SYNTHESIS AND MASS-SPECTRAL STUDY OF THE LUCIFERIN OF Luciola mingrelica

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By "luciferins" are understood various compounds produced by bacteria, fungi, invertebrates, and fish which are capable of luminescing on enzymatic oxidation [1, 2]. From the American glow worm Photinus pyralis a luciferin has been isolated with the structure of D-(−)-2-(6-hydroxybenzothiazol-2-yl)-3,4-dihydrothiazole-4-carboxylic acid (I) [2]. This substance is one of the few natural compounds containing a D-cysteine fragment (in the form of a thiazoline ring) [3], the L form usually being found in biological materials. Neither L- nor DL-luciferins luminesce on reaction with luciferase. For bioluminescence, in addition to oxygen and luciferase, the presence of magnesium ions and of adenosine triphosphate (ATP) is necessary. The intensity of luminescence is directly proportional to the concentration of ATP (during the first 10 sec) [5, 6]. The latter circumstance is used for the quantitative determination of ATP [6].

We have attempted to apply this method to the measurement of the level of ATP in the process of photosynthetic phosphorlyation in pea chloroplasts by making use of the luciferin–luciferase system of Luciola mingrelica (Caucasian glow worm). However, a rough homogenate gave a high background luminescence because of the presence in it of adenylate kinase, which is capable of converting ADP into ATP. Consequently, by means of molecular sieves, we separated a tris-acetate (pH 7.8) extract of the glow worm into individual protein fractions which enabled the adenylate kinase to be eliminated to a certain extent.

It can be seen from Fig. 1 that the adenylate kinase is most active in the first ten fractions, and the maximum activity of the luciferase is found in fractions 15 and 16, where adenylate kinase is already practically absent. However, in the purification of the luciferase a loss of luciferin is unavoidable. Thus, the necessity arose for the synthetic production of luciferin, since the isolation of the pure substance is associated with experimental difficulties and with the necessity for the consumption of large amounts of biological material [2, 7].

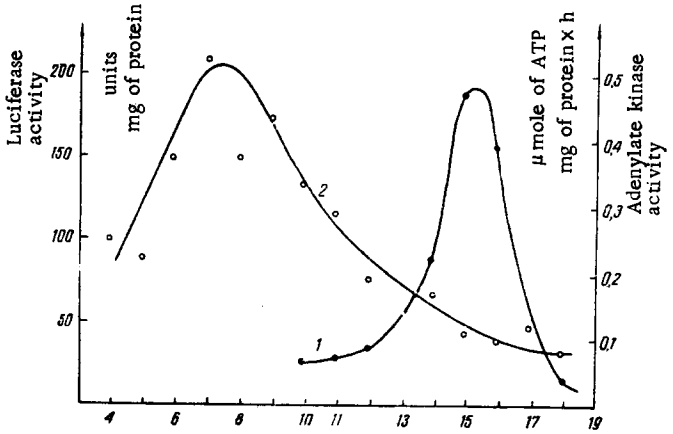

Fig. 1. Activity of luciferase (1) and of adenylate kinase (2) in protein fractions obtained in the chromatography of a homogenate of the glow worm Luciola mingrelica on a column of Sephadex G-25.

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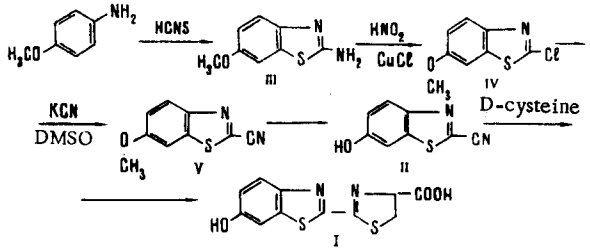
The luciferin from the Luciola mingrelica has not been studied previously; however, the identity of the luciferins of the various genera of American and Jamaican glow worms that is known from the literature [8] permitted the assumption of the possibility of an interaction of the luciferase of Luciola mingrelica with synthetic luciferin [1].

The syntheses of compound (I) that have been described include as the final stage the reaction of 2-cyano-6-hydroxybenzothiazole (II) with D-cystene [9-11]. We chose a method based on p-anisidine the thiocyanation of which takes place with simultaneous cyclization to the amine (III).



