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STUDIES ON ESTERIFICATION AND SULFONATION OF RIBOFLAVIN *VIA* SEMI-EMPIRICAL METHODS

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Abstract: The article presents extended computer investigations of various sulfate derivatives of riboflavin. A number of physicochemical parameters such as total energy, binding energy and formation heat were calculated *via* semi-empirical methods AM1 and PM3 for the different derivatives of riboflavin. Their analysis made it possible to determine the sequence of formation of sulfate derivatives - esterification is the easiest at hydroxyl groups at the farthest positions from the ring. This methodology may be used to study biologically active compounds.

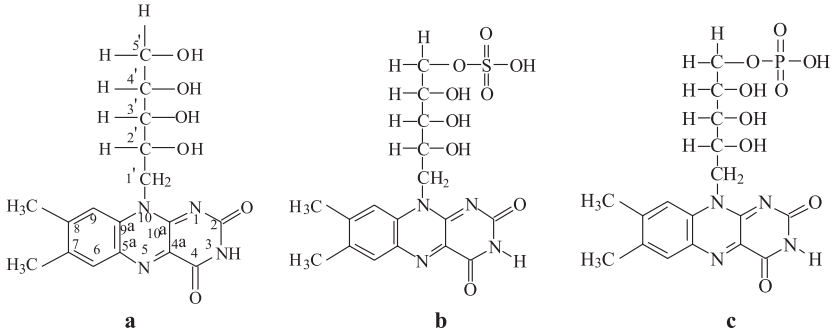
Key words: riboflavin, sulfate derivatives of riboflavin

In the course of our earlier experimental investigations we have studied the reactions of formation of esters and sulfate derivatives of riboflavin (1-3). These compounds were formed in the environment of sulfuric acid and the analysis of products was based mainly on TLC, HPLC and ^{1}H NMR methods. Using these methods both the nature of the products and the kinetics of their formation were studied. UV/VIS spectroscopy was not employed because the electron spectra of riboflavin esters are practically identical with those of riboflavin (4, 5).

Riboflavin (vitamin B_2) - Figure 1a, is a well-known compound of the vitamin group whose chemical and biological properties have been studied and described extensively (6, 7).

From the chemical point of view, this compound may undergo many chemical reactions, especially esterification. Both inorganic and organic acids may take part in these reactions. So far, apart from sulfate derivatives, butyric, palmitic and acetic acid esters have been obtained (6). These derivatives were obtained in order to improve riboflavin solubility in fats and to facilitate storage of its derivatives in the organism. These anticipations have not always been justified, for example, riboflavin tetrabutyrate is unstable and after quick degradation in the organism yields free riboflavin.

The most important studies on sulfate esters of riboflavin date back to 1970's and 1980's (8, 9) and are concerned mainly with riboflavin 5'-monosul-



a **b** **c**

Figure 1. Structural formulas of: **a**. riboflavin, **b**. riboflavin 5'-monosulfate (FMS), **c**. flavin mononucleotide (FMN)

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fate known in the literature as FMS (10) – Figure 1b. This ester is the main product of riboflavin sulfation in non-aqueous conditions with chlorosulfonic acid at 40°C. It was isolated as the calcium salt (8, 11).

FSM was considered interesting because this compound is a sulfated analogue of flavine mononucleotide (FMN) - Figure 1c, that acts as a coenzyme in oxidation processes of many amino acids, amines and carboxylic acids (12).

Contrary to FMN, riboflavin 5'-monosulfate inhibits the activity of D-amino acid oxidase (13). In biological studies, the effects of FMS upon higher organisms have been investigated. The results of this compound administration to mammals: the storage place in various organs, clearance path and rate as well as toxicity has been studied. In general terms, its activity is antagonistic to riboflavin (14). It has also been found that apart from the main mono-derivative, higher: di-, tri- and tetraesters are obtained. These derivatives may have various structures because the reaction may take place at four different hydroxyl groups of the ribityl side chain of the molecule. These groups undergo esterification in sulfuric acid only in the solutions of sufficiently high acidity. It begins practical when the solutions acidity on the Hammett scale is sufficiently high (H_o below -1) which corresponds to sulfuric acid concentrations not lower than 20% (w/w) (2, 3).

