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Invited Review

Progress in the Study of Bioluminescent Earthworms†

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ABSTRACT

Even though bioluminescent oligochaetes rarely catch people's eyes due to their secretive lifestyle, glowing earthworms sighting reports have come from different areas on all continents except Antarctica. A major breakthrough in the research of earthworm bioluminescence occurred in the 1960s with the studies of the North American *Diplocardia longa*. Comparative studies conducted on 13 earthworm species belonging to six genera showed that *N*-isovaleryl-3-aminopropanal (*Diplocardia* luciferin) is the common substrate for bioluminescence in all examined species, while luciferases appeared to be responsible for the color of bioluminescence. The second momentous change in the situation has occurred with the discovery in Siberia (Russia) of two unknown luminous enchytraeids. The two bioluminescent systems belong to different types, have different spectral characteristics and localization, and different temperature and pH optima. They are unique, and this fact is confirmed by the negative results of all possible cross-reactions. The bioluminescent system of *Henlea* sp. comprises four essential components: luciferase, luciferin, oxygen and calcium ion. For *Friderica heliota*, the luminescent reaction requires five components: luciferase, luciferin, ATP, magnesium ion and oxygen. Along with luciferin, more than a dozen analogues were isolated from worm biomass. These novel peptide-like natural compounds represent an unprecedented chemistry found in terrestrial organisms.

THE EARLIEST OBSERVATIONS OF BIOLUMINESCENT WORMS

The luminescence phenomenon is not uncommon among Oligochaeta. The light production by these worms was at different times reported in Europe, USA, Australia, New Zealand, India, Japan, South Africa and Russia (1). According to Vejdovský, luminous earthworms were first mentioned as early as 1670 when Grimm observed some forest worms glow on the Coromandel Coast of India (2). A century later, Flaugergues described earthworms at the period of copulation: "glowing like rotten tree, but much brighter" noting at the same time that the dead worms did not glow (3). In 1837, Antoine Louis Dugès assigned species rank to a luminous earthworm that he had discovered in abundance in the tan of the hothouses of the Jardin des Plantes in Montpellier. His *Lumbricus phosphoreus* emitted luminous fluid from the body surface, "a fluid undoubtedly similar to that released through the dorsal pores by many other earthworms." The species would be later classified as a nonlumbricid megadrile, *Microscolex phosphoreus*, introduced to France as to many other countries by human activity, probably from South America (4,5). From 1840, earthworm luminescence was reported more frequently and regularly and arrived to cover four different megadrile families. Among the authors of these reports were Allman (1844) (6), Cohn (1873) (7), Vejdovský (1884) (2), Atkinson (1887) (8), Lloyd (1897) (9), Friend (1893–1924) (10–12), Benham (1898) (13), Beddard (1899) (14), Gates (1925) (15), Skowron (1926) (16), Pickford (1937) (17) and others. Most of these records were in association with greenhouses or private gardens, but Skowron (1928) wrote about great numbers of *M. phosphoreus* having propagated in deep passages (about 230 m from the surface) of coal mines in Central Poland (18). It is notable that even an astrophysicist, Father Angelo Secchi, the first to classify stars by their spectra, director of the Pontifical Roman College Observatory in 1850–1878, was so excited with earthworm luminescence that he performed its spectral analysis (19). Pierantoni (1922) described chickens in New Zealand feeding in the twilight on *Octochaetus* worms resembling glowing macaroni (20). The luminosity of the marine littoral earthworm *Pontodrilus matsushimensis* was discovered by Kanda and Haneda when they stepped on it in the wet sand at the tidal line near Yokohama (21,22). In a recent review providing the list of terrestrial bioluminescent animals of Japan, the oligochaetes are represented by *Microscolex phosphoreus* and *Pontodrilus litoralis* (Syn. *Pontodrilus bermudensis* and *Pontodrilus matsushimensis*) (23).

Reports from Russia were not so numerous, and all of them concerned the microdrile family Enchytraeidae (potworms). Eversmann wrote in 1838 from Kazan about a new species of

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luminescent worm, *Lumbricus noctilucus*, that he had discovered in a flower pot. "Worms were so numerous that in darkness the soil seemed mixed with fire" (24). Later in 1863, Owsiannikow, on the basis of Eversmann's description and his own observations of the worms' luminescence in Kazan, identified the species as *Enchytraeus albidus,* noting "Its light is very weak. It is not associated with a specific organ but flickers here and there, now coming from the head, now from the tail, now from the whole body. Sometimes a faint blue glow remains on one's fingers upon handling the worm" (25). Walter in 1909 published an article which described in detail, with figures, the luminescence of small oligochaetes inhabiting Kaluga district and noted that a certain Ms. N.P. Trusova had observed the same phenomenon in Perm. The specimens were identified by W. Michaelsen as *Henlea ventriculosa*. The latter is a common, widespread enchytraeid species, whose luminescence, according to Walter, has gone unnoticed for a long time due to their habitat laying deep below the soil surface (26). After these early reports, no further investigation of luminous enchytraeids was made, until 1990. Just a sole short note appeared in 1976, concerning an accidental discovery of some bioluminescent freshwater oligochaetes (Enchytraeidae gen. sp.) while selecting a site for the construction of a hydroelectric power plant on the Bureya River (Khabarovsk Krai and Amur Oblast of Russia). The collected worms were immature; hence, it was impossible to identify their genus and species. When the worms were stimulated mechanically, they discharged a secretion emitting a greenish blue light for about a minute. With the decline of water level, the oligochaetes did not migrate and continued to live in damp soil, dying as it dried up (27).

FIRST LABORATORY STUDIES OF EARTHWORM LUMINESCENCE

For a long time, the study of glowing oligochaetes was descriptive, concerning the behavioral and physiological aspects of the phenomenon. Only Jean-Henri Fabre, the French naturalist, but also self-trained physicist and chemist, attempted some deeper inquiries. He observed the light of *M. phosphoreus* to extinguish in the vacuum and in "unbreathable gases" (i.e. hydrogen, carbon dioxide, etc.), whereas it kept unchanged brightness in aerated water, in ambient air and in pure oxygen. Fabre saw in earthworm luminescence a process of oxidation, a sort of respiration, especially active in certain tissues (28). The necessity of oxygen for luminescence of earthworms would be later confirmed by Gilchrist (1919) (29), Harvey (1926) (30), Skowron (1928) (18) and Komárek (1934) (31).

Many earlier researchers assumed luminescence of soil animals to derive from symbionts or parasites. However, numerous attempts to isolate luminous bacteria from worms were unsuccessful (26,32). Issatschenko, who succeeded in isolating *Photobacterium chironomi* from luminous mosquitoes, failed to replicate this with the *H. ventriculosa* potworms that Walter had sent to him (33). Similarly, Benham, Gilchrist, Skowron and Komárek demonstrated by microscopic examination in luminous megadriles of three different families that the source of light was not bacterial but contained in the worm's own cells. In the case of earthworms, all observations pointed to the granule-filled cells floating in the coelomic fluid, and the occurrence of light emission after these cells were osmotically lysed or mechanically disintegrated (13,18,29,31). This happened either spontaneously or following strong stimulation whenever the coelomic fluid was released to the exterior through the dorsal pores and/or from the mouth and anus in species lacking the dorsal pores, such as *M. phosphoreus, P. bermudensis*.

