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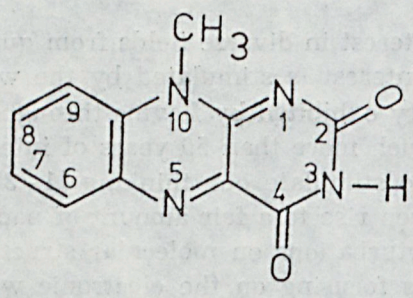
AN AM1 STUDY ON PROTONATION AND SOLVENT DEPENDENCE OF (ISO)ALLOXAZINE IN THE GROUND STATE

Flavins are still generating much interest in diverse fields from quantum chemistry to biochemistry. This interest is stimulated by the wide variety of chemistry and photochemistry exhibited by flavins throughout the energy metabolism realm. After much more than 50 years of intense research, this special field continues to stimulate our thinking [1 - 3].

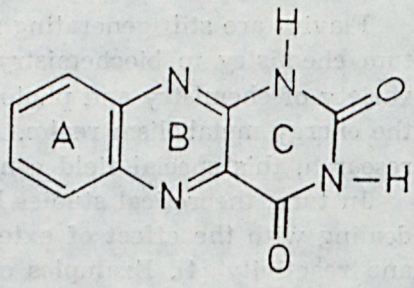
In turn, theoretical studies have given rise to a fair amount of papers dealing with the effect of external perturbations on molecular structure and reactivity [4]. Examples of studies focusing on the electronic wave function concern the changes made on Mulliken (or Löwdin) populations, electron densities, dipole moments, and many other properties. The theoretical studies have used a molecular orbital (MO) model within the quantum chemistry framework. However, it is nowadays universally accepted that the valence-bond (VB) method offers a more chemical description of the wave functions [5, 6]. The wave function can be visualized in terms of structural formulas that can be given rigorous quantitative weights. This structural formulas can be very helpful in understanding the reactivity. For instance, the zwitterionic and diradical character of 1,3-dipoles has been discussed by the VB method [4, 7]. This method can thus be considered as a suitable candidate for an alternate characterization of the wave-function changes under the effects of external perturbations.

It is well known that ionic terms of conjugated systems markedly manifest themselves under the influence of an electric field, substituents, or other factors [4]. The goal of the present work is to analyze the effect of external perturbations on alloxazinic and isoalloxazinic systems due to specific interactions with protic solvents. To keep up with simplicity and ease of interpretation, calculations have been carried out on the protonated species on pyridine-like nitrogen atoms only. It is assumed that protonation approaches the extremely strong, nonuniform electric field created by a positive point charge. The electron density redistribution upon protonation may be a useful probe into the electronic response of the (iso)-

alloxazine molecular system. As a result of the AM1 MO studies presented here, based on optimized geometries, computed changes in charge distribution upon protonation become the basis for comparing with available proton and carbon NMR data for oxidized forms of (iso)alloxazines in different polar media. It is also suggested that obtained good correlation with experimental data allows for a new interpretation of NMR spectra, first in terms of relative hydrogen bonds ordering in alloxazine and isoalloxazine systems, and secondly in terms of higher π bonds polarizations in benzoide moiety, which indicate increasing weights of ortopolar VB structures when going from apolar to more polar protic solvents.



10-methyl(iso)alloxazine



alloxazine

METHODS

Unsubstituted alloxazine and 10-methylisoalloxzine are used as models for flavins in this study because these molecules preserve the properties of the biological species and because they are of reasonable size for computation. The unsubstituted alloxazine molecule is given the symbol A, and for 10-methylisoalloxazine 10I. For protonated molecules the site of hydrogen attachment nitrogen atom is indicated, as $A5^+$, $A10^+$, $A5,10^{++}$, $10I1^+$, $10I5^+$ and $10I1,5^{++}$.