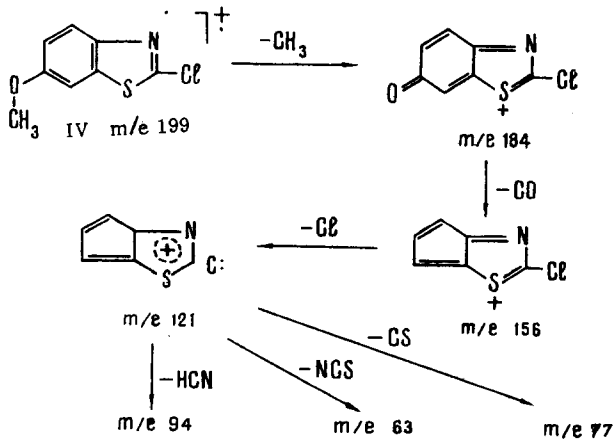
The following stage [the formation of the chloride (IV)] takes place with low yield, possibly because of the simultaneous formation of a methoxybenzothiazolinone, but so far as concerns the replacement of halogen by cyanogen, the conditions selected provided the possibility of performing the synthesis of the nitrile (V) without complications. The demethylation of (V) takes place fairly well when it is heated with pyridine hydrochloride, although special conditions are required. The last stage – the reaction of the nitrile (II) with cysteine – must be performed with careful protection from atmospheric oxygen: in an alkaline medium rapid oxidation takes place with the formation of a byproduct. On chromatography on Leningradskaya B ["Leningrad fast"] paper in the ethanol–1 M aqueous ammonium acetate (70 : 30, pH 7.5) system, in addition to luciferin with R_f 0.68 a spot with R_f 0.86 is found which apparently corresponds to the oxidized form of luciferin [9].

The luciferin (I) obtained in this way luminesces intensely in the presence of ATP and purified luciferase, which permitted the bioluminescence method of determining ATP to be used with a synthetic luciferin–luciferase system for studying the kinetics of the reactions of photosynthetic phosphorylation with various cofactors [12].

We checked the individuality of compounds (II-V) by chromatography in a thin layer of alumina and by means of UV and IR spectroscopy. To confirm the structure (having in view also the study of the pathways of the biosynthesis and metabolism of luciferin) we used mass spectrometry.

Condensed five-membered heterocycles (of the type of benzofuran, benzothiophene, benzothiazole, benzimidazole, etc.) with functional substituents give, under the action of electron impact, molecular ions which can decompose in two main directions: the first is connected with the loss of the functional substituent, and the second with the decomposition of the five-membered ring itself and the ejection of neutral molecules (CO, H_2S, HCN, etc., see for example [13-20]). The ratio of these directions is determined to a considerable degree by the nature of the substituents themselves.

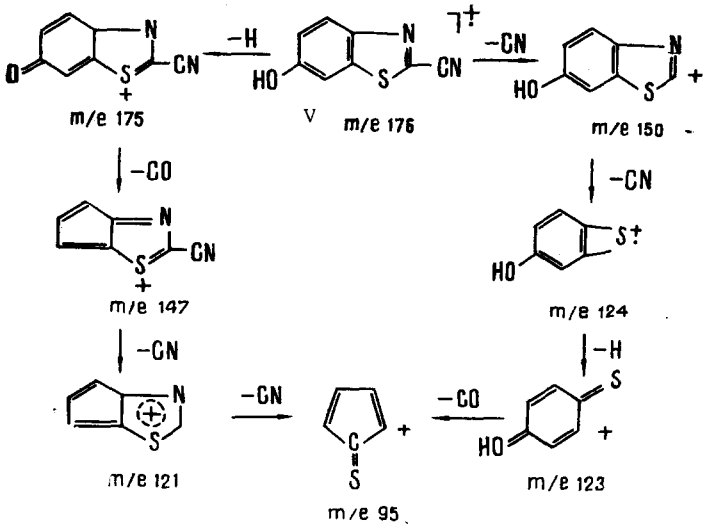
Scheme 1



In our case, the decomposition of the molecular ion of the chloride (IV) (Scheme 1) takes place with considerable selectivity and leads to a simple scheme of dissociative ionization with a small number of in-