A test for luciferin and luciferase had failed in *Parachilota* (29) and in *M. phosphoreus* (30), but Komárek & Wenig found evidence of heat-stable and heat-labile components in crude extracts of the native European luminous earthworm *Eisenia lucens* (34). In addition, the same Czech team later demonstrated the presence of riboflavin in unbound state (35,36), in the yellow coelomocytes of *Eisenia lucens*, as well as in those of its nonluminous congener, *E. fetida*. The coelomic fluid of *E. lucens* fluoresces yellow-green like riboflavin until the bioluminescence has disappeared, at which point the fluorescence color changes to blue, that of lumichrome, i.e. the product of UV irradiation of riboflavin. A corresponding change in the yellow-green fluorescence of the nonluminous *E. fetida* does not take place. Consequently, the Czech researchers postulated that (1) the bioluminescence of *E. lucens* is connected with a change from riboflavin to lumichrome, (2) in this species, riboflavin plays the role of luciferase and is changed into lumiflavin during the luminescence reaction, while (3) such a reaction does not occur in *E. fetida* due to the lack of some components of the oxidative system. Later they stated that the molecules of riboflavin "are believed to be absorbed in an oriented layer on the surface of granula of lipoid character… [and] the activation energy which brings them into an excited state is probably derived from an oxidative reaction in which molecular oxygen takes part (37)." In a most recent paper, Pes *et al.* report blue-green luminescence of *Eisenia lucens* upon stimulation with ethanol. These authors also state that riboflavin stored in coelomocytes plays an important role in the glowing reaction (38).

Beginning from the late 1960s, an increasing interest in photochemical reactions, intermediary metabolism and kinetics of biological processes, as well as the development of new methods of extraction, purification and measuring brought significant advances in the understanding of megadrile luminescence. Johnson *et al.* (39) found, as was previously shown by Komárek (1934) in *E. lucens* (31) and later documented by Wampler (1982) in *M. phosphoreus* (40), that the exuded coelomic fluid of *Octochaetus multiporus* fluoresces under UV with the same color of bioluminescence, which is orange-yellow orange-yellow in this species, but changes to blue upon cessation of luminescence, i.e. some product of the luminescence reaction affects the color of fluorescence. Unpurified extracts of the *Octochaetus* exudate gave a luciferin–luciferase reaction requiring molecular oxygen; yet none of various cofactors functioning in other bioluminescent systems (e.g. ATP, NADH, FMN) were found to be active in restoring light in a rehydrated exudate of the worm. The study by Cormier & coll. (1966–1972) (41–43) and Rudie, Wampler & coll. (1975–1981) (44–49) of the North American *Diplocardia longa*, a luminescent earthworm with nonfluorescent coelomocytes, showed the light production in this species to be likewise based on a luciferin–luciferase reaction, but to be inhibited by oxygen and enhanced by hydrogen peroxide *in vitro*. As supposed by Skowron (1928) for *M. phosphoreus*, the granules in these cells proved to be the site of both luciferin and luciferase activity and luciferase to make up about 5% of the total extractable protein from the cells (18).

CYTOLOGY OF EARTHWORM LUMINESCENCE

The size of the luminous coelomic cells varies across species. Cells are the largest in *Diplocardia longa*—30–50 μm, smaller and more uniform in *D. alba*-31 μm, *D. eigeni*—18 μm; *Spenceriella cormieri*—26 μm, *S. curtisi*—24 μm, *S. noctiluca*—18 μm; *Fletcherodrillus fasciatus*—23 μm, *M. phosphoreus* 27 μm and *P. bermudensis*—14 μm (41).

The luminescent cells were initially identified as free chloragocytes (44,50), but after comparisons of the specific ultrastructural traits, they were identified as coelomic mucocytes (51,52). For some time (1979–1980), in *Pontodrilus bermudensis*, the cellular source of luminescence could not be proved (50,53). However, in 1986, Wampler & Jamieson (52) explained that failure to demonstrate cell bounding in that marine littoral species was due to its high coelomic osmolarity and to the lysis of the cells by the hypotonic solutions used in the procedures, so that the luminescence components were then found free in solution. More appropriate procedures allowed to establish that, although smaller, the luminescent cells of *P. bermudensis* are similar to the bioluminescent mucocytes of other species.

Wampler & Jamieson summarized the cytological aspects of earthworm bioluminescence with these words (52): "In all species examined to date by our laboratories, the bioluminescence system is contained in coelomic cells and is specifically associated with a large, granule-filled cell type. In at least two species, this same cell type exhibits fluorescence which is spectrally quite similar to the bioluminescence. A careful study of the various types of coelomic cells in *P. bermudensis* (51), a thorough review of the literature and morphological characteristics (54) and a reinvestigation of the EM sections and photographs from our previous study of *D. longa* (J. E. Wampler and B. G. M. Jamieson, unpublished) suggest that the luminescent cells in these various species are morphologically similar and that they are not free chloragogen cells, as was previously reported (44). Instead, this cell type is identified as a mucocyte, an acidophil cell containing
β-glucuronidase and PAS-positive granules."

Does oligochaete luminescence always occur outside the body? In earthworms, light production always proceeds from a discharge of coelomic fluid. Only if the animals are dying, they become luminous within the body cavity (steady death glow), the coelomic cells breaking up inside the body (16). Instead, Walter so described the luminescence of the potworm *Henlea ventriculosa*: "In the dark, under the microscope, one can see that the worm's body is covered with a mass of small bright dots" (26). Walter suggested that the luminous material was allocated within the epidermal glands of the worm. On sections, he showed the head and tail of the worm to have a thicker epithelium and more glands.

COLOR OF OLIGOCHAETE LUMINESCENCE

Megadrile earthworms give emission spectra with λ_{max} ranging from 500 nm (*Diplocardia*, blue-green) to greater than 570 nm (*Octochaetus multiporus*, orange-yellow). Within one same genus, the color of bioluminescence is generally very similar (53). Spectral differences between the various species do not appear to be caused by different luciferins, but by the presence of different fluorescent entities, which act as the emitter. Bioluminescence and fluorescence are not always correlated: *Diplocardia* coelomocytes, unlike those of *Microscolex* (λ_{max} 538 nm) and *Pontodrilus* (λ_{max} 550 $\pm$ 10 nm), have no fluorescent component that matches the bioluminescent spectrum (52).

DISTRIBUTION OF LUMINESCENCE IN THE OLIGOCHAETA

Luminescence is scattered irregularly among oligochaete taxa, and reports of luminescence in natural and laboratory conditions (e.g. Gates 1925 (15)) imply considerable differences in the readiness of different species to luminesce. The list of luminous taxa increased in the 1970s by the addition of a new bioluminescent worm from Australia (55). Interestingly, that species does not show spontaneous luminescence nor could the light be elicited *in vivo* by electrical or mechanical stimulation. Luminescence was only obtained by the application of oxygen peroxide to the whole worm or its coelomic fluid. More importantly, the flash peak of luminescence declined to zero over a few minutes. This suggests that luminescence could be present in many species in a latent state. Furthermore, Jamieson in 1977 reported that Wampler had succeeded in isolating earthworm luciferin from some nonluminous species; thus, luciferin could have a more general biochemical function, and luminescence could be a by-product of other metabolic processes (55). In any case, Herring in his classification of 1978 pointed out that of 16 Oligochaeta families, three, namely Enchytraeidae, Lumbricidae and Megascolecidae, have luminous species (56). The involved genera were *Eisenia* (Lumbricidae); *Enchytraeus, Henlea* and *Michaelseniella* (Enchytraeidae); *Diplocardia, Microscolex* (including *Eodrilus* sp.), *Parachilota, Octochaetus, Pontodrilus, Eutyphoeus, Ramiella, Lampito, Digaster* (all of the latter classified in the "family Megascolecidae," which in Herring's acception is equivalent to the "superfamily Megascolecoidea," comprising several distinct families).