To arrive at the results and interpretations presented in this paper we carried out total geometry optimization (bond distance, bond angle, and torsion angle for all atoms) by AM1 method on the above mentioned molecules, in both oxidized and in cationic states. The advantages and weaknesses of AM1 have been described in several sources, some of which are of particular interest to the question of geometry optimization [8, 9]. Molecular properties are based on orbitals computed from the geometry determined at the computed energy minimum. In AM1 method the optimization is obtained using the Davidon-Fletcher-Powell algorithm. For

all structures studied the gradient and Herberts tests were satisfied. The idealized geometry obtained from standard bond distances and valence bond angles was the starting point for both oxidized and protonated forms.

RESULTS AND DISCUSSION

Protonated Oxidized (Iso)alloxazines:

In case of alloxazine geometry optimizations were carried out on molecule protonated at either the N(10) or N(5) position, or diprotonated at both nitrogens. The computed enthalpies of formation (Hf) of alloxazine and its cationic states, $A10^+$, $A5^+$ and $A5,10^{++}$ are as follows: 56.52, 723.99, 734.84 and 1791.69 kJ/mol, respectively. The predicted ΔHf for the $A10^+$ form is 10.85 kJ/mol more negative than for the $A5^+$ form. The attachment of a second proton to $A10^+$, on the basis of calculated Hf values, is less favourable by about 1070 kJ/mol.

Upon protonation at either N(10) or N(5) the positive charge is spread across all three rings to some extent. The computed net atomic charges of alloxazine and its protonated forms are given in Table 1. In $A10^+$ form all atoms become more positive except C(9). The most positive charge rich are the oxygen atoms, C(10a), N(5), C(8) and C(6). For A5+ form, the positive charge is mostly spread over O(12), N(10) (opposite to N(10) protonated form), C(7) and C(5a). The nitrogen N(5) itself loses only 0.02 of electron. Three important conclusions are to be noticed from perusal of Table 1:

1. the carbon atoms of benzoide ring, C(8) in $A10^+$ and C(7) in $A5^+$ forms, have the strongest capacity of excessive positive charge stabilization.

2. protonation at N(10) strongly decreases the negative charge on N(5) by about 0.08 of electron and, in turn, protonation at N(5) lowers the negative charge on N(10) by about 0.1 of electron,

3. Protonation at N(10) increases the electron density on C(9) by about 0.04 of electron and, again, protonation at N(5) causes a similar effect on C(6) carbon atom by 0.08 of electron.

In the case of diprotonated form we obtain such the picture that closely resembles a sum of two singly protonated forms. The only difference is C(9) atom which preserves the characteristics of N(5) cationic form. Except of both oxygens also C(10a) and, what is very surprising, C(8) and C(7) benzoide atoms are strongly engaged in stabilizing of electron deficient alloxazinic system.

In Table 2 charge redistribution upon protonation of 10-methylisoallo-

xazine is shown. The calculated trends of charge migration over all atoms though generally similar are in some way slightly different in comparison to those of alloxazine. In the case of N(1) protonation only the atoms

Table 1

Computed partial atomic charges (q) for alloxazine and its protonated forms

Atom	Molecule			
	q(A)	$\Delta q(A-A10^+)$	$\Delta q(A-A5^+)$	$\Delta q(A-A5,10^{++})$
C(7)	−0.138	0.019	0.101	0.075
C(6)	−0.067	0.047	−0.082	0.007
C(5a)	−0.083	0.012	0.073	0.029
C(9a)	0.024	0.015	0.032	0.062
C(9)	−0.117	-0.039	0.030	−0.021
C(8)	−0.093	0.069	0.016	0.122
H-C(7)	0.145	0.038	0.040	0.076
H-C(6)	0.160	0.034	0.017	0.046
H-C(9)	0.158	0.012	0.038	0.048
H-C(8)	0.144	0.036	0.042	0.076
N(5)	−0.006	0.079	0.022	0.092
C(4a)	−0.120	0.012	0.031	0.076
C(10a)	0.120	0.128	0.042	0.150
N(10)	−0.161	0.004	0.086	0.024
C(4)	0.367	0.001	−0.011	−0.008
N(3)	−0.370	0.014	0.030	0.044
C(2)	0.410	0.004	−0.003	−0.002
N(1)	−0.306	0.006	0.017	0.036
O(12)	−0.346	0.101	0.094	0.199
O(14)	−0.280	0.080	0.009	0.088
H-N(1)	0.282	0.012	0.029	0.042
H-N(3)	0.276	0.032	0.033	0.067
H-N(10)		0.286		0.322
H-N(5)			0.315	0.350
	$\sum q_i = 0.0$	$\sum q_i = 1.0$	$\sum q_i = 1.0$	$\sum q_i = 2.0$

