Bioluminescence chemistry of fireworm *Odontosyllis*

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Marine polychaetes *Odontosyllis undecimdonta*, commonly known as fireworms, emit bright blue-green bioluminescence. Until the recent identification of the *Odontosyllis* luciferase enzyme, little progress had been made toward characterizing the key components of this bioluminescence system. Here we present the biomolecular mechanisms of enzymatic (leading to light emission) and nonenzymatic (dark) oxidation pathways of newly described *O. undecimdonta* luciferin. Spectral studies, including 1D and 2D NMR spectroscopy, mass spectrometry, and X-ray diffraction, of isolated substances allowed us to characterize the luciferin as an unusual tricyclic sulfur-containing heterocycle. *Odontosyllis* luciferin does not share structural similarity with any other known luciferins. The structures of the *Odontosyllis* bioluminescent system's low molecular weight components have enabled us to propose chemical transformation pathways for the enzymatic and nonspecific oxidation of luciferin.

bioluminescence | *Odontosyllis* luciferin | oxyluciferin | heterocycles

Having appeared dozens of times during evolution, bioluminescence serves as one of the prime means of intraspecies and interspecies communication among marine organisms. The chemical nature and mechanisms of action of the few known types of bioluminescence substrates (luciferins) are as diverse as their phylogenetic distribution. Advances in the development of a wide diversity of applied procedures have been primarily the result of increased understanding of the basic biochemistry of bioluminescence from a very few well-characterized systems, including beetles and coelenterates. Important medical applications, especially those related to gene expression and calcium ion detection, have been spawned by characterizing bioluminescence systems at the molecular level.

Odontosyllis undecimdonta fireworms are small (12 to 20 mm long) but display a spectacular bioluminescence (Fig. 1*A*), correlated with the lunar cycle. Luciferin-luciferase reactions of *Odontosyllis phosphorea* and *Odontosyllis enopla* were first demonstrated by Harvey in 1952 (1). Further in-depth investigations conducted by Shimomura et al. (2, 3) and Trainor (4) established that the *Odontosyllis* bioluminescence reaction requires only oxygen, a luciferin, and a luciferase enzyme to emit greenish-blue light. During the enzymatic luminescent reaction, luciferin is converted into an oxyluciferin (light emitter), which we refer to here as "Green," a compound with fluorescence (λ_{abs} = 445 nm, λ_{em} = 507 nm) highly similar to *Odontosyllis* bioluminescence. Nonenzymatic autooxidation on air produces a pink-colored substance (λ_{abs} = 520 nm), which we refer to here as "Pink." The susceptibility of the luciferin to decomposition presented a serious challenge to investigations of the *Odontosyllis* bioluminescence system due to the difficulty obtaining the pure substrate.

The *Odontosyllis* luciferase has recently been sequenced and cloned (5, 6). This enzyme has proven to be unique among sequenced polychaetes and all other sequenced organisms, suggesting that chemical basis of *Odontosyllis* bioluminescence is not similar to that of any other species.

To elucidate the structures of the components of *O. undecimdonta* luminescence system, we designed the purification procedure from the polychaete biomass. We used 2 sources of target compounds: lyophilized *O. undecimdonta* worms and lyophilized substrate fractions of the *O. undecimdonta* biomass, collected and partially purified by Inoue et al. (7) via acetone extraction and thin-layer chromatography separation. The lyophilized worms produced bright luminescence on addition of water (Fig. 1*B*), indicating that all components of the bioluminescent system remained active. Lyophilized luciferin samples also demonstrated luminescence activity on addition of "cold extract" prepared from lyophilized worms as described previously (4). These observations