Later, more *Diplocardia* species in N. America (53) and three more genera of Megascolecidae in Australia were discovered to contain luminous species: *Spenceriella*, *Fletcherodrilus* (50) and *Diplotrema* (57).

In 1987, nine years after his first review, Herring revised the checklist of luminous organisms, deleting from it those representatives whose bioluminescence was not corroborated by recent reports and, consequently, he raised doubts about their authenticity. So, the family Enchytraeidae was not mentioned at all in his classification (58). No data on luminous Enchytraeidae were mentioned in the review by Hastings & Johnson, 2003, either (59). Thus, until recent studies, the list of luminescent oligochaetes comprised mostly representatives of the "Megascolecoidea superfamily" (about 30 luminous species, including the very common terrestrial *Microscolex phosphoreus* and the marine littoral *Pontodrilus bermudensis*), and all cases of luminescence in Lumbricidae family were classified in 2–3 species of the genus *Eisenia*. Surprisingly, even in the review by Osamu Shimomura, 2006, the confirmed existence of glowing enchytraeids passed unnoticed (60).

DETAILED RESEARCH ON THE MEGASCOLECID *DIPLOCARDIA LONGA*

Among all luminescent oligochaetes, the most extensively studied was *D. longa*. These are large worms up to 60 cm long,

1 cm in width and weighing up to 7 g, inhabiting the sandy soils of South Georgia, USA. Luminescence of this megascolecoid is localized, as in all megadrile earthworms, in the free cells of the coelomic fluid, discharged by worms during stimulation. Having isolated coelomic cells of *D. longa* by centrifugation in the saccharose density gradient, Bellisario (1972) confirmed the source of luminescence to be inside them, and the luminescence to depend on cellular lysis (41). First, the researchers worked with the lyophilized powder of worms, breeding it in potassium phosphate buffer (42), and then they simply "shocked" the worms by applying electrical stimulation, which caused copious exudation (45).

The luminescent system of *D. longa* includes the following components: a copper containing protein (luciferase) with a molecular weight of 300 kDa (41,46), an aliphatic aldehyde as the reaction substrate (luciferin), identified as N-isovaleryl-3-amino-1-propanal (45,47,61) and oxygen peroxide (43). In 1976, Rudie & Wampler (45) proposed the following schematic luminescent reaction for *D. longa* (Scheme 1).

Luminescence requires a two-step reaction: first, the formation of intermediate 3-isovalerylamino-1-hydroxypropane hydroperoxide, which is then degraded to form a product of unknown nature. The observed luminescence is bluish green, with a maximum at 490–500 nm.

The purified *Diplocardia* luciferase was identified as a 300 kDa, fairly unstable and highly asymmetrical protein of unusual aminoacid composition. As result of SDS-phoresis, subunits were revealed with molecular weights of 71 000, 58 000 and 14 500 Da. The early assumption of a heme group in the luciferase was not confirmed, whereas an atom of copper has been detected. But its functional role in the bioluminescence reaction according to Rudie, Mulkerrin and Wampler (46) is questionable, as 90% of its removal occurs without loss of luciferase activity (41). It was also shown that *D. longa* luciferin possesses Cu^{1+}-dependent chemiluminescence (48). In addition to copper, luciferase contains 6% of carbohydrates and 2% of lipids. Luciferase amino acid composition analysis showed unusual high content of proline and hydroxyproline (11% of total weight) (46).

Diplocardia longa luciferin was isolated, purified and identified in 1975 (49). Its structure was confirmed by chemical synthesis. The quantum yield for this substrate is 3% (47). The *in vitro* luminescence showed a broad optimum pH, from 7.0 to 8.5. The luminescence with partially purified luciferin and luciferase produced an emission spectrum (λ_{max} 503 nm) similar to the *in vivo* luminescence from freshly exuded slime (λ_{max} 507 nm) (41), but shifted to λ_{max} 490 nm when a pure sample of luciferin was used (47).

Subsequently, Jamieson and Wampler conducted comparative studies testing the cross-reactivity of the various components of the *Diplocardia* system on other bioluminescent earthworms and found that N-isovaleryl-3-aminopropanal, or its close analogue, is the common substrate for bioluminescence in 13 earthworm species belonging to six genera from the southern USA (three spp. of *Diplocardia* and *Pontodrilus bermudensis*), eastern Australia (*Diplotrema*, Fam. *Acanthodrilidae*; four spp. of *Spenceriella*, two spp. of *Fletcherodrilus*, Fam. *Megascolecidae*; and *Pontodrilus*) and New Zealand (*Octochaetus*). These species have different emission spectra between 500 and 570 nm. The *Diplocardia* luciferase was shown to be quite specific, being active with only a few simple analogues of luciferin and none of the straight chain aldehydes (50,53). On the basis of these works originated the hypothesis of a single mechanism for the bioluminescence of earthworms. The earthworm bioluminescence involves the reaction of hydrogen peroxide with N-isovaleryl-3-aminopropanal or its close analogue, and spectral distribution is determined by the luciferase or other luciferase-associated components.

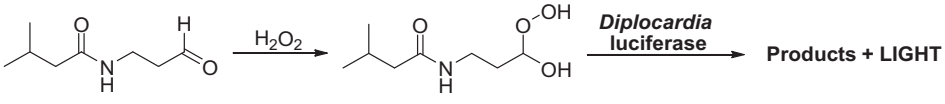
TWO NEW BIOLUMINESCENT ENCHYTRAEIDS—TWO NEW TYPES OF LUMINESCENT SYSTEMS

The luminous taiga floor enchytraeid *Fridericia heliota*

A first short report on the discovery of bioluminescent enchytraeids in Krasnoyarsk region (Siberia, Russia) was published in 1990 (62). The specimens were assigned to the genus *Fridericia*, and the majority of them, based on their morphological characteristics, were allocated to a new species named *Friderica heliota*, from the Greek *hēliōtis*, meaning "of the sun." A decade later, this luminous worm from the Siberian taiga was redescribed in more detail (63). The body of the worm is semitransparent of white yellowish color; a mature individual weighs about 2 mg, with live length 15–20 mm, width about 0.53 mm at clitellum, having 50–58 segments (Fig. 1A). Lateral chaetae are most often one per bundle; ventral chaetae most often two per bundle, sometimes one, rarely three. The peptonephridia give off many short branches. Coelomocytes are oval or elliptical; *in vivo,* they contain small peripheral granules. *Fridericia heliota* is also peculiar for the uncommon structure of the spermathecae, with paired diverticula lying backward, alongside the ental part of ampulla.