tense ion peaks. In the first stage, the elimination of the methyl radical of the $6=OCH_3$ takes place with the formation of a stable ion having m/e 184. Lowering the energy of the ionizing electrons increases the relative intensity of the peak of this ion approximately twofold. The subsequent ejection of CO probably takes place with contraction of the benzene ring to a cyclopentadiene ring and the formation of a likewise extremely stable completely conjugated system (ion m/e 156). The relative probability of the processes is sufficiently high to suppress the possible elimination of chlorine from the molecular ion [14, 17]. This elimination takes place only in the third stage, and its high-energy nature is confirmed by the marked decrease in the intensity of the peaks of the ion m/e 121 when the energy of the ionizing electrons is reduced.

Scheme 2



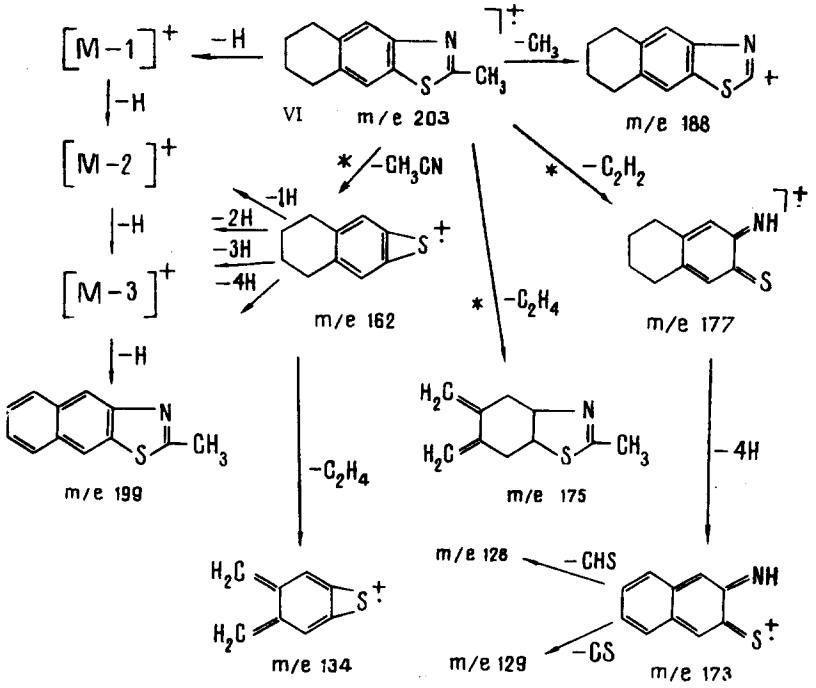
The replacement of the chlorine in position 2 by a cyane group [compound (V)] doubles the number of most probable decomposition pathways of the molecular ion (Scheme 2). The decomposition of the molecular ions of compounds (II) and (V) differs only by the first stage of one of the directions (the ejection of H and of CH_3, respectively). It is extremely characteristic that in both cases the peak of $[M-CO]^+$ ions, which is generally so strong in the mass spectra of phenols [21], is absent, and in the first stage of decomposition the ions $[M-H]^+$ and $[M-CN]^+$ arise. The sequence of decompositions of $[M-H]^+$ is generally similar to that for compound (IV): the elimination of the radical from position 2 generally also takes place in the third stage of the decomposition. However, the proportion of ions formed in the total ion current is considerably smaller than the proportion of the corresponding ions of the successive decomposition of the primary ion $[M-CN]^+$. The ion $[M-CN]^+$ with m/e 150 corresponds to the strongest peak of the fragmentary ions in the ordinary and the low-voltage spectrum.

As a model structure having no OH or OCH_3 group in the benzene ring (which excludes the possibility of "phenolic" fragmentation) we took 2-methyl-5,6-tetramethylenebenzothiazole (VI). For this substance, as was to be expected, the maximum peak at 50 eV corresponds not to the molecular ion but to a fragmentary ion with m/e 134. The main processes proved to be dehydrogenation (peaks of the ions $[M-1]^+$, $[M-2]^+$, etc.) and the retrodiene decomposition of the cyclohexane ring (Scheme 3). With a reduction of the energy of the ionizing electrons to 17 eV, the spectrum was obtained in which the maximum peak was that belonging to an ion with m/e 162, which corresponds to the elimination of CH_3CN from the molecular ion that is characteristic for this type of structure (confirmed by a metastable transition). However, in this case, as well, in spite of the low selectivity of the decomposition it is possible to detect elimination of the substituent from position 2 (ion with m/e 188).