Significance

We report the identification and characterization of the small molecule aspects of the *Odontosyllis undecimdonta* bioluminescence system. The chemical structures of the 4 best-known marine luciferins are as diverse as their phylogenetic distribution. The unique structure of *Odontosyllis* luciferin provides a key insight into a completely novel chemical basis of bioluminescence. *Odontosyllis* oxyluciferin is the only green primary emitter described for any known bioluminescent marine organism. Together with *Odontosyllis* luciferase, our recent findings provide insight into the biochemistry and photochemistry of a new light-emitting system. Our studies represent a crucial step in the development of orthogonal luminescence-based analytical methods for a variety of applications, including live animal imaging and pharmaceutical development.
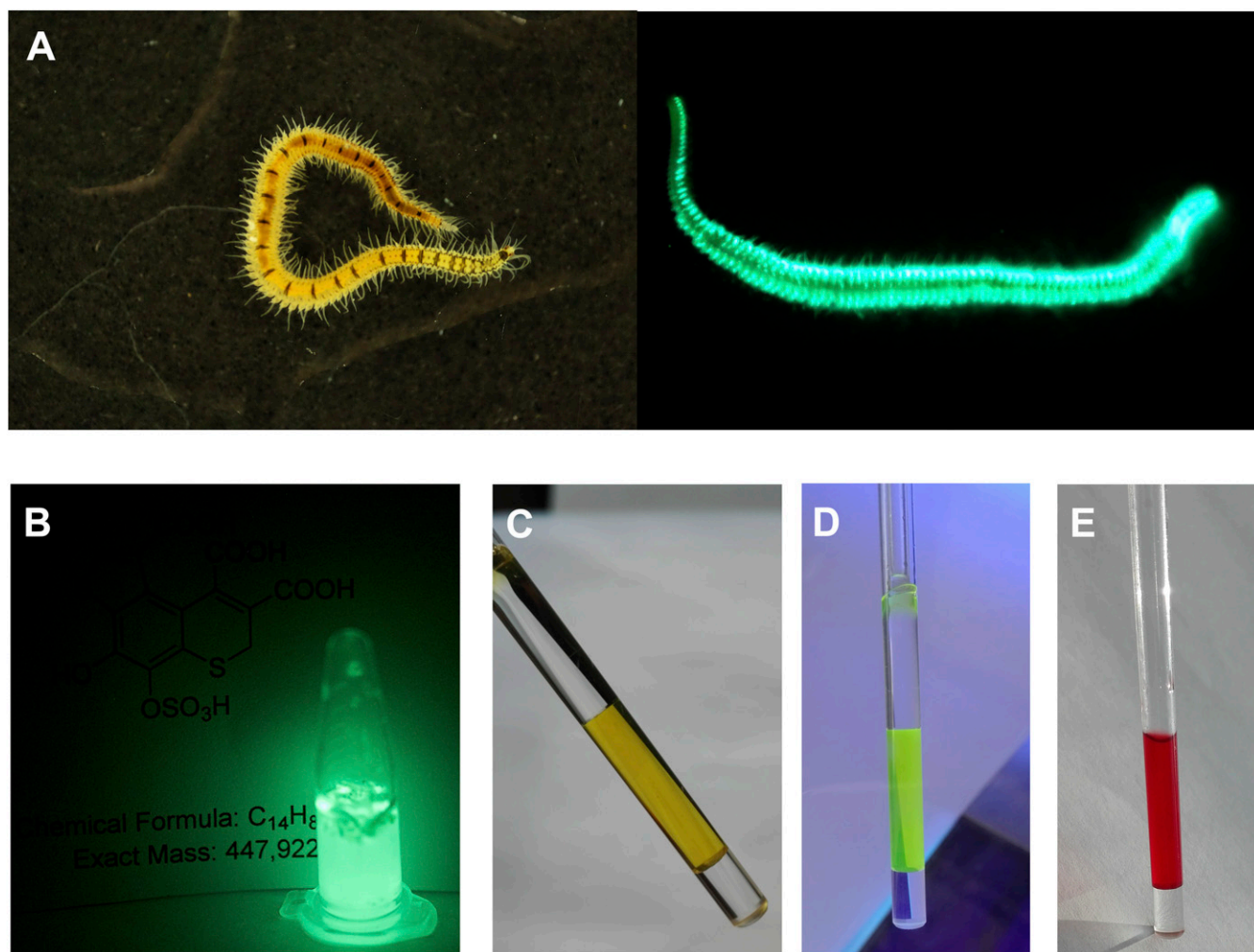
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Fig. 1. *O. undecimdonta* worms and components of its bioluminescence system. (*A*) The fireworm in daylight and in the dark. (*B*) Light emission of ly-ophilized worms in water. (*C*) NMR tube with the purified oxyluciferin (Green), visible light. (*D*) Fluorescence of Green, UV light. (*E*) NMR tube with the purified product of nonenzymatic oxidation of luciferin (Pink), visible light.

allowed us to conclude that we had functionally active luciferin for further structural and mechanistic studies.