C(9a), N(1) and methyl group show an increase in negative charge. For $10I5^+$ form slight charge increase at C(6) (by 0.04 electron) is predicted — this effect also in dicationic state is found. The benzoide carbons C(7) and C(8) of 10I play the stabilizing role in the same degree as in the alloxazine counterpart. Crystal structures show that N(1) is the usual site for proton location. The computed ΔHf for the N(1) protonated form is 76.72 kJ/mol more negative than for N(5) protonated of 10I form (747.37 and 824.09 kJ/mol). The Hf value for dicationic state is 1801.11 kJ/mol. In MINDO/3 calculations of Hall [1] the enthalpy difference between N(1)

and N(5) protonated forms is about 42 - 50 kJ/mol in several isoalloxazines substituted with methyl groups. The next value for comparison is that of Palmer, Simpson and Platenkamp [10], who computed the largest difference, 167.6 kJ/mol (unoptimized geometry derived from crystal structures). It is also predicted, in this study, that geometry of the protonated

Table 2

Computed partial atomic charges (q) for 10-methylisoalloxazine and its protonated forms

Atom	Molecule			
	q(A)	$\Delta q(10I - 10I1^{+})$	$\Delta q(10I - 10I5^{+})$	$\Delta(10I - 10I1,5^{++})$
C(7)	−0.165	0.046	0.052	0.106
C(6)	−0.033	0.012	−0.037	−0.035
C(5a)	−0.104	0.031	−0.013	0.055
C(9a)	0.082	−0.038	0.049	0.004
C(9)	−0.179	0.021	0.014	0.038
C(8)	−0.063	0.032	0.045	0.084
H-C(7)	0.149	0.033	0.036	0.071
H-C(6)	0.165	0.028	0.008	0.039
H-C(9)	0.149	0.018	0.031	0.052
H-C(8)	0.145	0.033	0.037	0.072
N(5)	0.008	0.060	0.049	0.069
C(4a)	−0.111	0.002	0.093	0.071
C(10a)	0.176	0.064	−0.003	0.074
N(10)	−0.176	0.071	0.024	0.103
C(4)	0.357	0.009	−0.017	0.002
N(3)	−0.395	0.040	0.033	0.070
C(2)	0.370	0.044	−0.009	0.038
N(1)	−0.277	−0.024	0.077	0.002
O(12)	−0.304	0.052	0.104	0.147
O(14)	0.292	0.089	0.015	0.096
H-N(3)	0.263	0.044	0.035	0.078
H-N(1)		0.291		0.318
H-N(5)			0.316	0.348
C-N(10)	−0.092	−0.043	−0.012	−0.058
H_1-C-N(10)	0.091	0.057	0.026	0.078
H_2-C-N(10)	0.146	−0.020	0.017	0.006
H_3-C-N(10)	0.091	0.045	0.029	0.073
	$\sum q_i = 0.0$	$\sum q_i = 1.0$	$\sum q_i = 1.0$	$\sum q_i = 2.0$

forms varies somewhat from corresponding molecules in neutral form. Protonation at N(1) exerts its principal effects on geometry within the bonds N(1)—C(10a)—N(10) (similarly to protonation at N(10) in the case of alloxazine). By contrast the computed changes upon N(5) protonation

are smaller, principally lengthening the C(4)—C(4a) bond. Additionally, N(1) protonated form of 10I tends to be nonplanar, what is consistent with experimental data revealing nonplanarity in riboflavin hydrobromide monohydrate crystal structure [11]. This can be easily explained by repulsive interactions of positively charged methyl group and aromatic protons folding the isoalloxazine ring system along N(5)—N(10) axis.