In the environment of the highest possible activity (H_0 ca. -9, which corresponds to ca. 95% sulfuric acid) the reaction of ring sulfonation at 6 and 9 positions may also occur. We have already analyzed this problem by ^{1}H NMR spectroscopy. The changes in the intensity of proton signals in the ring at the positions 6 and 9 were registered. Their location has been described in the paper by Kasai (9). For riboflavin, the chemical shifts of δ signals (ppm) for these two protons are 7.90 and 7.88, respectively, and that of the NH proton in the ring is 11.34 (spectra in DMSO-δ_6). The ratio of intensities of these signals is 2 : 1, which is the consequence of the number of these protons in the molecule.

In the course of spectroscopic investigations of the reaction products in the environment of the highest acidity we noticed that this ratio is much lower. This suggests that the molecule after the side chain esterification undergoes the ring sulfonation. The confirmation of this are changes in the UV/VIS spectrum. The spectrum of riboflavin in the aqueous solution consists of four strong bands whose maxima λ_{max} are: 223, 267, 375 and 445 nm (4). In the spectrum of the product, however, the bands change their location, which reflects the visible color change of the solution from yellow to red. Such changes may only result from the change in the chromophore group of the molecule which is the isoalloxazine ring. Sulfonation in the ring is the reason for these changes and in consequence for changes in the UV/VIS absorption spectrum.

Further investigations of esterification products of riboflavin were completed with TLC experiments (1). Silanized silica-gel $60F_{245}$ was used as stationary phase. The mobile phase consisted of benzyl alcohol, water and ethanol (3 : 2 : 1, v/v/v).

After development, the chromatogram consisted of a few groups of spots. R_f values for this chromatogram were within the following ranges: 0.44–0.58; 0.25–0.35 and 0.14–0.16. The last spot had R_f = 0.07. It must be added that for riboflavin itself R_f = 0.69. This value is significantly different than those for the analyzed products.

It should be underlined that particular group of spots have appeared on the chromatograms subsequently along with the increase in the acidity of solutions and the reaction time. It is obvious that mono derivatives appear first and higher derivatives follow until the fully esterified tetra-form is obtained. It is clear that in fact a complicated mixture of many derivatives is analyzed. Studies of esterification kinetics were carried out with HPLC (2, 3).

In the process of chromatograms interpretation, the first group of spots was linked to mono derivatives that appeared first. Four compounds are possible as the result of esterification of 2', 3'or 4' hydroxyl group in the ribityl side chain.

Similarly, the second group of spots corresponds to diester derivatives (6 possible compounds), the third one to tri derivatives (4 derivatives). The last spot (R_f = 0.07) corresponds to the single tetra derivative.

So far, no detailed data have been available for the order of the chain hydroxyl group esterification apart from the information that the esterification begins at 5'-hydroxyl group of the ribityl side chain (8). The reason for this is probably the experimental difficulty of such studies.

In our latest theoretical-experimental studies, we have employed the computational semi-empirical methods to analyze the acid-base properties of biologically active substances: flavonoids (15–17) and riboflavin (18). Calculations were made with HyperChem 7.0 (19). We analyzed the order and place of dissociation of subsequent protons from the neutral molecule. Similar analysis was performed for the cationic forms obtained in the acidic environment. With the positive experience from the analysis of these problems in mind, we applied the

same procedure to the analysis of the order of formation of riboflavin sulfate esters.

EXPERIMENTAL

Several physicochemical parameters of riboflavin and its all possible sulfate esters were calculated with HyperChem 7.0 (HyperCube Inc.). The geometry of analyzed molecules was optimized by two methods: AM1 and PM3 (20, 21).