We observed that aqueous extracts from lyophilized worms emit bright light at 25 °C, with the emission significantly decreasing at 4 °C but then increasing back to the initial intensity on thawing. This means that low temperatures reversibly inhibit the lumines-cent reaction, thereby enabling the extraction and separation of bioluminescence system components from *Odontosyllis* biomass. The highly labile nature and extremely low content of *Odontosyllis* luciferin dictated that the protocol for purification should include low temperatures, fast lyophilization, and shielding from light at all times. Failure to comply with the protocol led to the rapid visible conversion of colorless luciferin to Pink and to the irre-versible loss of bioluminescence activity.

Ion-exchange chromatography of a chilled water extract of *O. undecimdonta* worms allowed the separation of substrate and enzyme-containing fractions in the first chromatographic step (*SI Appendix*, Fig. S1). None of the fractions alone possessed bio-luminescence activity. Pairwise combination of all the fractions allowed the identification of those containing luciferin and lu-ciferase. Luciferin-containing fractions showed visible yellow-green coloration (Fig. 1*C*) and were fluorescent under UV light (Fig. 1*D*). Taking into account the earlier reports by Trainor (4), we considered that the observable yellow-green color was

likely caused by the presence of oxyluciferin, which necessitated further purification steps (*SI Appendix*, Fig. S2). Fractions con-taining luciferin or Green (*SI Appendix*, Fig. S2) were identified by bioluminescence assay or absorbance at 410 nm and were collected separately, lyophilized, and used for further analysis. The UV spectrum of Green (Fig. 2), while strongly pH-dependent, was similar to that of oxyluciferin reported by Shimomura et al. (3).

To confirm Green as the oxyluciferin, the purified *Odontosyllis* luciferin and luciferase preparations were mixed together in re-action buffer and monitored by high-performance liquid chro-matography (HPLC). The reaction mixture produced visible light, and the Green concentration increased over the course of the reaction (*SI Appendix*, Fig. S3 *A* and *C*). HPLC and high-resolution mass spectroscopy (HRMS) analyses showed that the product of enzymatic luciferin bioluminescence oxidation and Green had similar absorption spectra (*SI Appendix*, Fig. S3*B*) and identical molecular ions and fragmentation patterns in mass spectra (*SI Appendix*, Fig. S4). These data indicate that Green is in fact the *Odontosyllis* oxyluciferin.

Meanwhile, the HPLC analysis of lyophilized luciferin samples revealed that the most abundant compound (*SI Appendix*, Fig. S5) corresponded to Pink—the product of nonenzymatic oxida-tion of luciferin (Fig. 1*E*), whose absorption spectrum (Fig. 2) was similar to that described by Shimomura et al. (3).
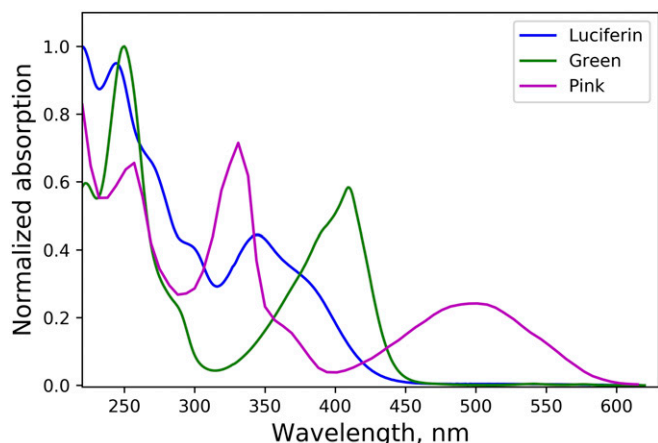
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Fig. 2. UV-visible spectra of *Odontosyllis* luciferin (blue line), *Odontosyllis* oxyluciferin (Green; green line), and the product of luciferin nonspecific oxidation (Pink; magenta line). Spectra were measured in the following conditions: luciferin in MeCN/H$_2$O 35%/65% containing 0.1% trifluoroacetic acid; Green in MeCN/H$_2$O 50%/50% containing 0.1% trifluoroacetic acid; Pink in MeCN/H$_2$O 25%/75% containing 0.1% trifluoroacetic acid.

