SYNTHESIS OF DYNAMIC RIBOFLAVIN DERIVATIVES AND THE STUDY OF THEIR ABILITY TO UREASE PHOTOINACTIVATION

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Riboflavin or vitamin B2 refers to the flavin group. Its special feature is involved in many biological processes, as well as a high photodynamic activity [1]. Riboflavin in native form is widely used for photodynamic sterilization of blood products [2]. Additionally, a number references shows that riboflavin demonstrated the ability to inactivate toxins and enzymes [3].

In one patents demonstrated the riboflavin ability to inactivate vaccine strains of viruses and bacteria, as well as cancer cells during the production of vaccines [4]. DNA and RNA within living organisms have specific aptamers or flavin places where Riboflavin and its derivatives are able to selectively join [5]. Upon further blue light irradiation 440-450 nm or ultraviolet light at 280-350 nm such complexes between nucleic acids and riboflavin observed selective nucleolysis for nucleic acids in the field of riboflavin attachment [6].

In fact, light with riboflavin combination behaves as selective photo-nuclease [7]. This property of riboflavin can be successfully used to replace toxic formalin and propiolactone in vaccine / antitoxins production [8]. The main disadvantage of native riboflavin is its low solubility in almost all solvents [9]. Maximum solubility in acidified water is 0.2%, whereas the dosage forms of riboflavin in the form of eye drops containing 0.02% riboflavin. Although the sale is sometimes found injectable form of 1% riboflavin mononucleotide. This drug is the riboflavin phosphate ester sodium salt. A disadvantage of this compound is a lower sensitivity to visible light than riboflavin [10]. As the model toxins for photo-inactivation we was chosen urease. Urease inactivation under riboflavin derivatives leads to irreversible blockade of its ability to urea degrade [11].

Accordingly, the research aim was to synthesize a series of riboflavin derivatives, including dynamic derivative having greater photodynamic activity (higher sensitivity to light) in the visible region spectrum at lower concentrations.

Materials and methods

Used for the synthesis: riboflavin (Fluka, Austria), succinic anhydride (Sigma, USA), maleic anhydride (Azot, Ukraine). For HPLC analysis were used: acetonitrile (Sigma, USA) and lithium perchlorate, perchloric acid in a kit for chromatograph BD2003 (Econova, RF). For fixing the UV / spectrum of the synthesized compounds was used spectrophotometer Gene-quant - 1300 (General Electric, USA). To analyze riboflavin succinyl-dynamic systems was used HPLC- system Milichrome A-02 (Econova, RF). To study the ability of riboflavin derivatives for urease's photo-inactivation reagents were used: test-system Urease-U (Ukrmedsnab, Ukraine) and spectrophotometer Gene-quant – 1300 for fixing end of reaction.

In addition, we used a set of chromatography conditions for analysis of riboflavin derivatives: gradient separation of acetonitrile (from 0% to 100%) / 0.05 M lithium perchlorate buffer + 0.01 M perchloric acid at 40 ^{0} C, and fraction detection in UV region. As a comparison, we used substance (I).

Synthesis of dynamic combinatorial riboflavin derivative
A dynamic self-organizing system based on riboflavin was designed with TRIZ techniques and used to create a variety of other dynamic structures with high biological activity [12]. For dynamic structures in the initial compound (like (I)) must be present for several similar substitution (in our case - acylation) groups (n=4). In the structure of the carbohydrate moiety of riboflavin available four hydroxyl groups for modification. Binary combinatorial modification of the structure (I) at the correct calculation according to the formulas F1-F3 leads to the formation of 44 compounds which not only react with the target, but also able to interact with each other, as indicated by a single band on the chromatogram.

