Bioluminescence

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A Novel Type of Luciferin from the Siberian Luminous Earthworm *Fridericia heliota*: Structure Elucidation by Spectral Studies and Total Synthesis**

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Abstract: *The structure elucidation and synthesis of the luciferin from the recently discovered luminous earthworm Fridericia heliota is reported. This luciferin is a key component of a novel ATP-dependent bioluminescence system. UV, fluorescence, NMR, and HRMS spectroscopy studies were performed on 0.005 mg of the isolated substance and revealed four isomeric structures that conform to spectral data. These isomers were chemically synthesized and one of them was found to produce light when reacted with a protein extract from F. heliota. The novel luciferin was found to have an unusual extensively modified peptidic nature, thus implying an unprecedented mechanism of action.*

Bioluminescence is a fascinating phenomenon in which visible light is emitted by living organisms. Hundreds of species of bioluminescent animals, fungi, protists, and bacteria are known, and there are estimated to be 30 different chemical mechanisms underlying the generation of "cold light".[1] In all known cases, the energy required for light production is generated by the oxidation of a small organic molecule, luciferin, facilitated by a specific enzyme, luciferase. To date, the structures of only seven natural luciferins are known (Figure S1 in the Supporting Information).[1] The last structural characterization of a novel luciferin, from dinoflagellates, dates back 25 years.[2]

Recently, a novel bioluminescent species was discovered in Siberia.[3] *Fridericia heliota* is a small (ca. 15 mm in length, 0.5 mm in diameter, and ca. 2 mg in weight) white-yellowish oligochaete worm that inhabits forest soils and emits blue light (with a luminescence maximum at 478 nm) when mechanically stimulated. The luminescence of *F. heliota* is located in the region of the epidermal cells (Figure 3B).

The general concept of the common nature of luminescence in earthworms was based on the results of comparative studies of the physiology and biochemistry of 12 species belonging to 6 genera (*Diplocardia, Diplotrema, Fletcherodrilus, Octochaetus, Pontodrilus*, and *Spenceriella*).[4] All of these bioluminescent oligochaetes secrete a luminescent liquid containing coelomic cells, in the granules of which the luminescence is localized. Bioluminescence in oligochaetes is characterized by a common feature: the involvement of hydrogen peroxide. The luciferin of *Diplocardia longa*, *N*-isovaleryl-3-amino-propanal, serves as a substrate for the luciferases of all bioluminescent earthworms. In addition, *D. longa* luciferase is active in cross reactions with luciferins from other worms.[5]

The bioluminescence of potworms (of the family Enchytraeidae) has been known since 1838, although in lesser detail than that of megadriles, and it is confined to the genera *Henlea* and *Fridericia*.[6]

We demonstrated the light-production mechanism of *F. heliota* to be unique, since cross-reaction experiments with the luciferase or luciferin from *Fridericia* with luciferins and luciferases from other organisms were all negative. We found five components to be essential for *F. heliota* luminescence: *Fridericia* luciferase, *Fridericia* luciferin, ATP, Mg^{2+}, and atmospheric oxygen.[7,8]

The isolation and structural characterization of *Fridericia* luciferin has been seriously hindered by the scarcity of earthworm biomass (manual harvesting gave about 30 g/year), and the low content of luciferin (ca. 0.1 μg per gram of wet biomass).[9] In the course of our extensive efforts aimed at purification of *Fridericia* luciferin, we isolated CompX, a component of *Fridericia* biomass that is similar to luciferin by chromatographic and UV spectral behavior.[10] Spectral

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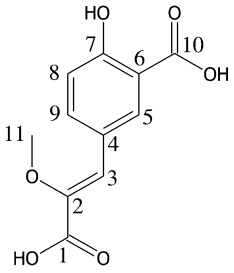

Figure 1. CompX, a structural analogue of *Fridericia* luciferin found in *Fridericia* biomass.

studies supported by total synthesis revealed CompX to be an unusual derivative of tyrosine (Figure 1).