HPLC-purified fractions containing Pink were combined (yielding 7.2 absorbance units), lyophilized, and subjected to nuclear magnetic resonance (NMR) and HRMS analyses. Only 3 hydrogens (Fig. 3C) and 12 carbon atoms were observed in ^{1}H, ^{13}C, and ^{1}H-^{13}C heteronuclear multiple quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) NMR spectra of Pink (*SI Appendix*, Fig. S6 and Table S1). The analysis of NMR correlation data revealed an aromatic core of Pink containing 2 sulfur atoms. The HRMS spectra of Pink showed [M+H]$^+$ with m/z = 402.9196, which corresponds to the formula C$_{13}$H$_7$O$_9$S$_3^+$ (calcd m/z = 402.9245), and an [M−H]$^-$ ion peak at m/z = 400.9123 closely matching the formula C$_{13}$H$_5$O$_9$S$_3^-$ (calcd m/z = 400.9101). The MS fragmentation pattern of deprotonated Pink unambiguously showed the presence of a sulfate group and 2 carboxylic acid groups (*SI Appendix*, Fig. S7).

A consecutive series of chromatographic experiments allowed the isolation of 10 and 2.6 absorbance units of pure oxyluciferin (Green) and luciferin, respectively, from 6 g of lyophilized worm biomass. The following NMR spectra were acquired for Green: ^{1}H, ^{13}C, 2D ^{13}C-HSQC, and 2D ^{13}C-HMBC (*SI Appendix*, Fig. S8 and Table S1). The observed NMR chemical shifts (Fig. 3B) and correlations coincided with the Pink carbon core, except for the loss of 1 aromatic singlet at position 7 (Fig. 3B) and the appearance