Synthesis of combinatorial derivative (IV). Initially, the number of available modifying groups into the riboflavin's structure n = 4 (Formulas F1 and F2). Dynamic derivative (IV) is synthesized by adding to (m) moles of riboflavin (in the riboflavin structure are available four groups for substitution m = 44) calculated by the combinatorial formulas (above) (k) moles of the modifier (II) and (III) (k for n = 4 was 60) in a formic acid concentrated solution. The solution was stirred at room temperature until complete riboflavin dissolution (I), succinic anhydride (II), maleic anhydride (III). The solution was then heated on a steam bath for 40 minutes. The solution was cooled to 15 ^{0} C. The precipitate (IV) was separated by centrifugation, washed with acetonitrile and dried. Recrystallization (IV) from a water- formic acid mixture under temperature 15 ^{0} C was conducted. The result of the synthesis is a dynamic combinatorial self-assembled structure comprising 44 different derivatives with different combinations and variations by substituent groups. The resulting derivative (IV) is easily dissolved in phosphate buffered saline to a concentration of 2%. UV/Vis spectrum of (IV) is shown in Fig. 1. HPLC-chromatogram shown in Fig. 2.

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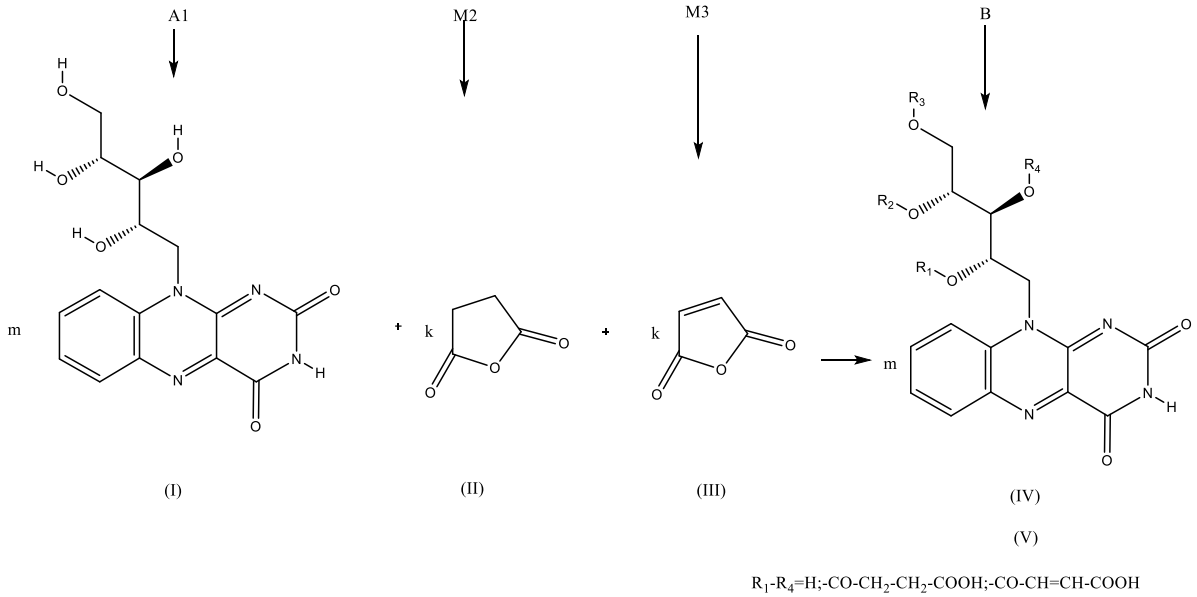

$$(F1) \qquad m\,A1 + k\,M2 + k\,M3 = m\,B$$

$$(F2) \qquad k = n*(2^n - 1) = 4*(2^4 - 1) = 60$$

$$(F3) \qquad m = 4*(3*2^{n-2} - 1) = 44$$

Figure 1. Synthesis scheme of the dynamic riboflavin (IV) and the formula for calculating the combinatorial synthesis (fully substituted derivative thereof, where m = 1, k = 4, only one modifier (II) is tetrasuccinyl-riboflavin (V).

Synthesis of combinatorial derivative (V). Full modified derivative (V) is synthesized by adding to (m) moles of riboflavin (in the riboflavin structure are available four groups for substitution) calculated by the combinatorial formulas 4 moles of the succinic anhydride (II) in a formic acid concentrated solution. The solution stirred at room temperature until complete riboflavin dissolution (I), succinic anhydride (II). The solution then heated on a steam bath for 40 minutes. The solution was cooled to 15 ^{0} C. The precipitate (V) separated by centrifugation, washed with acetonitrile and dried. Recrystallization (V) from a water- formic acid mixture under temperature 15 ^{0} C was conducted. The result of the synthesis is individual full substituted derivative (V) – tetra-succinylated riboflavin. The resulting derivative (V) easily dissolved in phosphate buffered saline like (IV) to a concentration of 2%.