The total amount of luciferin obtained from 90 g of biomass was 0.005 mg, and this small amount allowed us to obtain only the ^{1}H, COSY and partial ^{13}C-HSQC NMR spectra (Figure S4–S6). 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 us to obtain 1D ^{13}C and HMBC spectra, which would have revealed the connectivity of these three fragments and the presence of non-hydrogenated carbon atoms. Obviously, the most plausible way for these fragments to form a stable compound while leaving all of their C–H bonds intact (a requirement imposed by available NMR data) is the formation of peptide bonds between some of their four carboxylic acid and three amino groups. Therefore, we performed an ^{1}H NMR titration of luciferin in the pH range 3.1–7.5 in order to discriminate between the free carboxylic groups and those forming peptide bonds with the amino groups of the lysine and GABA residues. The pH dependence of the chemical shifts of the protons adjacent to titratable carboxy groups indicated that the carboxy groups of lysine and GABA are free, whereas the two carboxy groups of the CompX moiety are probably involved in peptide bonds (Figure S7).

The HRMS spectra of luciferin showed a molecular ion with $m/z = 524.1851$, for which the closest molecular formula is $C_{23}H_{30}N_3O_{11}^+$. The difference between this formula and (CompX + Lysine + GABA − 2 H_2O) is C_2O_3. In this calculation, the loss of two water molecules is assumed to result from the formation of two peptide bonds between the three specified fragments. Furthermore, the MS spectra showed two abundant peaks corresponding to the loss of CO_2 and of both CO_2 and CO, but not of CO alone (Figure S8). Taken together, these data suggest a monosubstituted oxalic acid residue to be a missing structural fragment of luciferin.

Four isomeric structures (**1**–**4**; Figure 2) were consistent with the NMR and mass spectroscopy data summarized above. These isomers differ only by the order of the peptide bonds connecting the four residues identified as the building blocks of *Fridericia* luciferin: CompX, lysine, GABA, and oxalate. Derivatives of L-lysine and racemization-preventing conditions were used throughout all of the syntheses.

We synthesized the isomeric peptides **1**–**4** and compared their NMR spectra with those of the natural sample (Table S1 in the Supporting Information). The synthesis started from CompX monomethyl ester[10] and utilized common peptide chemistry methods (Schemes S1–S4 in the Supporting Information). All of synthetic compounds gave similar NMR spectra in D_2O at pH 5.0. However, only **1** showed ^{1}H and ^{13}C chemical shifts completely matching those of the natural luciferin (Figure 2B, Table S1).