Comprison with NMR spectra:

Except for NMR data, there is relatively little additional experimental information that bears dependence directly on the electron distribution in flavins. ^{1}H and ^{13}C NMR data for several methyl derivatives of (iso)-alloxazines have been reported by Grande and Müller [12, 13]. The Authors have assigned resonances in the ^{1}H NMR spectra by methods of unambiguous organic syntheses. Their assigments suggest that C(6) has the lowest electron density (811.4 Hz) and C(7) (760.3 Hz) the highest in benzene ring, similarly in the case of alloxazine: C(6) (820.1 Hz) and C(7) (779.6 Hz). Their correlation of NMR frequency for aromatic protons with computed σ and π total partial charges, using the MINDO/2 results of Fox [14], did not give good results. Again, it is well known that ^{13}C resonances reflect the electron density distribution in a molecule in much higher degree than proton resonances. Based on their ^{13}C NMR data Grande and Müller [13] have shown that in the case of 10I, C(8) (136.4 ppm) is the most shielded and positively charged as compared to C(9) (115.8 ppm), which is the most upfield shifted, suggesting a negative charge, whereas C(6) (133.5 ppm) and C(7) (127.2 ppm) are located at relatively lower field. In the case of parent alloxazine molecule the following picture is obtained: C(8) (134.7 ppm) as the most positively charged and C(9) (128.2 ppm) as the most negatively charged benzoide atom, the C(7) 131.0 ppm) and C(6) (129.7 ppm) are intermediately located. So, the discrepancy of ^{13}C and that of ^{1}H NMR data is obvious. It was found by Hall et al. [1] that little correlation exists between ^{13}C resonances and π-electron density calculations. Also our AM1 results can not serve as guidance for ^{13}C resonance signal assigments. However, in the case of alloxazine, we have found that surprisingly better correlation exists between ^{13}C resonances and enthalpy of methylation ΔHf (as difference between Hf of alloxazine substituted with one methyl group and the unsubstituted). The obtained ΔHf for 8-, 7-, 6- and 9-methylalloxazines are: -33.35, -31.88, -26.94 and -29.87 kJ/mol.

These results indicate that relative stabilization energies for substitution with electrondonating methyl group can serve as "probe" test of a non-local character of electron density distributions on particular car-

bon atom. It seems that the response of the electronic system to an excess of negative charge introduced by external perturbation, is more consistent with Karplus-Pople theory of NMR spectra (e.g. because of involvement of non-local terms in screening constance). In the isoalloxazine system, for instance, difference between C(7) and C(8) in methylation enthalpy is more pronounced, C(8) is being even more electronpositive, what is excellently reflected in ^{13}C resonances.

Solvent effects introduce inevitably some perturbations of the ground state. Grande and Müller [12] have studied also the solvent dependence of ^{3}H NMR spectra of (iso)alloxazines. The concentration dependence

Table 3

The influence o] the polarity of the solvent on the resonances (in Hz) of (iso)alloxazines (Table 6 in [12])

% CD_3OD	3,7,8,10-tetramethyl-isoalloxazine (Ring positions)					
	Aromatic proton resonances		Methyl resonances			
	6	9	7	8	3	10
1.2	806.8	743.8	245.6	255.8	352.8	412.6
11.1	806.5	754.0	248.0	258.7	351.8	414.1
29.8	806.1	762.2	249.7	260.7	351.4	416.0
50.0	803.1	769.2	249.2	260.7	350.0	416.3
68.6	798.7	774.0	248.2	259.8	347.7	414.7
88.0	798.2	772.2	248.0	259.5	346.2	413.8
% CD_3OD	1,3,7,8-tetramethyl-alloxazine (Ring positions)					
	Aromatic proton resonances		Methyl resonances			
	6	9	7	8	3	1
1.2	806.4	779.7	250.8	253.4	359.4	380.8
11.1	803.6	782.0	252.1	254.9	358.6	381.6
29.8	801.3	782.7	253.3	255.3	357.6	380.9
50.0	799.5	783.0	254.0	256.8	357.1	380.7
68.6	793.3	777.7	251.8	254.0	354.2	375.7
88.0	792.7	778.2	252.0	254.3	352.7	375.0