UV / Vis spectrum of (IV) is shown in Fig. 3. HPLC-chromatogram shown in Figure 2.

The biological activity of the riboflavin derivatives in their ability to inactivate urease under visible light

The study used urease from the standard urea test-system for Urea-U (Ukrmedsnab, Ukraine). The initial enzyme activity was 5000 U/ml. Standard urease solution before adding to the reaction medium was diluted with distilled water to 1000 times. As a substrate was used a 0.2% aqueous solution of urea. The reaction temperature was 37 ° C, incubation time - 10 minutes. Urease activity determined by color reaction with a hypochlorite reagent (Nessler reactive). Photometry of samples was performed on Gene-Quant -1300 spectrophotometer at a wavelength of 590 nm.

We prepared (I), (IV), (V) dilution in concentrations of 0.1 to 10 μg / ml.

Pre-prepared two-fold dilutions (I), (IV), (V) in a 96-well plate was left in the well with 100 μL solution of appropriate dilution (I), (IV), (V), was added 100 μL 5 U/ml urease solution. All solutions were irradiated with 450 nm and 10 W/cm^2 blue light using dental polymerizer for 3 minutes per well. Then was determined the ability of irradiated urease to degrade urea by the final reaction product - ammonia with Nessler's reagent according to the test system instructions . The color intensity is proportional to the released ammonia concentration. Table 1 shows a relative activity falling in % against non-inactivating control. Research duplicated 5 times, the data processed using the variation statistics, namely the Student's t test.

Results and discussion

Riboflavin poor solubility causes difficulty of its application for vaccine production at a high concentration

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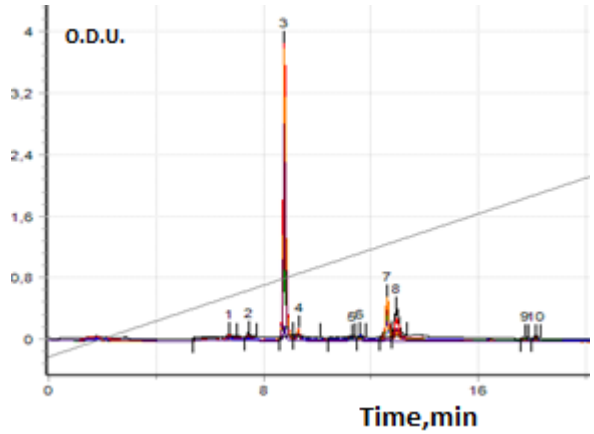
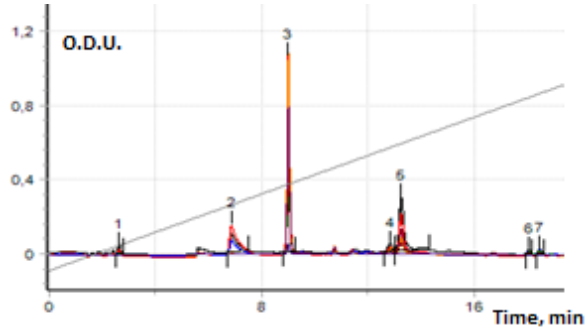
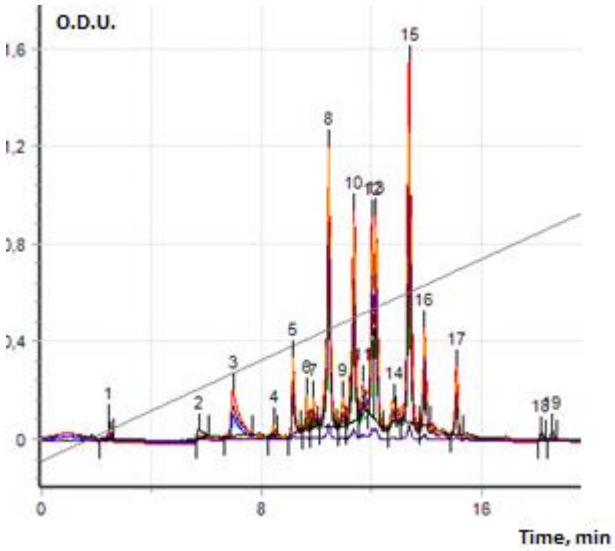
of bacteria in the reactor. Modified riboflavin really better dissolved up to a 2% concentration and allowed to use it as photo-inactivator of bacteria. The use of riboflavin for viruses and bacteria photoinactivation are relatively well studied, whereas about toxins inactivation are only single publication. A feature of our proposed synthesis is the preparation of 44 riboflavin combinatorial derivatives in a dynamic mixtures. In fact, this derivative (IV) is succinylated at the carbohydrate residue. The large number of derivatives with different positions of a succinic acid residues allows penetrate globule protein in various ways and modified protein within. We are used urease as a microbial toxin model. Most microbial toxins, like urease, also are enzymes [13].