of deshielded carbon atom in the same position (*SI Appendix*, Fig. S8). Electrospray ionization (ESI)-HRMS spectra of Green showed an [M+H]$^+$ ion at m/z = 418.9207 for which the closest molecular formula is C$_{13}$H$_7$O$_{10}$S$_3^+$ (calcd m/z = 418.9196). HRMS spectra of oxyluciferin recorded in negative-ion mode showed an [M−H]$^-$ ion with m/z = 416.9067, closely matching the formula C$_{13}$H$_5$O$_{10}$S$_3^-$ (calcd m/z = 416.9050) (*SI Appendix*, Fig. S9).
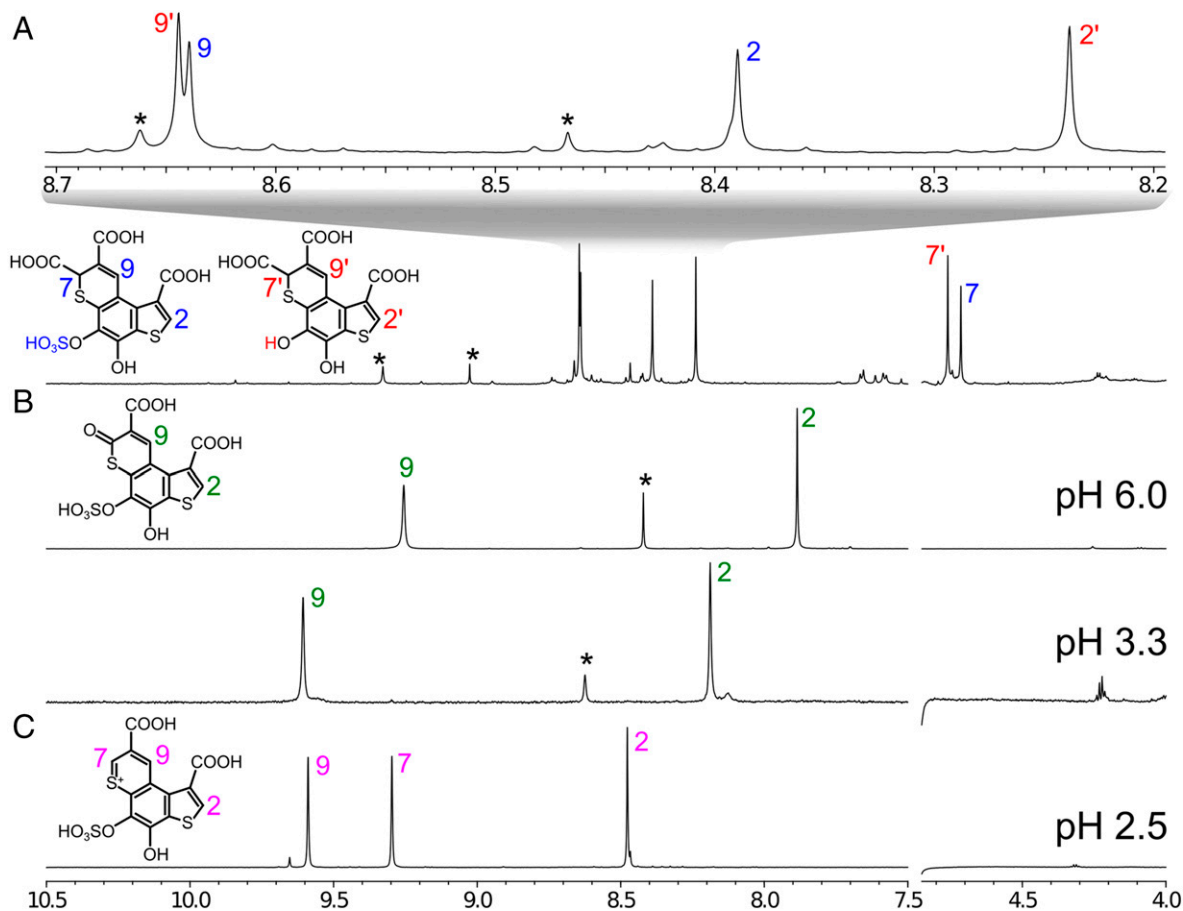
Fig. 3. ^{1}H NMR spectra of compounds from *Odontosyllis*. (*A*) Luciferin in methanol-d$_4$ at 10 °C, 800 MHz. (*B*) Oxyluciferin in H$_2$O + 10% D$_2$O at 15 °C, pH 3.3 (800 MHz) and pH 6.0 (600 MHz). (*C*) Pink in H$_2$O + 10% D$_2$O at 15 °C, pH 2.5 (800 MHz). Impurities are labeled by asterisks; signals of the compounds are labeled with numbers (*SI Appendix*, Table S1 and Figs. S6, S8, and S14).
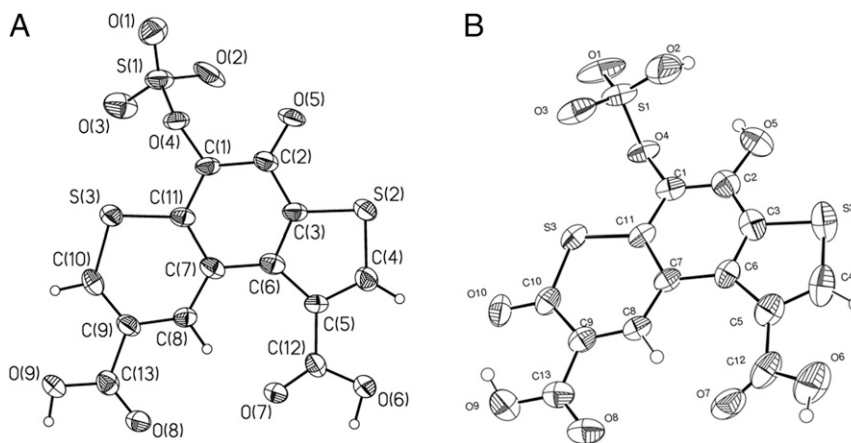
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Fig. 4. Structures of *Odontosyllis* oxyluciferin (Green) and product of nonspecific luciferin oxidation (Pink). (*A*) General view of *Odontosyllis* luciferin nonenzymatic oxidation product (Pink) as revealed by X-ray diffraction; nonhydrogen atoms are shown as thermal ellipsoids (*P* = 50%). (*B*) General view of oxyluciferin (Green) as revealed by X-ray diffraction; nonhydrogen atoms are shown as thermal ellipsoids (*P* = 50%).