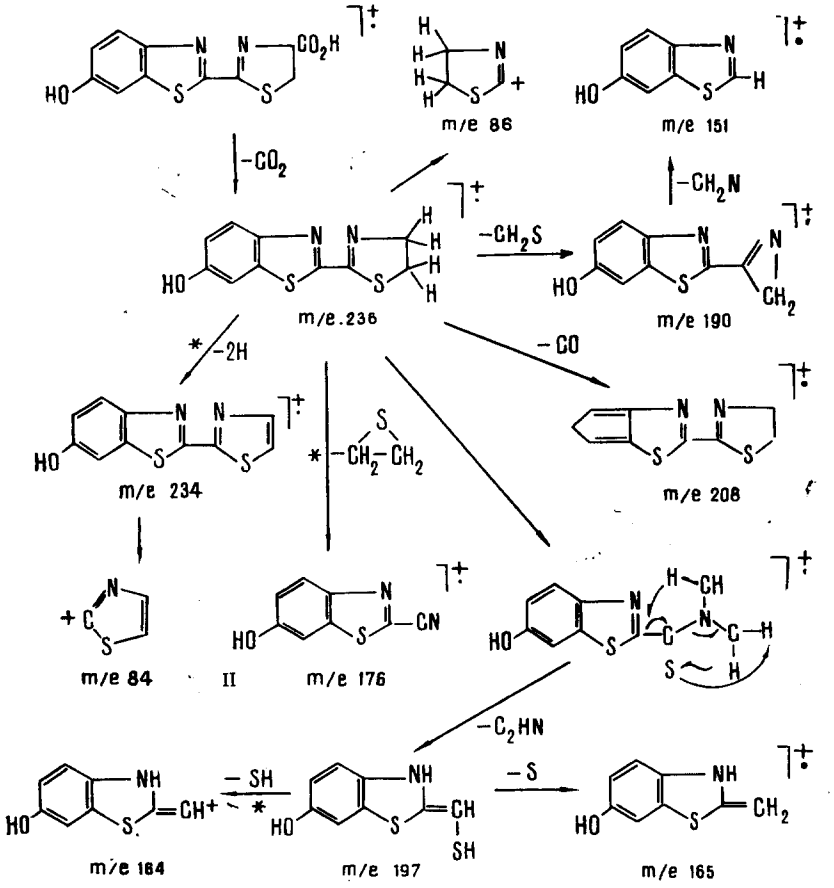
It is natural that the molecular ion of luciferin (I) is extremely unstable (its intensity is only 0.2% of the maximum ion) and easily undergoes decarboxylation (possibly thermally) with the formation of an ion with m/e 236, giving the strongest peak. The latter either loses 2H, giving a stable ion with m/e 234, which is shown by a metastable peak (Scheme 4) or it eliminates a molecule of ethylene sulfide with the formation of the strong peak of an ion with m/e 176 having structure (II) (see Scheme 4). This process is also confirmed by a metastable transition.

The third route for the dissociative ionization of the ion with m/e 236 apparently consists in cleavage at the CH_2-S bond with the redistribution of three hydrogen atoms and the splitting out of C_2HN, also lead-

Scheme 3



Scheme 4



ing to the appearance of a fairly strong peak of an ion with m/e 197. It then loses sulfur, giving an ion with m/e 164. The latter process is confirmed by a metastable peak.

"Phenolic" fragmentation (the splitting out of H or CO) is not of great importance in the mass spectrum of luciferin and appears, for example, in the conversion of an ion with m/e 236 into an ion with m/e 208 with the loss of carbon monoxide). Of the ions with low mass numbers we must mention fragments with masses 86 and 84.