was studied in detail employing 3,7,8,10I in CH_3CN over a wide range of concentrations. The results showed that only the resonances due to H-C(6) and H_3C-N(3) are affected by concentration. The signal due to

H-C(6) shifts from 805.5 Hz to 807.5 Hz at infinite dilution, whereas the signal due to H_3C-N(3) shifts from 335.0 Hz to 332.5 Hz. The solvent polarity affects the resonances much more. To illustrate this effect 3,7,8,10I and 1,3,7,8A were investigated using mixtures of $CDCl_3$ and CD_3OD [12]. The results, for our comparison, are given in Table 3.

The data in Table 3. are clearly showing that the influence of the solvent polarity on the aromatic protons C(6) and C(9) is different for the alloxazine and isoalloxazine derivatives. The different behaviour of the H-C(9) resonances of 3,7,8,10I as compared to that of 1,3,7,8A suggests, that the electron density distributions in both molecules are affected in different ways by the polar solvents. Grande and Müller [12] have given such an explanation: first, they noticed that a high electron density is localized on the carbonyl groups. In the case of isoalloxazine, they supposed, this can lead to a shift of electron density from C(9) and C(9a) towards two carbonyl groups and withdrawal of electron density from positions C(6) and C(8). Some decrease of ring current is, in their explanation [12], compensated by some additional delocalization of the N(10) π-electron pair. This would result in a downfield shift of H-C(9) and a upfield shift of H-C(6). As the second possibility, more unlikely, was based on assumption that both a decrease of the ring current and of electron density of C(6) and C(8) should lead to an upfield shift of the resonances due to positions C(7) and C(9) and a downfield shift of the resonances due to positions C(6) and C(8) caused by further electron density reduction at these sites. Therefore, the second interpretation of these Authors was supported by MINDO/2 (unoptimized geometry for neutral form of I) results of Fox [14], who found relatively high excess of electron density at C(9) and C(7), while the C(8) and C(6) atoms being positive. In the case of alloxazine, as they explained [12], the influence of the carbonyl groups is less important than in isoalloxazines.

We propose, basing on already described AM1 results for protonated forms, a new interpretation of data shown in Table 3. At first let us take into account 3,7,8,10I. When going from aprotic solvent (1.2% of methanol) to more polar (say, 50% of methanol) only resonances of H-C(9), H_3C-C(7), H_3C-C(8) and H_3C-N(10) are the most affected but not H-C(6), what indicates, most probably, hydrogen bonding to N(1) atom. From about 50% of methanol concentration strong shift of H-C(6) to higher field takes place (increase in negative charge), further shift of H-C(9) to lower field, shifts of H_3C-N(3) and H_3C-N(10) to higher field, but not further significant shifts of H_3C-C(7) and H_3C-C(8) protons - what indicates hydrogen bonding to N(5) and/or to oxygens of carbonyl groups.