It should be mentioned that earlier nobody had ever noted luminescence in the genus *Fridericia*. The luminescence of *F. heliota* is bright and constant. There is no difference between the adults and the juveniles, even the earliest life stages being luminescent. Following (even slight) tactile, chemical or electrical stimulation, each specimen produces a continuous bright glow for 1–3 min. The production of light is confined to the body wall with a pattern corresponding to that of the epidermal gland cells, although it does not involve discharge of luminous mucus (Fig. 1C). This contrasts with observations on other enchytraeids and on the oligochaetes in general (52).

The molecular phylogenetic analysis of the family Enchytraeidae carried out by Erséus *et al.* (64) placed *F. heliota* closest to *Fridericia parathalassia*, a species inhabiting European coastal habitats from northern Spain to Sweden (65) The two species are morphologically quite different, both externally, with regard to their respective abundance of epidermal glands, the presence/absence of subneural glands and, internally, in terms of shape/size/



Scheme 1. Luminescent reaction for *Diplocardia longa.*

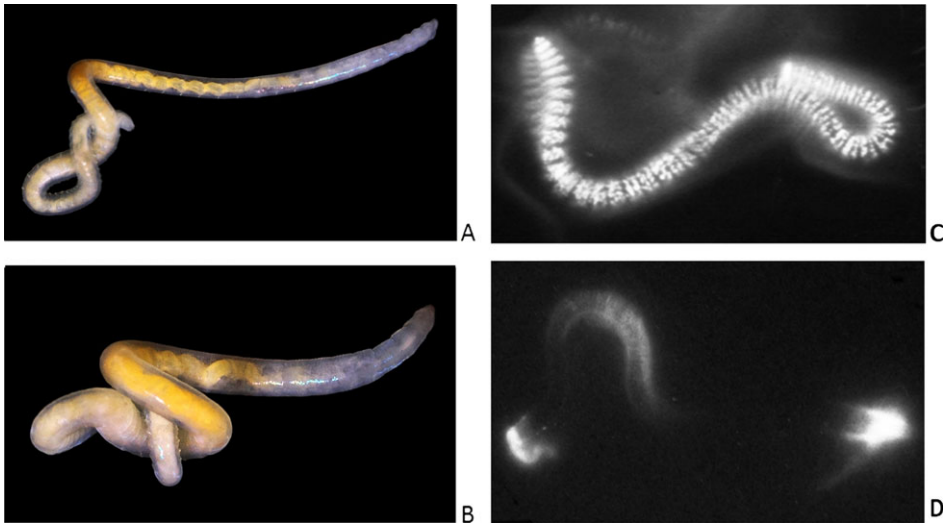


Figure 1. Bioluminescent enchytraeids: live specimens under light microscope and their lumography (photograph taken by direct contact printing worm to film through thin polyethylene film in the dark room): *Fridericia heliota* Zalesskaja, 1990 (A, C) and *Henlea* sp. (B, D).

number of somatic and reproductive structures (63,65). The only common trait seems to be their association with soils right above or close to the waterline—in one case (*F. heliota*) of rivers, in the other (*F. parathalassia*) of sea shores—and consequently their high tolerance of dehydration (65).

The luminous soil enchytraeid *Henlea* sp

In the same soil samples where *F. heliota* occurred, larger luminous worms of a different species were found (Fig. 1B). On the average, their number was 1% *versus* 99% of *F. heliota* (66). At first, they were erroneously identified as *Fridericia ratzeli* (62), but they were later found to belong to the genus *Henlea* (1). The correct attribution of this *Henlea* worm to one of the known species or its recognition as a novel taxon is not an easy task as its morphology widely overlaps with many described *Henlea* species. Furthermore, as reviewed in Rota *et al.* (1,63), bioluminescence has been reported several times in *Henlea*, but at a time when enchytraeid taxonomy was in a very preliminary stage, thus associated with wrong species names. In contrast, modern zoologists practically always work in well-lighted work areas; hence, even if a very well-described enchytraeid had the ability to luminesce, this property would probably pass unnoticed.

In a 1929 paper by Burov, an extensive description of a morphologically similar species *Henlea irkutensis* was provided, but nothing was mentioned about its ability to produce light (67). In any case, the data published in this paper later enabled the discovery of bioluminescent *Henlea* sp. in the Baikal Lake area (Irkutsk region, Russia).

The specimens of *Henlea* sp. are bigger than *F. heliota*—the length of the worms varies from 20 to 35 mm (Fig. 1B), a mature individual weighs up to 10 mg. In response to various irritants, the worms of both species emit bluish green light visible well in darkness. Specific and unique feature of *Henlea* sp. is the duplicity of luminescence localization. On the one hand, these worms, such as *D. longa* and all other known luminous megadriles (but not *F. heliota*), in response to irritation release mucus containing luminescent coelomic cells (Fig. 1D, Table 1). On the other hand, *Henlea* sp., like *F. heliota*, has luminescent formations localized in the body wall: A worm with collapsed, empty body wall continues glowing, and under high magnification, it distinctly shows small luminescing points (66). The intensity of luminescence in response to irritation is 2–5 times higher for *Henlea* sp. than for *F. heliota*. Kinetics of luminescent signals of *F. heliota* and *Henlea* sp. *in vivo* was not found to have radical differences; the intensity decay curve in both cases is close to exponential.

Table 1. Comparative characteristics of bioluminescent oligochaetes.

Class/Subclass	*Clitellata/Oligochaeta*		
Family	*Enchytraeidae*		*Megascolecidae*
Species	*Fridericia heliota*	*Henlea* sp.	*Diplocardia longa*
Secretion	–	+	+
Body wall	+	+	–
Luminescence, λ_{max}, nm	478	464	500–530
pH_{opt}	8.2	7.3	7–8.5
T_{opt}	33°C	20°C	–
Luciferase, MW, kDa	~60 2 × 28	2 × 36	300
Luciferin	*Fridericia* luciferin $C_{23}H_{29}N_3O_{11}$	n.d.	N-isovaleryl-3-aminopropanal $C_8H_{15}NO_2$

n.d. not determined

Features of *F. heliota* and *Henlea* sp. luciferin–luciferase reactions

The maximum of luminescence spectrum for *F. heliota* is around 478 nm, for *Henlea* sp. 464 nm (Table 1) (68). These are the shortest wave length values among all known luminescent oligochaetes: *Octochaetus multiporous*—more than 570 nm, *Diplotrema heteropora*—545 ± 10 nm, *Diplocardia longa*—530, 500 ± 5 nm, *D. alba*—501 ± 5 nm, *D. eigeni*—505 ± 5 nm, *Pontodrilus bermudensis*—550 ± 10 nm, *Spenceriella curtisi*—535 ± 5 nm (53,69).

To better characterize the BL systems of each of the newly discovered worm species, elaborate procedures for isolation of their luciferins and luciferases from biomass homogenates and for identifying optimal conditions for their further chromatographic purification were set up.