EXPERIMENTAL

The mass spectra of compounds (II, IV-VI) were taken on a MKh-1303 instrument with modified recording systems and the introduction of the sample at the temperature of the ion source and of the inlet flask of 250°C, accelerating voltage 2 kV, and energies of the ionizing electrons of 50 and 17 eV, and the spectrum of luciferin on a MKh-1303 instrument with direct introduction into the source at an energy of the ionizing electrons of 30 eV (emission current 100 mA, 200°C). The UV spectra were obtained on a SF-4 instrument and the IR-spectra on a UR-10 instrument in paraffin oil.

A homogenate of the glow-worms was fractionated on a column of Sephadex G-25 in 0.005 M tris-acetate buffer (pH 7.8), 0.5-ml fractions being collected. The protein content was determined by Lowry's method [23]. The activity of the adenylate kinase was measured as described by Colowick and Kaplan [24], and the activity of the luciferase was judged from the intensity of luminescence [5, 6] of a system containing the protein, the synthesized luciferin, and ATP.

2-Amino-6-methoxybenzothiazole was synthesized by Stuckwisch's method [22] with a yield of 90%, mp 163-165°C; it was used subsequently without additional purification. Literature data: mp 161-162°C [22].

2-Chloro-6-methoxybenzothiazole (IV) was obtained by Stuckwish's method [22] with a modified purification procedure. The crude substance (IV) was dried in the air and sublimed in vacuum (~60°C, 20 mm Hg).

The yield of chromatographically pure compound (IV) was 8%, mp 52°C. Literature data: mp 52.5-50°C [11], 43-44°C [22].

2-Cyano-6-methoxybenzthiazole (V) was synthesized by the method of White et al., [11]. The crude nitrile (V) was purified by preparative thin-layer chromatography on Al_2O_3 [activity grade (II)] in the cyclohexane–ethyl acetate (9 : 1) system, R_f 0.40, yield 54%, mp 129-131°C. UV spectrum (methanol): 322, 265 nm (log ε 4.30, 3.98); IR spectrum: 2240 cm^{-1}. Literature data: mp 129-130°C [11].

2-Cyano-6-hydroxybenzothiazole (II). A solution of 100 mg (0.5 mmole) of 2-cyano-6-methoxybenzothiazole in benzene was added to 260 mg (2.2 mmole) of pyridine hydrochloride (obtained immediately before the synthesis in a molybdenum tube from absolute pyridine and gaseous HCl in absolute benzene with subsequent removal of the benzene together with the excess of pyridine by distillation in vacuum), and the benzene was evaporated in vacuum. The tube was evacuated (~15 mm Hg), sealed, and heated at 170-190°C for 1.5 h. The cooled tube was opened and the vitreous mass that had been formed was treated with 10% sodium bicarbonate solution in the cold, and was repeatedly extracted with ethyl acetate. The combined extracts were dried with magnesium sulfate, the ethyl acetate was evaporated, and the oily residue was treated with benzene. This gave substance (II). Yield 30 mg (32%), mp 195-200°C (from benzene). UV spectrum: λ_{max}, 264, 324 nm (log ε 3.56, 3.82). IR spectrum: 2240, 3230 cm^{-1}. Literature data: mp 205-207°C [10], 203-206°C [11].

Luciferin (I) was synthesized by the method of Seto et al., [10] and isolated by the method of White et al., [11]. Yield 47%, mp 204°C, UV spectrum (ethanol): λ_{max} 268, 331 nm (log ε 3.83, 4.21); IR spectrum: 3350, 2589, 1710 cm^{-1}. Literature data: mp 202°C [10], 196°C [2].

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CONCLUSIONS

1. The synthesis of the luciferin of Luciola mingrelica, i.e., D(−)-2-(6-hydroxybenzothiazol-2-yl)-3,4-dihydrothiazole-4-carboxylic acid, has been performed with some modifications.

2. It has been shown that in the mass spectrum of luciferin the most characteristic ions are those that correspond to decarboxylation, aromatization, and, finally, the elimination of the thiazoline part of the

molecule. The spectra of the intermediate products of synthesis are characterized by competing processes of the elimination of the substituent from position 2 of the benzothiazole molecule, and, to a small extent, the "phenolic" fragmentation of the benzene ring.

3. Synthetic luciferin in combination with purified luciferase of the glow worm Luciola mingrelica can be used for the luminescence determination of the concentration of ATP in biological materials.

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