In the case of alloxazine (1,3,7,8A) the picture is quite different: when going from aprotic solvent (1.2% of methanol) to a more polar one (50% of methanol) the significant shifts of H-C(6) and of H_3C-N(3) to higher field can be noticed, for H-C(9) only slight downfield shift, whereas that of H_3C-C(7), and of H_3C-C(8) (but not this of H_3C-N(1)) are significantly shifted to lower field, what clearly indicates hydrogen bonding principally to N(5). From 50 per cent of methanol it can be observed that all protons considered are upfield shifted, what builds up a more complex picture. However, it can be assumed that in a highly polar solvent all heteroatoms of the solute are involved in specyfic interactions. It means that conclusions drawn by Grande and Müller [12], in relation to carbonyl groups, are still valid, but it seems reasonable to assume, that carbonyls play an important role in more polar solvents. Some additional arguments to the above suggestion is supported by ^{13}C NMR spectra of tetra-acetylriboflavin in dioxan/D_2O mixtures. The data [13] showed, that ^{13}C resonances due to atoms C(7), C(8), C(2) and C(4) (for both carbonyls in higher concentration of water) are most affected by polarity of the solvent (all are shifted to lower field). But the Authors [13] did not notice the change of resonance of C(6) which got shifted upfield by about 0.5 ppm when going from 0.0% to 30.0% of water (see Table 3 in [13]).

Our idea is to show the importance of nitrogen heteroatoms in modeling of (iso)alloxazine overall electronic structure. We have theoretically shown, that in highly polar enviroment the electronic systems of alloxazine and isoalloxazine are strongly polarized along longer axes of the molecules. The most important difference between iso- and alloxazine systems is that the alloxazine is more symmetric (near quinoxaline) so the electronic "status" of both pyridine-like nitrogens N(10) and N(5) is very similar, thus C(6)=C(7) and also C(9)=C(8) double bonds are more polarized. On the other hand, isoalloxazine is a less symmetric molecule with a greater difference in electronic density on both N(1) and N(5), thus polarized C(6)=C(7) and (from computations) C(5a)=C(9a) double bonds are obtained.

In terms of valence-bond theory it can be proposed that, in the case of alloxazine system, protonation (or strong hydrogen bonds) enhances the weights of the ortopolar $^{(-)}C(6)-C(7)^{(+)}$ and then $^{(-)}C(9)-C(8)^{(+)}$ VB structures. In the case of isoalloxazine system, due to asymmetry, protonation (or strong hydrogen bonds) enhances the weights of the ortopolar $^{(+)}C(5a)-C(9a)^{(-)}$ or $^{(+)}C(10a)-N(1)^{(-)}$ and then $^{(-)}C(6)-C(7)^{(+)}$ valence-bond structures.

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PROTONOWANIE I ZALEŻNOŚĆ ROZPUSZCZALNIKOWA (IZO)ALLOKSAZYNY W STANIE PODSTAWOWYM. OBLICZENIA ZA POMOCĄ METODY AM1

Streszczenie

Przy pomocy półempirycznej metody AM1 przeprowadzono obliczenia pełnej optymalizacji geometrii alloksazyny, 10-metyloizoalloksazyny oraz ich form protonowanych. Z obliczonych entalpii tworzenia kationów alloksazyny wynika, że protonacja przy N(5) jest o 10,85 kJ/mol mniej korzystna niż protonacja przy N(10). W przypadku 10-metyloizoalloksazyny entalpia tworzenia formy protonowanej przy N(5) jest o 76,72 kJ/mol bardziej dodatnia niż entalpia protonacji przy N(1).

Obliczono zmiany rozkładu gęstości elektronowej w cząsteczkach form protonowanych w stosunku do cząsteczek obojętnych. Z uzyskanych wyników teoretycznych wynika, że efektem protonacji cząsteczki alloksazyny przy N(10) jest, paradoksalnie, wzrost gęstości elektronowej na atomie C(9), natomiast przy protonacji N(5) następuje wzrost gęstości elektronowej na atomie C(6). W przypadku 10-metyloizoalloksazyny, dla formy protonowanej przy N(5), przewiduje się wyraźny efekt wzrostu gęstości elektronowej na C(6), natomiast przy protonacji N(1) następuje niewielki wzrost gęstości elektronowej na C(9a). Otrzymane wyniki porównano z doświadczalnymi widmami ^{1}H NMR 1,3,7,8-tetrametyloalloksazyny i 3,7,8,10-tetrametyloizoalloksazyny zmierzonymi w serii roztworów mieszaniny $CD_3OD/CDCl_3$ oraz doświadczalnymi widmami ^{13}C NMR tetraacetyloryboflawiny zmierzonymi w serii roztworów mieszaniny D_2O/dioksan. Uzyskano dobrą zgodność przewidywanych zmian gęstości elektronowej na atomach węgla w układach protonowanych (izo)alloksazyn z obserwowanymi wielkościami przesunięć chemicznych NMR w rozpuszczalnikach protonodonorowych.