A luciferin–luciferase reaction was demonstrated for *Henlea* sp., and participation of air oxygen was also established. Hydrogen peroxide was shown to have no effect on the activity of purified luciferase in contrast to crude preparations (worm homogenate). Purified *Henlea* luciferin turned out to be highly unstable and lost activity quickly. For this reason, its molecular mass has not yet been established. However, it was found to be less than 10 kDa, as far as the luciferin activity could pass through a 10 kDa ultrafiltration membrane. Luciferase molecular weight was estimated as ~72 kDa by gel filtration (66). Along with the enzyme itself, luciferase preparations contained a thermostable component which could activate the BL reaction. Later, it was found to be calcium ions (70). Thus, the BL system of *Henlea* was found to include four components (Scheme 2):

For *F. heliota* too, it was shown that its BL system does not utilize hydrogen peroxide, but requires molecular oxygen. Native molecular weight of luciferase changed from 60 to 28 kDa upon purification without loss of activity. Luciferin from *F. heliota* was shown to be thermostable, as it sustained heating to 100°C for 1 min with full retention of activity, and its molecular weight was estimated as ~0.5 kDa. Magnesium ions were found to enhance the BL reaction (71). After many experiments, it was established that ATP is another required factor in the BL reaction of *F. heliota* and serves as second substrate along with *Fridericia* luciferin (72). Thus, the following scheme was proposed for the BL reaction of *F. heliota* (Scheme 3):

A similar set of components was recorded for bioluminescence in fungus gnats of the genus *Arachnocampa* (73), a millipede *Luminodesmus sequoiae* (74,75) and a deep-sea squid *Watasenia scintillans* (76). The most comprehensively investigated ATP-dependent luminescence is that of fireflies (*Photinus pyralis, Luciola mingrelica*) (77). *F. heliota* represents the only example of ATP-dependent BL among oligochaetes.

An important feature of enzymatic BL reactions is their pH dependency. The optimal pH for *F. heliota* BL system was found to be 8.21, whereas for *Henlea* sp., the value is 7.25. At the same time, the interval of pH values sustainable for BL reaction was significantly broader in the case of *Henlea* sp. system compared to that of *F. heliota*. In other words, *F. heliota* luciferase is more pH sensitive than *Henlea* luciferase. The pH-induced loss of *F.heliota* luciferase activity is irreversible, which may be caused by protein denaturation. The optimal pH value of *F. heliota* luciferase coincides with that of other ATP-dependent BL systems: 8.0 for fireflies and *Arachnocampa flava* and 8.3 for millipede *Luminodesmus sequoiae*. Probably, this coincidence might be related with the participation of ATP in the reaction.

$$\text{luciferin} + O_2 \xrightarrow{\textit{Henlea}\ \text{luciferase},\ Ca^{2+}} \text{Products} + \text{LIGHT}$$

Scheme 2. Bioluminescent system of *Henlea* sp.

$$\text{luciferin} + \text{MgATP} + O_2 \xrightarrow{\textit{F. heliota}\ \text{luciferase}} \text{Products} + \text{LIGHT}$$

Scheme 3. Bioluminescence reaction of *Fridericia heliota*.

Neutral optimal pH of *Henlea* sp. may be explained by the fact that the BL reaction proceeds outside of *Henlea* body, where pH is usually close to neutral.

Temperature dependencies of these two BL systems also differ significantly (Table 1). For the *F. heliota* system, the activity rises gradually from 0 to 33°C, and then, falling is observed due to irreversible inactivation of luciferase. For the *Henlea* system, the optimal temperature is 20°C and temperature inactivation by short-term heating is reversible.

Henlea luciferin and luciferase do not cross-react with the reaction components from *Fridericia*. Both luciferases are inactive with N-isovaleryl-3-aminopropanal and other known luciferins.

In summary, the BL systems of *Fridericia heliota* and *Henlea* sp. have totally different structural and functional organization regardless of close geographical and ecological relation between these two species (Table 1) (1,68). Morphologically, the two genera may appear closely related, by sharing a superficially similar shape and arrangement of the chaetae, and initially, the Krasnoyarsk luminous worms were in fact all assigned to *Fridericia*. But the chaetal arrangement, as much as the bioluminescence system, has undoubtedly arisen independently in the two genera, as shown by recent molecular phylogenetic analyses (64,78).

STUDY OF *F. HELIOTA* BIOLUMINESCENT SYSTEM

Effect of salts and detergents on luciferin–luciferase luminescence of *F. heliota*

Collection of luminescent oligochaetes turned out to be a challenging task, as the worms are very small; their natural habitat is unexplored, while all attempts at cultivation proved unsuccessful. Only the protocol for all-season sustaining of their population in the laboratory incubators could be developed. Therefore, all biochemical experiments had to be carried out under pressing deficiency of biological material.

To optimize chromatographic separation and purification of the BL system components of *F. heliota*, it was necessary to understand the factors that inactivate luciferase. For this purpose, the effect of different inorganic salts and detergents (SDS, Triton X-100, Twin series) on bioluminescent reaction *in vitro* was studied. In the luciferin–luciferase system purified from endogenic ions, practically, all added bivalent cations in concentration range 0.1–1 mM are capable of stimulating activity of luciferase. But only three of them such as Ca^{2+}, Mg^{2+} and Mn^{2+} stimulate luciferase activity at concentrations varying within a wide range; at that, Mn^{2+} replaces Mg^{2+} with 100% efficiency in the luminescence reaction. The nature of anion did not matter at all. By maximum stimulation, the cations can be ranked as follows: $Hg^{2+} \sim Cu^{2+} \sim Ba^{2+} \sim Sn^{2+} \sim Be^{2+} \sim Sr^{2+} \sim Ni^{2+} < Cd^{2+} \sim Co^{2+} \sim Ca^{2+} < Zn^{2+} < Mn^{2+} \sim Mg^{2+}$. The inhibitory effect of monovalent metal salts on luminescence is largely determined by the action of the anion. Inhibiting efficiency of anions increases in the following order: $Cl^- < CO_3^{-2} \sim SO_3^{-2} \sim Br^- < SO_4^{-2} \sim PO_4^{-3} < NO_3^- < I^- \ll Cr_2O_7^{-2} \ll Fe(CN)_6^{-3}$.

Among sodium salts, the inhibitory effect on BL reaction is the strongest for dodecylsulfate which is an anion detergent. Nonionic detergents, on the contrary, stimulate activity of *F. heliota* luciferase. They can be ranked as follows: Triton X-100 > Tween 80 > Tween 60 > Tween 20. Triton X-100,

the most effective of them, intensifies luminescence of the cell-free extract by five times. It is determined by its ability to eliminate the lipid barrier for substrates to access the active center of the enzyme, releasing the inhibition of the luciferase (79).

Separation and purification of various fractions from biomass of *F. heliota*: luciferase, luciferin and inactive luciferin-related compounds

For the first time, the pure luciferin of *F. heliota* was obtained in 2007 (80). The isolation procedure included the separation of active luciferin and luciferase, followed by the purification of luciferin: At the first stage, a cell-free biomass extract was prepared; then, anion-exchange chromatography was used to separate luciferin and luciferase; concentration of luciferin fraction by solid-phase extraction; two consequent reversed phase HPLCs. As a result of purification, the specific activity of luciferin was concentrated 4000-fold.