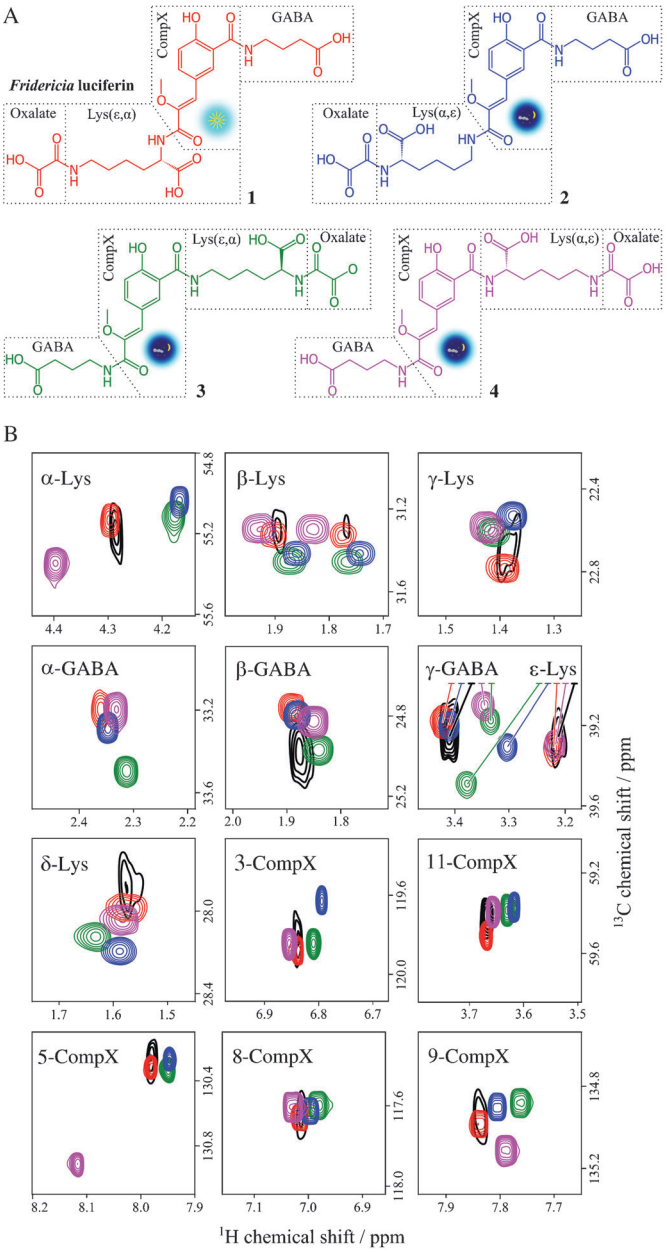

Figure 2. A) Structures of the synthetic isomeric peptides **1**–**4**. Only **1** produced light when mixed with *Fridericia* luciferase. B) A comparison of selected fragments of the ^{13}C-HSQC NMR spectra of *Fridericia* luciferin and compounds **1**–**4** (D_2O, 30 °C, pH 5.0). The colors of the peaks correspond to the compound colors shown in (A); the peaks for natural luciferin are shown in black.

We explored the ability of synthetic compounds **1**–**4** to produce light upon addition to the crude *Fridericia* luciferase in the presence of ATP and $MgSO_4$. Only synthetic compound **1** produced luminescence under these conditions, with a luminescence spectrum and intensity-concentration dependence identical to those of the natural luciferin (Figure 3 and Figure S3).

In summary, we report the structure of a novel luciferin, which represents a key component of a novel ATP-dependent bioluminescence system from the Siberian earthworm *Fridericia heliota*. This luciferin probably represents a fundamentally

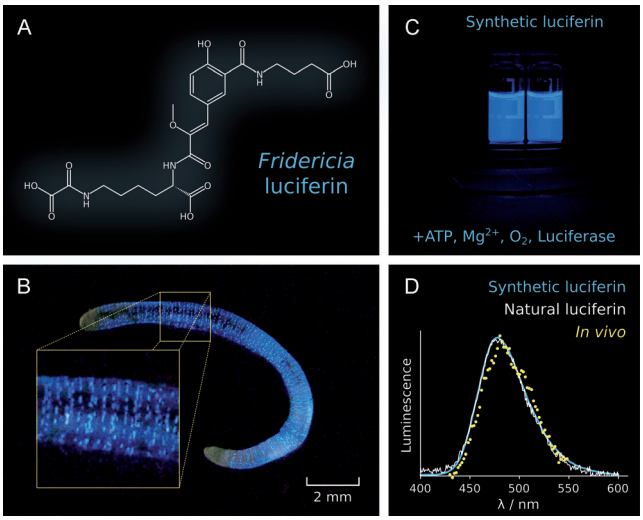

Figure 3. A) The structure of *Fridericia* luciferin. B) The bioluminescence of *Fridericia heliota*. The photograph is courtesy of Alexander Semenov (White Sea Biological Station, Biology Department of Lomonosov Moscow State University). C) The luminescence of synthetic Fridericia luciferin. D) A comparison of the in vivo bioluminescence spectra of the worms (yellow dots) with the in vitro bioluminescence spectra of natural (white line) and synthetic (blue line) samples of the luciferin.

novel chemical mechanism of luminescence, which will be evaluated in the near future. Presumably, an oxalate moiety is oxidized in the course of the luminescence reaction while a fluorescent CompX moiety serves as a light emitter, in a manner similar to the chemistry underlying the "glow stick" chemiluminescence toys. The role the of the CompX moiety as the light emitter is supported by the close similarity of luciferin fluorescence emission spectrum to the bioluminescence spectrum of *Fridericia heliota* (λ_{max} 466 and 480 nm, respectively).[10] An interesting question concerns the biosynthetic route that leads to *Fridericia* luciferin. In a recent study by Clardy et al., a carbonylated analogue of tyrosine similar to the CompX fragment was identified through genome screening for the members of the ATP-grasp enzyme family.[11] Considering the nonribosomal peptidic nature of the novel luciferin, its biosynthesis might utilize the enzymes of this family. A set of unusual peptides containing the CompX moiety is found in *F. heliota* biomass. One such peptide (a tyrosine–CompX–lysine derivative designated AsLn2) was described in our recent paper.[10b] The structures of other members of this family and their role in luciferin metabolism are presently under investigation. Our further efforts will be focused on the structural characterization of luciferin biosynthetic precursors and oxyluciferin, evaluation of the role of ATP, and on sequencing and cloning *Fridericia* luciferase.

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