AM1 (Austin Model 1) is a semi-empirical method of quantum chemistry. It is based on the Neglect of Differential Diatomic Overlap integral approximation (NDDO) and enables calculation of molecular electronic structure. This method was developed by M.J.S. Dewar and co-workers and was published in 1985 (22). The PM3 (Parameterized Model number 3) was developed by J.J.P. Stewart (23, 24) and is similar to the AM1 method.

AM1 takes some of parameter values from spectroscopic measurements, while PM3 treats them as utilizable values. In these methods, geometry optimization is carried out by using a conjugate gradient method (Polak-Ribiere algorithm). In our calculations, the SCF (self-consistent-field) convergence limit was set to 0.0001 kcal/mol and the RMS was set to 0.001 kcal/(Å mol).

Parameters employed in the analysis were: total energy, binding energy and heat of formation. The structures for which these parameters reached the lowest values after the optimization were assumed to be most likely.

RESULTS AND DISCUSSION

Semi-empirical calculations were made for riboflavin and its all possible esters from the mono to the tetra form. The values of energies calculated by AM1 and PM3 methods are presented in Tables 1 and 2.

Following the analysis of data in Table 1 it may be concluded that the minimum values of total energy, binding energy and heat of formation are calculated by the AM1 method for the 4'-monoester. Similar sequence of these values is obtained by the PM3 method – Table 2. Analysis of the results shows that similar values of total energy, binding energy and heat of formation are received for the 5'-monoester. The maximum values refer to 2'- and 3'-monoesters. In particular, the 2'-form reaches the highest values in all cases. These results prove that hydroxyl groups at the farthest positions from the isoalloxazine ring of the molecule of riboflavin are esterified first. This order is confirmed by the analysis of diester forms. In both methods the forms of the lowest energy are 4',5'-diesters. The highest energies are obtained for the compounds esterified at groups close to the ring - 2',3'-diesters. It is particu-

Table 1. Physicochemical parameters of riboflavin esters calculated by AM1 method (the lowest values for each form are given in italics).

Form	Total energy (kcal/mol)	Binding energy (kcal/mol)	Heat of formation (kcal/mol)
Riboflavin	-119971.08	-4942.26	-185.74
2'-monoester	-146541.19	-5222.09	-220.49
3'- monoester	-146543.12	-5224.02	-222.42
4'-monoester	***-146635.45***	***-5316.35***	***-314.75***
5'-*monoester*	-146629.36	-5310.26	-308.66
2',3'-diester	-173251.68	-5642.30	-395.63
2',4'-diester	-173233.33	-5623.95	-377.27
2',5'-diester	-173226.02	-5616.64	-369.97
3',4'-diester	-173287.90	-5678.52	-431.84
3',5'-diester	-173276.88	-5667.51	-420.83
4',5'-diester	***-173296.52***	***-5687.15***	***-440.47***
2',3',4'-triester	-199881,94	-5982.28	-490.53
2',3',5'-triester	-199898,73	-5999.08	-507.32
2',4'5'-triester	-199882,33	-5982.68	-490.92
3',4',5'-triester	***-199957.22***	***-6057.56***	***-565.81***
2',3',4',5'-tetraester	-226539.20	-6349.27	-612.44

Table 2. Physicochemical parameters of riboflavin esters calculated by PM3 method (the lowest values for each form are given in italics).