As the NMR data were insufficient for unequivocal structure determination, single crystals of purified Pink and oxyluciferin were grown and subjected to X-ray diffraction studies (Fig. 4) to unequivocally establish the chemical structures of nonenzymatic luciferin oxidation product and oxyluciferin. Both Pink and oxyluciferin have identical tricyclic thieno[3,2-*f*]thiochromene skeletons, carboxylated at the C1 and C8 positions. The sole difference between the 2 molecules is the absence of a carbonyl group in the α-position with respect to the sulfur atom in thiopyran moiety of Pink compared with oxyluciferin. The NMR data for Pink and oxyluciferin were fully consistent with the X-ray structures (*SI Appendix*, Figs. S6 and S8).

The X-ray structures of Pink and Green played a key role in the subsequent elucidation of the parental *Odontosyllis* luciferin structure. ESI-HRMS spectra of luciferin showed an [M−H]⁻ion with m/z = 446.9167, while in positive mode an [M+H]⁺ molecular ion was observed with m/z = 448.9246. These data are consistent with the molecular formula $C_{14}H_8O_{11}S_3$ (calcd [M−H]⁻ m/z = 446.9156 and [M+H]⁺ m/z = 448.9301). HPLC-MS analysis also revealed a second compound in the luciferin sample with an [M−H]⁻ion at 366.9594, for which the closest molecular formula is $C_{14}H_7O_8S_2^-$. Both compounds showed similar retention times and almost identical fragmentation patterns (*SI Appendix*, Fig. S10). The tandem mass spectrometry (MS/MS) fragmentation pattern of deprotonated luciferin (m/z = 446.9156) showed a characteristic loss of SO_3 followed by elimination of water and sequential triple decarboxylation, leading to a heterocyclic core (*SI Appendix*, Fig. S11). In the mass spectrum of the second compound (m/z = 366.9594), an identical fragmentation pattern

was observed, differing only in the absence of the elimination of SO_3. It seemed reasonable to conclude that the second compound was O-desulfated luciferin (*SI Appendix*, Figs. S10–S13).

The HRMS analysis was further supported by NMR data (*SI Appendix*, Fig. S14 and Table S1). ¹H NMR of luciferin revealed 2 distinct molecules with a 1:1.25 mol/mol ratio (Fig. 3*A*). Both compounds had 3 singlets in ¹H NMR. Their ¹³C correlations in HSQC and HMBC NMR spectra were coincident (*SI Appendix*, Fig. S14), and the fragmentation patterns in HRMS identified the presence of SO_3 group as the only difference between these substances. The signals at 4.713/4.759 ppm in the ¹H NMR spectrum that were absent in Pink and Green support the loss of aromaticity in the core of luciferin otherwise shared with Pink and Green. The pattern of 2D NMR correlations assigned these signals to position 7 of the luciferin core (*SI Appendix*, Fig. S14). The integrals in ¹H NMR and the cross-peak sign in multiplicity-edited ¹³C-HSQC unambiguously demonstrated that the nonaromatic carbon at position 7 has only one directly attached proton, and thus the last C7 valence should be occupied with the third carboxylic group.