1- riboflavin (I) 0,02 %



2- tetrasuccinylated riboflavin (V) 0,02%



3- dynamic riboflavin (IV)

Figure 2. HPLC- chromatogram riboflavin (I), dynamic riboflavin (IV),] tetrasuccinylriboflavin (V)

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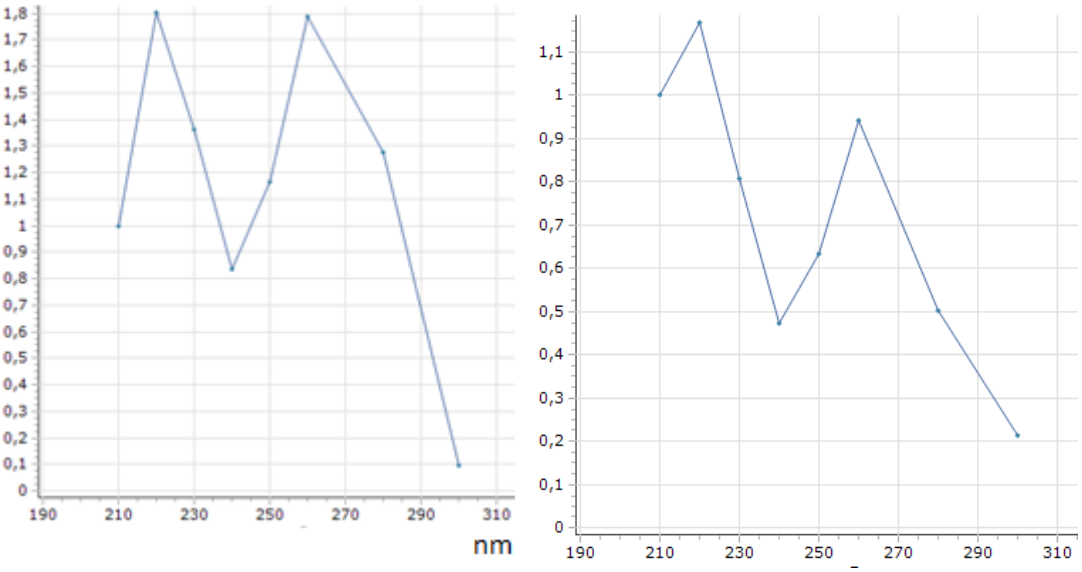
As can be seen from Fig. 1 with comparative chromatograms, riboflavin after acylation peak shifts to heavier derivatives. The derivative (IV) consists of 44 compounds, and they are all similar in weight and form many absorption peaks (peak 2-17). Flavin heterocycle structure itself does not change, therefore UV spectrum (IV) from (I) differs not significantly - spectral ratios very close to the native riboflavin (Fig. 3). Differences observed in the absorption region 260-280 nm, showing little change coupling system in secondary heterocycle and the supporting molecular structure change.