The content of luciferin in the worm biomass was extremely low: 0.5–0.7 μg per 1 g (~500 individuals). For that reason only at the final chromatographic stage, the peak corresponding to luciferin could be observed. Its UV absorption maximum was 294 nm with a local minimum at 262 nm. Along all the purification stages, a major peak corresponding to a compound with a similar retention time was observed and designated as CompX. Within the range 280–360 nm, the UV spectra of CompX and luciferin were nearly identical (80). Later, another group of alleged luciferin analogues designated AsLn(1–3) was found. Their UV–Vis absorption spectra showed maxima at 235 nm for AsLn1, 228 and 294 nm for AsLn2, 241 and 295 nm for AsLn3 (81). Similarly to luciferin itself, all of these analogues were fluorescent with emission maxima in the visible range. However, neither CompX nor AsLn(1–3) exhibited activity in the BL reaction with luciferase. The similarity of chromatographic and spectral properties of CompX and AsLn(1–3) with luciferin suggests their structural similarity. Moreover, in nonluminescent oligochaetes and in *Henlea* sp., which possesses a different BL system, these compounds were not detected. The authors hypothesized that CompX and AsLn (1–3) might represent biosynthetic precursors of *F. heliota* luciferin or its derivatives.

Structure elucidation of luciferin-related compounds

The content of CompX and AsLn2 in the biomass of *F. heliota* turned out to be 1–2 orders of magnitude higher than that of luciferin. This prompted the start of structural studies of *Fridericia* BL system with these alleged luciferin analogues.

In the year 2014, a 150 μg of pure CompX (82) and 100 μg of pure AsLn2 (83) were isolated from 90 g of *F. heliota* biomass (~45 000 worms). The ESI-HRMS of CompX revealed a molecular formula $C_{11}H_{10}O_6$. Based on the analysis of NMR spectra, CompX structure was determined as (Z)-5-(2-carboxy-2-methoxyvinyl)-2-hydroxybenzoic acid (Fig. 2a). This compound appeared to be an unusual derivative of tyrosine, three modifications of which (deamination, O-methylation and carboxylation) lead to the formation of CompX. Configuration of the CompX double bond was determined by total synthesis and comparative analysis of the 2D NMR spectra of both *E*- and *Z*-isomers. Synthetic *Z*-isomer was found to be identical to the natural sample, while (*E*)-CompX (Fig. 2b) exhibited considerably different properties, the most notable of which was the absence of fluorescence ability (82).

AsLn2 turned out to be an unusual peptide containing a CompX moiety, to which the residues of tyrosine and lysine are attached through amide (peptide) bonds (Fig. 2c), as supported by four-three-bond $^{1}H–^{13}C$ HMBC connectivities (83). Interestingly, lysine residue is connected to CompX carboxylic group via N(ω). The configuration of the double bond in AsLn2 was postulated to be the same as in CompX based on the chemical shift of vinylic double bond proton. The proposed structure, (Z)-N^6-(5-(3-((1-carboxy-2-(4-hydroxyphenyl)ethyl)amino)-2-methoxy-3-oxoprop-1-en-1-yl)-2-hydroxybenzoyl)lysine, is in agreement with all the NMR data and the observed molecular ion (83). The structure of AsLn2 was later confirmed by total synthesis. Synthesis was accomplished in six steps starting from (Z)-5-(2,3-dimethoxy-3-oxoprop-1-en-1-yl)-2-hydroxybenzoic acid (84).

Structure elucidation of *F. heliota* luciferin

Along with CompX and AsLn2, from the same 90 g portion of *F. heliota* biomass, 5 μg of pure luciferin was isolated (85). This small amount allowed to obtain only the ^{1}H, COSY and partial ^{13}C-HSQC NMR spectra. These data revealed the following three fragments of luciferin structure: substituted CompX, lysine and γ-aminobutyric acid (GABA). The scarcity of luciferin did not allow to collect 1D ^{13}C and HMBC spectral data, which could have revealed the connectivity of these three fragments and the presence of nonhydrogenated carbon atoms.

HRMS spectra of luciferin revealed the formula $C_{23}H_{29}N_3O_{11}$, with the atomic difference between this formula and the sum of the fragments, previously determined in 1H spectra being C_2O_3H. It was hypothesized that this difference might

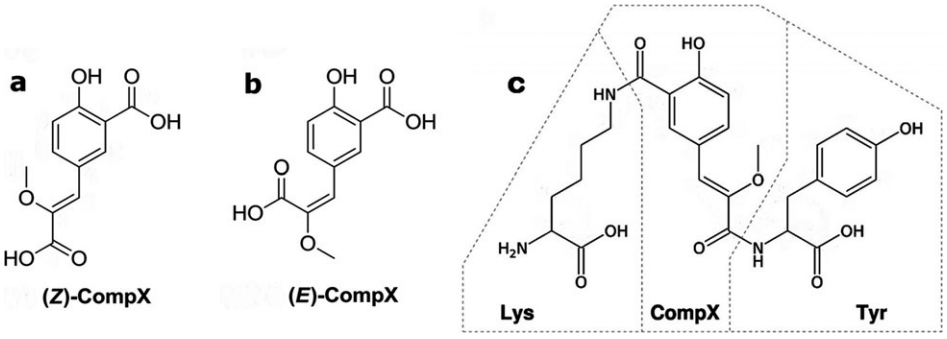


Figure 2. Structures of CompX, its unnatural *E*-isomer and AsLn2.

correspond to a residue of oxalic acid, attached to one of the amino groups of luciferin. Thus, four alleged fragments of luciferin structure were proposed: CompX, lysine, GABA and oxalate (85). ^{1}H NMR titration of luciferin in the pH range 3.1–7.5 indicated that the carboxylic groups of lysine and GABA were free, whereas the two carboxylic groups of the CompX moiety were involved in peptide bonds. Altogether, these spectral data were consistent with four isomeric structures, which differed only in the connectivity of four fragments (Fig. 3). All these four isomers were synthesized and assayed for the ability to produce light upon addition to the *Fridericia* protein extract in the presence of ATP and $MgSO_4$. Only synthetic compound **1** (Fig. 3) was able to generate light *in vitro* under biomimetic conditions. Moreover, only NMR spectrum of synthetic compound **1** matched with that of natural substrate. Thus, the structure of the novel 8th luciferin in the world list has been elucidated after a ~25 year interval.

Structure elucidation of new unusual peptides from *F. heliota*

Apart from the previously isolated AsLn2 (tyrosine–CompX–lysine), a range of other luciferin analogues was isolated from *F. heliota*: AsLn5, AsLn7, AsLn11 and AsLn12 (86). All new analogues are unusual peptides, built by the combination of one of the modified tyrosine residues (CompX or CompY) with the residues of γ-aminobutyric acid, threonine, homoarginine, of