The resulting chemical structure of *Odontosyllis* luciferin (Fig. 3*A*) is in full agreement with the $C_{14}H_8O_{11}S_3$ molecular formula, the neutral loss of 3 CO_2 molecules observed in MS/MS fragmentation pattern (*SI Appendix*, Fig. S11), and the structures of Pink and oxyluciferin (Fig. 4 *A* and *B*). The proposed structure of luciferin is supported by literature data on the bioluminescence mechanisms of fireflies (8) and earthworms *Fridericia heliota* (9), where enzymatic decarboxylation of luciferins leads to the formation of a keto group in the corresponding oxyluciferins, as is seen in the chemical structure of Green.
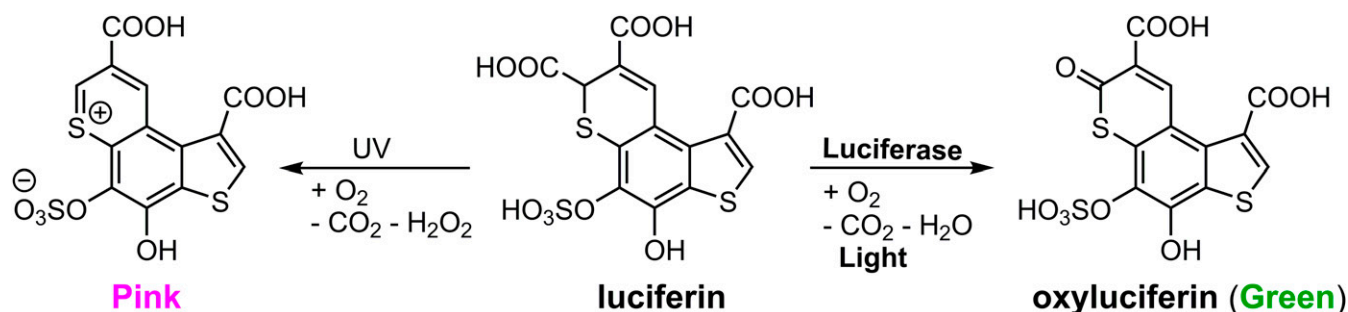

Fig. 5. Chemical structures of *Odontosyllis* luciferin, oxyluciferin (Green), and a product of nonspecific luciferin oxidation (Pink). Biochemical and chemical transformations lead to the oxyluciferin bioluminescence emitter and the nonspecific oxidation product.
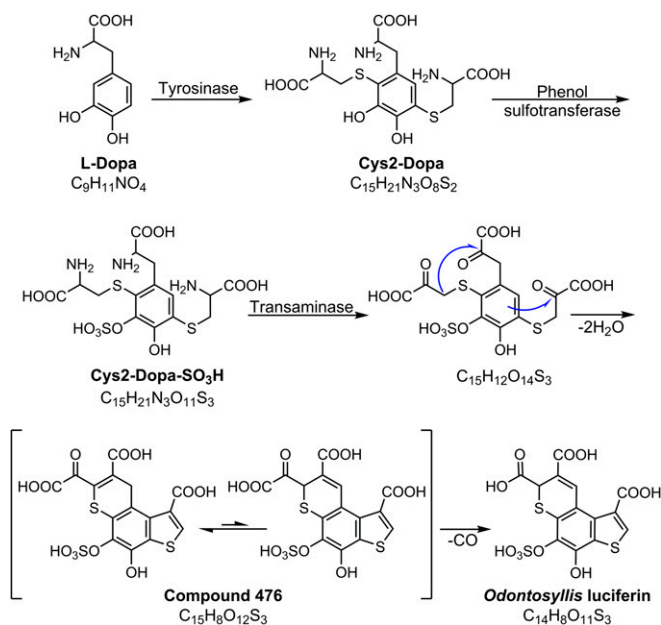
Fig. 6. Proposed pathway of *Odontosyllis* luciferin biosynthesis.

Based on the chemical structure of Green, we hypothesize that *Odontosyllis* luciferin undergoes an *Odontosyllis* luciferase-catalyzed oxidative decarboxylation at the α-position of thiopyran moiety, thereby generating oxyluciferin (Fig. 5). The structure of the nonenzymatic oxidation product suggests a visible-light–induced oxidative decarboxylation in the presence of O_2 as the oxidant (Fig. 5). Presumably, photoinduced oxidation of luciferin accompanied by the generation of H_2O_2 leads to formation of an *Odontosyllis* luciferin thiopyrylium cation (*SI Appendix*, Fig. S15), the α-decarboxylation of which affords Pink, as was previously reported for pyrylium salts (10, 11).