1- riboflavin (I) 0,02 % (peak 3) 2- acylated riboflavin (IV) 0,02% (peak 12)

Figure 3. UV spectra of riboflavin (I) and dynamic acylated riboflavin (IV)

The main difference of (I) from (IV) is much higher water solubility (up to 2%) for (IV). To compare the efficiency photoinactivation by riboflavin (I) and dynamic riboflavin (IV) was studied photoinactivation efficiency of urease as a model of microbial toxin and its transformation into a toxoid. The photoinactivators effectivity criterion is the ability to inactivate urease by disrupt performance of the active center of the enzyme. In our case - to slow down or completely block the urease ability to catalyze the hydrolysis of urea.

Figure 4 shows the 2x dilutions of both substances which inactivate urease.

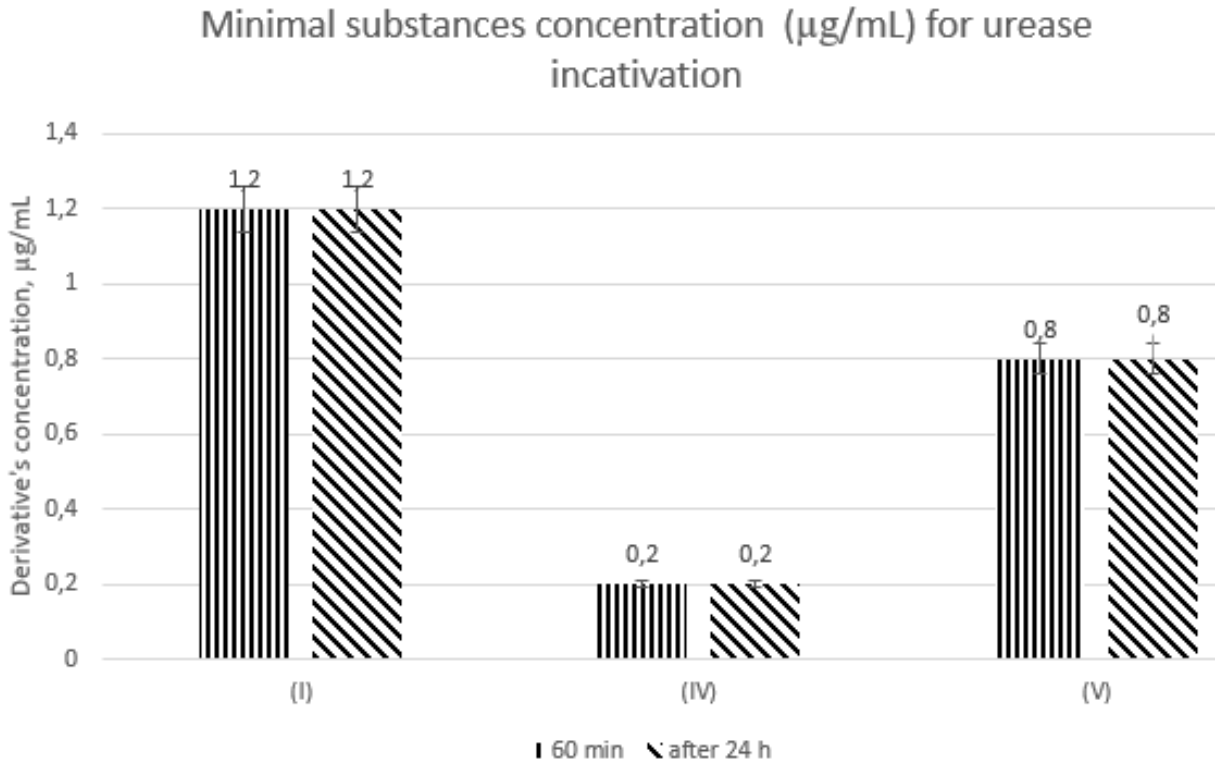


Figure 4. Biological Activity of riboflavin Derivatives at ability for urease's photoinactivation

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As seen in Fig. 4, the least active succinylated turned derivative (V), that was fully succinylated by available hydroxyls alcohol residues and initial riboflavin (I). Under used (V), within 60 minutes after the dissolution of its minimum effective concentration was 0,8 ± 0.1 μg/mL, while after day was not observed degradation (hydrolysis) (V) and its activity was not changed after 24 h. Dynamic derivative (IV) with an immediate use along 60 minutes showed activity at a concentration 0.2 ± 0.05 μg/mL and after 24 hours also not changed. The starting riboflavin (I) was also active, regardless to storage time and they active concentration in solution was 1.2 ± 0.2 μg/mL. The effective concentration statistically differed between compounds (I), (IV) and (V) at p≤ 0,05.