Form	Total energy (kcal/mol)	Binding energy (kcal/mol)	Heat of formation (kcal/mol)
Riboflavin	-109174.65	-4964.80	-208.28
2'-monoester	-133719.08	-5261.05	-259.45
3'-monoester	-133791.92	-5333.90	-332.29
4'-monoester	***-133794.90***	***-5336.88***	***-335.27***
5'-monoester	-133793.83	-5335.80	-334.20
2',3'-diester	-158366.24	-5660.04	-413.36
2',4'-diester	-158415.34	-5709.13	-462.45
2',5'-diester	-158411.07	-5704.87	-458.19
3',4'-diester	-158409.15	-5702.94	-456.26
3',5'-diester	-158410.94	-5704.74	-458.06
4',5'-diester	***-158417.59***	***-5711.38***	***-464.70***
2',3',4'-triester	-182981.29	-6026.91	-535.15
2',3',5'-triester	-182970.24	-6015.85	-524.10
2',4',5'-triester	***-183032.81***	***-6078.41***	***-586.66***
3',4',5'-triester	-183027.36	-6072.97	-581.22
2',3',4',5'-tetraester	-207601.80	-6399.23	-622.40

Table 3. Results of calculations of physicochemical parameters of the tetra ester by AM1 method (the lowest values for each form are given in italics).

Form	Total energy (kcal/mol)	Binding energy (kcal/mol)	Heat of formation (kcal/mol)
6-Monosulfone tetraester	***-253201.31***	***-6721.10***	***-739.19***
9-Monosulfone tetraester	-253186.60	-6706.38	-724.48
6,9-Disulfone tetraester	-279822.67	-7052.18	-825.19

Table 4. Results of calculations of physicochemical parameters of sulfonated derivatives of riboflavin tetra ester by PM3 method (the lowest values for each form are given in italics).

Form	Total energy (kcal/mol)	Binding energy (kcal/mol)	Heat of formation (kcal/mol)
6-Monosulfone tetraestrer	***-232200.89***	***-6750.14***	***-768.23***
9-Monosulfone tetraestrer	-232197.80	-6747.05	-765.14
6,9-Disulfone tetraestrer	-256793.40	-7094.47	-867.48

larly visible for the results calculated by the PM3 method where the values of particular energies for the 2',3'-diester are significantly higher. This suggests again that esterification of these hydroxyl groups is most difficult.

The results of calculations for triesters are not entirely consistent. For the AM1 method the lowest energies are calculated for the 3',4',5'-triester form. For the PM3 method, the lowest values are received for the 2',4',5'-triester. It must me noted, however, that for this method the calculated energies are very close to those for the 3',4',5'-triester. Taking into account that for the AM1 method results calculated for the 2',4',5'-triester are clearly lower than the

energies for other possible forms it should be concluded that this is the most energetically favorable triester form.

The results of calculations are generally consistent and make it possible to draw a conclusion that esterification is easiest at hydroxyl groups at the farthest positions from the ring. These conclusions are consistent with the experimental results published by Yagi (8), who, in the conditions studied in his work, received the 5' monoester first although it was possible to obtain other forms as well. These appeared with more difficulty, later and in smaller quantities. Sulfonated forms are obtained in the environment of the highest acidity, practically after the full esterification of riboflavin. These derivatives are connected with the tetraester form. Results presented in Tables 3 and 4 show that more like is the form where sulfonation takes place at C6 position in the ring. Both in the AM1 and PM3 methods the energy values are clearly lower than those for sulfonation at C9 position in the ring. In fact sulfonation can take place at both positions but the reaction yield would be higher for the 6-monosulfate form.

It may be concluded that the computational methods of quantum chemistry offered by HyperChem are useful tools for the analysis of the direction of multi-step reactions. In the situation when the experimental analysis of a compound is difficult, theoretical calculations may give valuable data on its reactivity. We have already encountered such problems during the research on acid-base properties of riboflavin. Using AM1 and PM3 methods we could study the possible cationic forms of riboflavin and draw conclusions regarding the order of proton addition to the neutral molecule. Similar calculations were made for various anionic forms to find out about the order of their formation in the basic environment (18). The potential of experimental methods is significantly broadened and completed.

In this study, we employed the above mentioned methods to solve similar problems concerned with the order of substitution – in this case of $-SO_3H$ groups, to the neutral form of the compound. The results made it possible to draw conclusions regarding the reactivity of riboflavin in sulfuric acid, especially with respect to the formation of its esters and sulfonated forms.

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