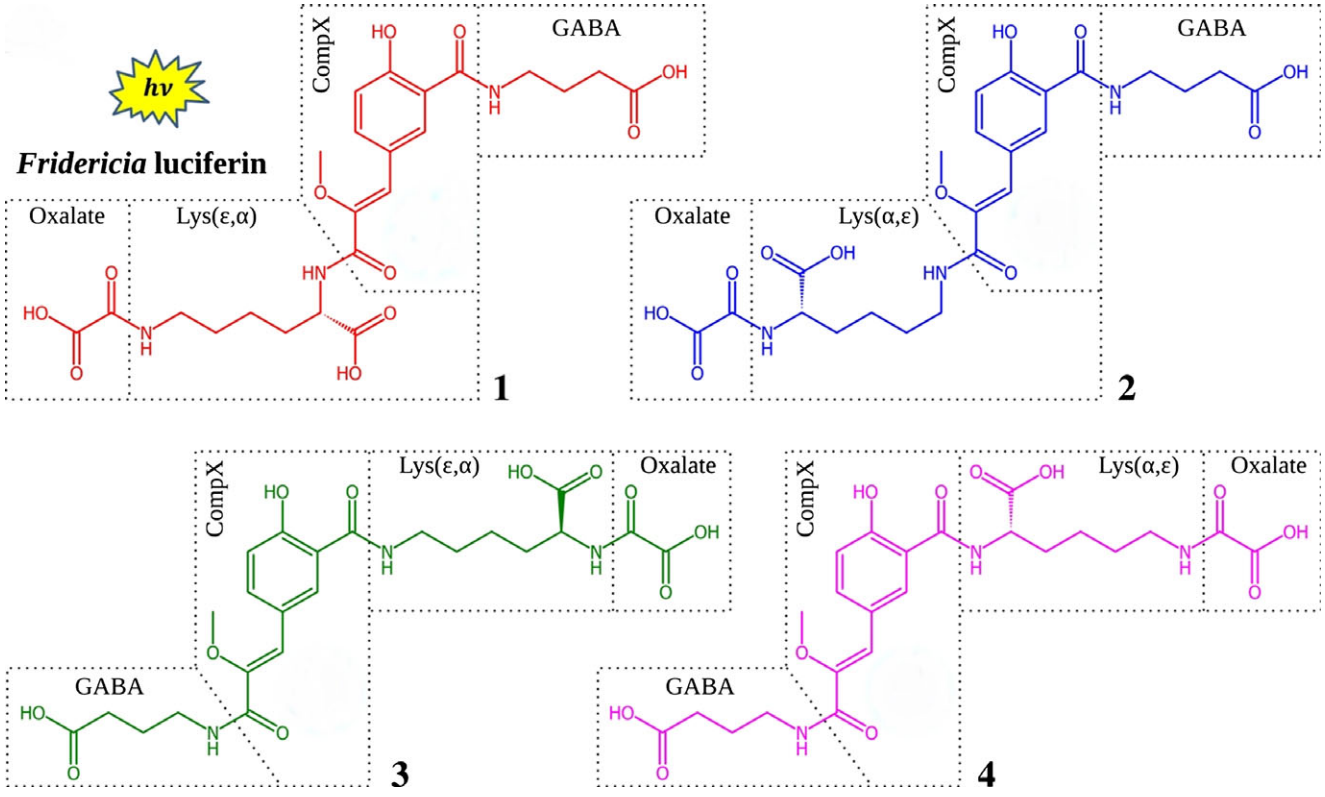


Figure 3. Structures of the synthetic isomeric peptides 1–4. Only one produced light when mixed with *Fridericia* luciferase. Adapted from (84). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

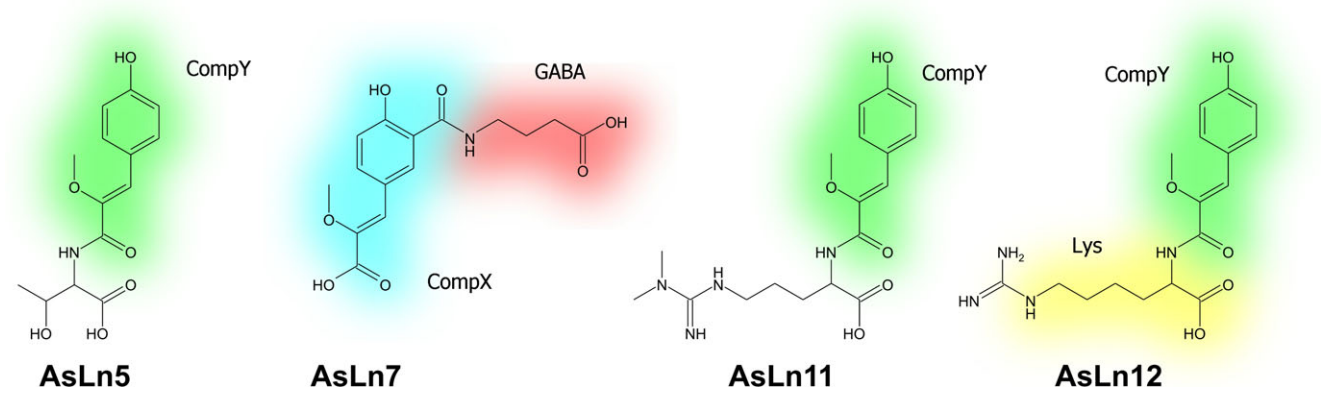


Figure 4. Novel unusual peptides from *Fridericia heliota*. Adapted from (78). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

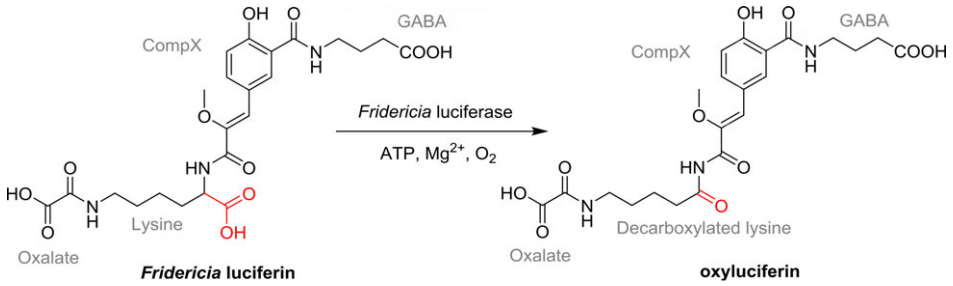
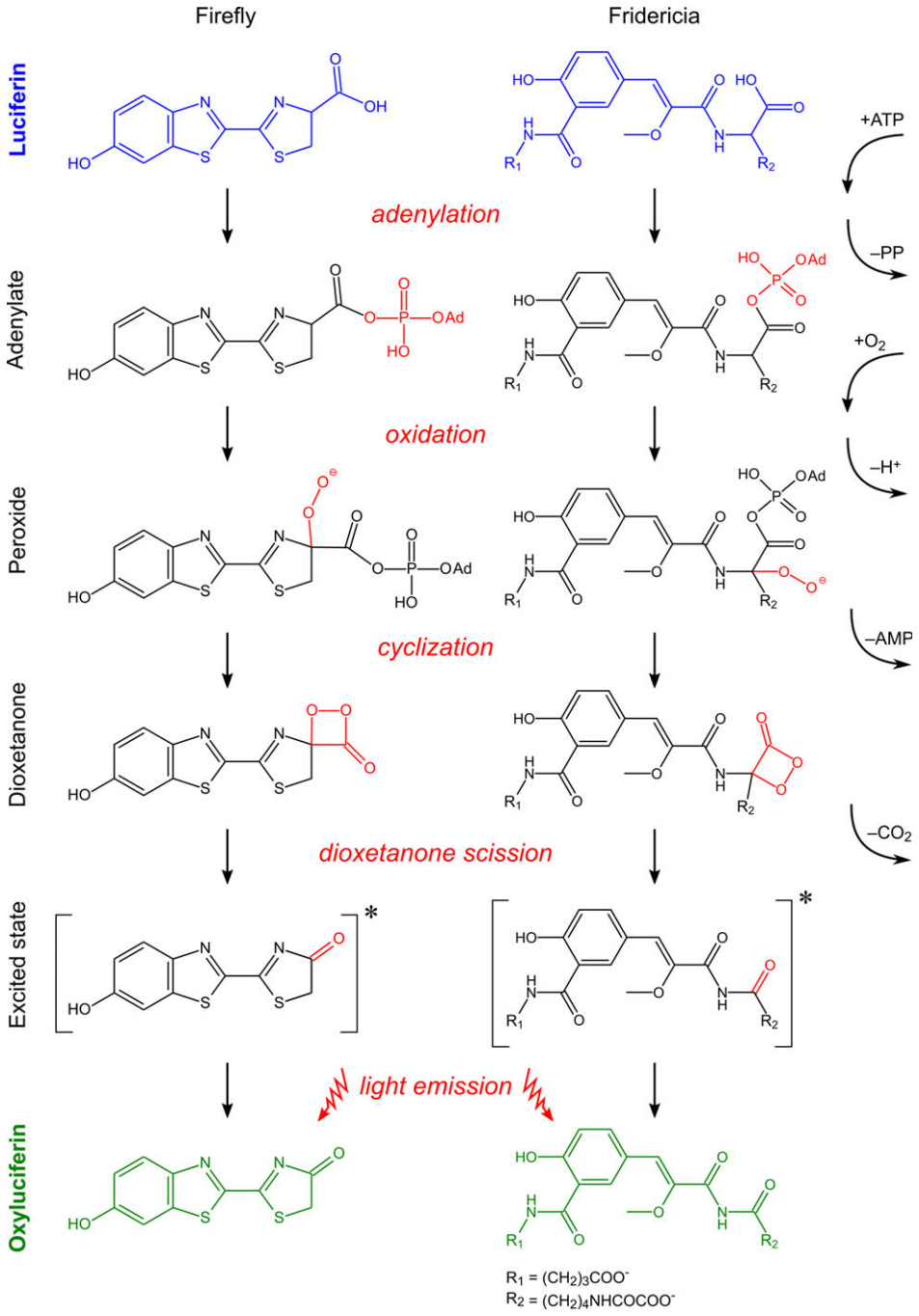