Conclusion

1. The first compound obtained is dynamic combinatorial - maleylated / succinylated riboflavin (IV).
2. The synthesized compound (IV) and (V) retain photodynamic activity. The minimum effective concentration of the compound (IV) with an immediate application amounted to 0.2 ± 0.05 μg/mL, whereas for the (V) this value was 0.8 ± 0.1 μg/mL.
3. Active dynamic structure (IV) and even exceed the original riboflavin activity at immediate use after dissolution.
4. Riboflavin (I) active concentration is not dependent on the storage time of the aqueous solution and was 1.2 ± 0.2 μg/mL.

Abstract

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Introduction. In one patents demonstrated the riboflavin ability to inactivate vaccine strains of viruses and bacteria, as well as cancer cells during the production of vaccines . DNA and RNA within living organisms have specific aptamers or flavin places where Riboflavin and its derivatives are able to selectively join . Upon further blue light irradiation 440-450 nm or ultraviolet light at 280-350 nm such complexes between nucleic acids and riboflavin observed selective nucleolysis nucleic acids in the field of riboflavin attachment .Research aim was to synthesize a series of riboflavin derivatives, including dynamic derivative having greater photodynamic activity (higher sensitivity to light) in the visible region spectrum at lower concentrations. **Materials and methods**. For gradient HPLC analysis were used: acetonitrile and lithium perchlorate, perchloric acid in a kit for chromatograph BD2003. For fixing the UV / spectrum of the synthesized compounds was used spectrophotometer Gene-quant - 1300. To analyze riboflavin succinyl-dynamic systems was used HPLC- system Milichrome A-02. To study the ability of riboflavin derivatives for urease's photo-inactivation reagents were used: test-system Urease-U and spectrophotometer Gene-quant – 1300 for fixing end of reaction. In addition, we used a set of chromatography conditions for analysis of riboflavin derivatives: gradient separation of acetonitrile (from 0% to 100%) / 0.05 M lithium perchlorate buffer + 0.01 M perchloric acid at 40 ^{0} C, and fraction detection in UV region. As a comparison, we used substance riboflavin (I). **Results and discussion**. The main difference of (I) from dynamic riboflavin (IV) is much higher water solubility (up to 2%) for (IV). To compare the efficiency photoinactivation by riboflavin (I) and dynamic riboflavin (IV) was studied photoinactivation efficiency of urease as a model of microbial toxin and its transformation into a toxoid. The photoinactivators effectivity criterion is the ability to inactivate urease by disrupt performance of the active center of the enzyme. In our case - to slow down or completely block the urease ability to catalyze the hydrolysis of urea. Full succinylated derivative (V) acts in minimum effective concentration 0,8 ± 0.1 μg/mL. Dynamic derivative (IV) with an immediate showed activity at a concentration 0.2 ± 0.05 μg/ml and after 24 hours also not changed. The starting riboflavin (I) was very active, regardless to storage time and they active concentration in solution was 1.2 ± 0.2 μg/mL. The effective concentration statistically differed between compounds (I), (IV) and (V) at p≤ 0,05. **Conclusion.** The first compound obtained is combinatorial - maleylated / succinylated riboflavin (IV). The synthesized compound (IV) and (V) retain photodynamic activity. The minimum effective concentration of the compound (IV) with an immediate application amounted to 0.2 ± 0.05 μg/mL, whereas for the (V) this value was 0.8 ± 0.1 μg/mL. Active dynamic structure (IV) and even exceed the original riboflavin activity at immediate use after dissolution, it is still unstable and gradually hydrolyzed. The most stable activity possessed source riboflavin (I), its active concentration is not dependent on the storage time of the aqueous solution and was 1.2 ± 0.2 μg/mL.

Keywords: riboflavin, photodynamic, vaccine, urease, dynamic combinatorial derivatives

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