Zaproponowano nową interpretację danych doświadczalnych NMR, dotyczących efektów rozpuszczalnikowych, uwzględniającą udział pirydyno-podobnych atomów azotu w tworzeniu wiązań wodorowych. Przewidywany teoretycznie i obserwowany doświadczalnie efekt polaryzacji wiązań podwójnych C(6)=C(7) i C(8)=C(9) w układzie alloksazynowym i polaryzacji wiązania C(6)=C(7) w układzie izoalloksazynowym zinterpretowano jako wzrost populacji odpowiednich ortopolarnych struktur walencyjnych. Podkreśla się, że podstawową różnicą między układem izo- i alloksazynowym jest odmienny rozkład gęstości elektronowej na pirydyno-podobnych atomach azotu, gdzie znacznie większą asymetrią charakteryzuje się cząsteczka izoalloksazyny.

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ПРОТОНИРОВАНИЕ И РАСТВОРЯЕМАЯ ЗАВИСИМОСТЬ /ИЗО/АЛЛОКСАЗИНА В НАЧАЛЬНОМ СОСТОЯНИИ. ВЫЧИСЛЕНИЯ С ПОМОЩЬЮ МЕТОДА AM1

Содержание

С помощью полуэмпирического метода AM1 проведено подсчёты полной оптималиэации геометрии аллоксазина, IO-метилизоаллоксазина а также их протонированных форм. Из подсчитанных энталпий образованния катионов аллоксазина вытекает, что протонация при N(5) на 10,85 кД/моль менее выгодна чем протонация при N(10). В случае 10-метилизоаллоксазина энталпия образования протонированной формы при N(5) на 76,72 кД/моль более положительна чем энталпия протонации при N(1).

Были подсчитаны изменения разложения электронной плотности в молекулах протонированных форм по отношению к нейтральным молекулам. Из полученных теоретических результатов вытекает, что эффектом протонации молекул аллоксазина при N(10) является, парадоксально, увеличение электронной плотности на атоме C(9), а при протонации N(5) происходит увеличение электронной плотности на атоме C(6). В случае 10-метилизоаллоксазина для протонированной формы при N(5) предвидится отчёгливый эффект роста электронной плотности на C(6), а при протонации N(1) происходит небольшой рост электронной плотности на C(9a). Полученные результаты сравнили о экспериментальным спектрами ^{1}H NMR 1,3,7,8-тетраметилоаллоксазина и 3,7, 8,10-тетраметилизоаллоксазина измеренными в серии растворов смеси $CD_3OD/CDCl_3$ а также экспериментальными спектрами ^{13}C NMR тетраацетилорибофлавины измеренными в серии растворов смеси D_2O/диоксан. Была получена хорошая совместность предвидимых изменений электронной плотности на атомах углерода в протонированных системах (изо)аллоксазин с наблюдаемыми величинами химических смещений NMR в протонодонорных растворителях.

Была предложена новая интерпретация экспериментальных данных NMR относящихся к растворяемым эффектам учитывающая действие пиридино-сходных атомов азота в создавании водородных связей. Теоретически предвидимый и экспериментально наблюдаемый эффект поляризации двойных связей C(6)=C(7) и C(8)=C(9) в аллоксазиновой системе и поляризации связи C(6)=C(7) в изоаллоксазиновой системе был истолкован как рост популяции соответствующи ортополярных валенционных структур. Подчёркивается, что основным отличием между изо- и аллоксазиновой системой является разное распределение электронной плотности на пиридино-сходных атомах азота, где значительно большей асимметрией характеризуется молекула изоаллоксазина.