Figure 5. The bioluminescence reaction of *Fridericia heliota*, including structures of the luciferin and oxyluciferin.



Scheme 4. ATP-dependent mechanism of *Fridericia* bioluminescence in comparison with that of firefly bioluminescence. Adapted from (88) Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

unsymmetrical *N,N*-dimethylarginine (Fig. 4). Probably, CompX serves as a chromophore and fluorophore in *Fridericia* luciferin molecule. Analysis of NMR and UV spectra of novel luciferin analogues revealed the same chromophore in AsLn7, but a different chromophore in three other analogues AsLn5, AsLn11 and AsLn12. This new chromophore designated CompY differs from CompX by the lack of aromatic carboxylic group. Its identity and the configuration of trisubstituted double bond were also established by chemical synthesis.

Other structural fragments of AsLn5, AsLn11 and AsLn12 were established by the combination of NMR and mass spectrometry employing NMR titration approach to distinguish between free and amidated carboxylic groups (85,86).

The structures of AsLn2, AsLn5, AsLn7, AsLn11 and AsLn12 seem very unusual for terrestrial animals. Interestingly, CompX fragment is unique for *F. heliota*, whereas substituted CompY has been reported as a structural fragment of natural products isolated from ascidians (87,88).

At the moment, it does not seem possible to unequivocally determine the *Fridericia* luciferin biosynthetic pathway. However, the profusion in worm biomass of peptide-like compounds structurally similar to luciferin suggests that these modified peptides might be precursors or by-products of *Fridericia* luciferin biosynthesis.

Mechanism of *F. heliota* bioluminescence

After the establishment of *Fridericia* luciferin chemical structure, the question of its mechanism of action in the BL reaction arose. The first step toward answering this question is isolation and structure elucidation of the reaction product—oxyluciferin. The synthetic *Fridericia* luciferin was mixed with partially purified luciferase and excessive amounts of ATP and $MgSO_4$ (Fig. 5). HPLC reaction monitoring showed the formation of one major product of luciferin oxidation. Upon conversion of 62% of the luciferin, the reaction mixture was subjected to solid-phase extraction with subsequent HPLC (89).

The mass spectra of the resulted substance revealed its molecular formula to be $C_{22}H_{27}N_3O_{10}$. Taken together with ^1H, COSY and HSQC NMR, the data of oxyluciferin suggest that its formation proceeds through oxidative decarboxylation of the lysine moiety of luciferin.

Based on the structure of *Fridericia* oxyluciferin, obtained by enzymatic oxidation, a new light-generation mechanism, greatly resembling that of the fireflies, was proposed (Scheme 4) (89). Presumably, in the first step, *Fridericia* luciferase catalyzes the reaction between luciferin and ATP, leading to the formation of luciferin–adenylate conjugate at the lysine carboxyl group. The resulting conjugate then undergoes oxygenation and cyclization, yielding dioxetanone adduct. Subsequent electrocyclic scission of the dioxetanone ring accompanied by the release of CO_2 molecule produces the electronically excited oxyluciferin (90). In the final stage, the relaxation of excited oxyluciferin to the ground state results in the emission of blue light.

The structure of *Fridericia* oxyluciferin also suggests that CompX moiety is the light emitter in *Fridericia* bioluminescence. This hypothesis is supported by close similarity between the luciferin fluorescence emission spectrum and its bioluminescence emission spectrum (λ_{max} 466 and 480 nm, respectively) as well as with the fluorescence emission spectrum of oxyluciferin (λ_{max} 460 nm). The direct measurement of oxyluciferin fluorescence quantum yield (FQY) in water at pH 5.7 leads to a low value of 0.16%, which contradicted the observed bright luminescence of the live worms. Most likely, the high FQY of oxyluciferin *in vivo* occurs as a result of the steric stabilization of substrate when bound to the active site of luciferase.

The role of the luciferin adenylate as an intermediate in *Fridericia* bioluminescence was confirmed by a chemiluminescent study of a model luciferin-*tert*-butyl ester at the lysine carboxy group (89). The observed chemiluminescence of a model compound under the action of bases suggested that the formation of ester at the lysine carboxyl group facilitates the deprotonation of lysine α-H and further oxidation to form peroxide intermediate.

Thus, in the suggested BL mechanism, the energy for light generation is supplied by the decarboxylation of a lysine fragment of luciferin, while a fluorescent CompX moiety serves as light emitter. Further structural research on *Fridericia* BL mechanism is currently in progress. The next most important step will be isolation, sequencing and cloning of *Fridericia* luciferase.

CONCLUSION

The current efforts in studying the second Siberian enchytraeid *Henlea* sp. include the collection of sufficient amounts of biomass, detailed morphological description of the worm and specification of its precise position in the taxonomy of Enchytraeidae family. At the same time, the authors work on the optimization of chromatographic conditions for isolation and purification of the components of its novel Ca^{2+}-dependent BL system.

The discovery and structural investigation of novel BL systems provide new possibilities in tracing the evolution of different groups of organisms, and in unraveling the enigma of the biochemical origin of BL as well as its role in natural selection. Detailed comprehension of different chemical mechanisms of BL is important for understanding the basic principles of the conversion of the energy of chemical reactions into light quanta. From a practical point of view, each new basic finding in the field of BL leads to the creation of a diversity of applied techniques in analytics, technology and medicine.

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