The structures of the newly identified compounds imply 2 possible pathways of *Odontosyllis* bioluminescence. In the first pathway, the reaction is initiated by the deprotonation at the C7 position of luciferin under luciferase catalysis, followed by addition of O_2 via single electron transfer (SET) oxidation (8), leading to the formation of a dioxetanone intermediate and concomitant elimination of water (*SI Appendix*, Fig. S16). In an alternative possible mechanism, luciferase catalyzes the formation of cyclic anhydride intermediate, thereby increasing the acidity of neighboring α-hydrogen and facilitating its deprotonation. The resulting carbanion is readily oxygenated by O_2 to form a peroxide anion, the cyclization of which leads to dioxetanone (*SI Appendix*, Fig. S16). In the final stage of both pathways, the release of CO_2 generates oxyluciferin in an electronically excited state, which produces blue-green light on relaxation to the ground state.

Based on the reasonable structural homology of *Odontosyllis* luciferin with benzothiazine moieties characteristic of pheomelanins (12), it is possible to propose a sequence of biosynthetic steps leading to the formation of luciferin (Fig. 6). It is likely that biosynthesis of luciferin starts with oxidation of a common tyrosine metabolite, L-DOPA, which undergoes oxidative coupling with 2 cysteine molecules, generating 2,5-dicysteinyldopa intermediate (Cys2-DOPA) (13). In turn, Cys2-DOPA may further undergo

sulfation and pyridoxal-catalyzed transamination, followed by spontaneous dehydrative cyclization via aldol condensation and electrophilic thiophene ring closure to form a tricyclic luciferin precursor, compound 476 (Fig. 6). In the final step, oxidative decarboxylation of the latter yields *Odontosyllis* luciferin (14, 15).

A strong argument in favor of this proposed mechanism is the presence of one of the predicted intermediates, compound 476, in the *Odontosyllis* biomass. The liquid chromatography–mass spectrometry profiles of the luciferin-containing fraction (*SI Appendix*, Fig. S17) show a major peak at m/z 476.9216 with a fragmentation pattern similar to that of the luciferin (*SI Appendix*, Fig. S18). The observed molecular ion corresponds to the molecular formula $C_{15}H_9O_{12}S_3^+$ (calcd m/z = 476.9251), with the difference between this formula and that of the luciferin being CO. Further analysis of NMR spectra of the HPLC-purified substance (^{1}H, ^{13}C-HSQC and ^{13}C HMBC) showed that compound 476 is a mixture of 2 tautomers (at a 3:1 ratio) with characteristic ^{1}H and ^{13}C chemical shifts and 2D cross-peak patterns coincident with the tricyclic thieno[3,2-*f*]thiochromene cores of Green, Pink, and luciferin (*SI Appendix*, Fig. S19). The significant change of chemical shift at the C7 position (155.3 ppm) of the major tautomer's heterocyclic core results from conjugation of the double bond to the carbonyl group, indicating the presence of oxalyl moiety at this position. Thus, the proposed structure of compound 476 shown in Fig. 6 is supported by both MS/MS fragmentation pattern and NMR data.

In summary, we report the isolation and structural characterization of the novel luciferin, a key component of the fireworm *O. undecimdonta* bioluminescence system, *Odontosyllis* oxyluciferin (Green), and the product of luciferin nonspecific oxidation (Pink). The elucidation of *Odontosyllis* luciferin and oxyluciferin structures is a key step in the study of a new bioluminescent system, allowing us to propose the possible mechanisms of the luminescent and nonenzymatic oxidative decarboxylation pathways of this bioluminescent system, as well as shed light on the luciferin biosynthesis pathway. The unusual structures of these molecules present a difficult challenge for synthetic chemists. Once obtained, a new synthetic luciferin together with the recombinant luciferase (5) will further promote investigations of the biosynthetic pathway of luciferin and the chemical origin of the *Odontosyllis* bioluminescence, as was recently accomplished for fungal bioluminescence system (16). The structures and mechanisms presented here have the potential to stimulate the development of new bioluminescence-based applications in the future.

Materials and Methods

Details of the materials and methods used in this study, including experimental procedures, materials and equipment used for structural characterization of *Odontosyllis* luciferin, oxyluciferin (Green), and the product of luciferin nonspecific oxidation (Pink), and supplementary figures and tables, are provided in *SI Appendix*.

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