from Table I.

Based on the calculation results, we can confirm the previously proposed hypothesis [4], that the excited state order affects the Σk_{nr} value and hence the emission lifetime. In fact, shorter lifetimes and the largest non-radiative excited state decay rates correspond to those of the compounds where the lowest excited singlet state has the $n - \pi^*$ character, providing extra non-radiative decay channels for the optically visible lowest excited $\pi - \pi^*$ state (compare Fig. 4 and Table I). On the contrary, the compounds with longer lifetimes and lower Σk_{nr} values have their lowest excited state of the $\pi - \pi^*$ character, with the respective $n - \pi^*$ states out of play. The good

correlation observed between the predicted excited state order and the emission lifetimes provides additional evidence in proof of the currently obtained TD-DFT results.

CONCLUSIONS

We have shown through a systematic study of the effect of methyl substitution on iso- and alloxazines that various properties of these compounds, including their spectral and photophysical properties, are a sensitive function of the number and position of these substituents. TD-DFT calculations were used for the first time to correlate the electronic structure changes with the substitution patterns. Good correlations between calculated and theoretical band positions and oscillator strengths were obtained. These calculations lend weight to the assignment of the reduction in fluorescence quantum yields relative to isoalloxazines as being due to closely spaced $n - \pi^*$ and $\pi - \pi^*$ states in these compounds, with substitution patterns being responsible for the energy order inversion between these states.

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