Supplementary Information for

Bioluminescence chemistry of fireworm *Odontosyllis*: molecular mechanisms of enzymatic and non-enzymatic oxidation pathways.

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Sample collection

Worms *Odontosyllis undecimdonta* were collected by Prof. S. Inoue in 1993 (7), lyophilized and maintained at -20°C.

Luciferin extraction protocol by Inoue

Lyophilized *Odontosyllis undecimdonta* worms were washed with methanol and then luciferin was extracted by 70% acetone. Extract was applied to a TLC on silica-gel with MeOH:CH_2Cl_2 (2:1) or EtOAc:acetone:MeOH:H_2O (5:2:2:1). The samples of pink or purple color were collected, lyophilized and store at -20°C.

Extraction, separation and purification of luciferin and oxyluciferin

Water extraction of native luciferase, luciferin and oxyluciferin from lyophilized Odontosyllis undecimdonta worms

5 ml of phosphate buffer (5 mM sodium phosphate buffer, pH 7.4) was added to 200 mg of lyophilized worms. The mixture was pipetted into liquid nitrogen to create small drops of frozen material, which were ground in a mortar. Frozen powder was added to 15 ml of phosphate buffer (5 mM sodium phosphate buffer, pH 7.4) and incubated for 40 min with ice bath cooling and stirring. The mixture was centrifuged at 40000 g (4 °C) for 40 min. The supernatant, containing luciferase, luciferin and oxyluciferin, was collected and the cell-free extract was frozen and stored at -70°C.

Anion exchange chromatography of water extract

A water extract of *Odontosyllis undecimdonta* was applied to a DEAE Sepharose (GE Healthcare, Uppsala, Sweden) HiTrap Fast Flow column (1.6 x 2.5 cm) on an Akta Prime chromatography system (GE, USA) and was equilibrated and washed with 5 mM sodium phosphate buffer, pH 7.4 at rate of 5 ml/min. The column was eluted with a linear NaCl gradient from 0 to 0.4 M (80 ml) and 5 ml fractions were collected. To prevent bioluminescent reaction, the solvent, fractions and the column were maintained at 4°C. Analysis was performed on Akta Prime chromatography system (GE, USA). In this step specific activities of luciferase and luciferin were separated and detected by pairwise mixing of all fractions. The bioluminescence was monitored with a custom made luminometer Oberon-K (Krasnoyarsk, Russia). Luciferin and oxyluciferin (visibly green fluorescent) were not separated at this stage. All fractions were frozen. Luciferase fractions, obtained as described in (5), were used for luminescence measurement assay.

Concentration on Strata C18 solid phase extraction cartridge

Fractions that contained both luciferin and oxyluciferin were acidified by adding trifluoroacetic acid (0.05% trifluoroacetic acid) and applied to a 3 ml Strata C18 solid phase extraction cartridge (Phenomenex, Torrance, USA), which was previously equilibrated with 0.05% trifluoroacetic acid in water. After washing with 0.05% trifluoroacetic acid, the cartridge was eluted with acetonitrile containing 0.05% trifluoroacetic acid. Bioluminescence activity was monitored (see below for BL assay protocol) and fractions were lyophilized.

Reverse-phase chromatography

Further separation of luciferin and oxyluciferin was performed using a TSK ODS 120T 5 um column (4.6 mm x 250 mm, LKB-producter AB, Bromma, Sweden) with a Shimadzu chromatography system (Shimadzu corporation, Kyoto, Japan). Solvent A was 0.1% trifluoroacetic acid in water and solvent B was 0.08% trifluoroacetic acid in acetonitrile with a 10-50% B gradient over 20 min and a 1 ml/min flow rate. Absorbance was monitored at 220, 250, 330 and 410 nm. Fractions containing luciferin and oxyluciferin were collected separately and lyophilized.

Luminescence measurement assay

Reactions were monitored with a custom made luminometer Oberon-K (Krasnoyarsk, Russia). For each measurement 100 µl of reaction mixture (10 mM sodium phosphate buffer, 150 mM NaCl, 2 µl of luciferase fraction, pH 7.4) were used. Measurements were corrected for background luminescence of luciferase based on monitoring reactions for 20 s prior to luciferin addition.

Reverse-phase HPLC analysis of lyophilized substrate fractions of *O. undecimdonta* biomass

Each preserved sample vial was rinsed twice with 1 ml of water to dissolve the powder and the solutions were lyophilized. For HPLC analysis, samples were dissolved in 300 µl of 0.1 % aqueous trifluoroacetic acid. A column (5 µm, 9.2 x 150 mm) ZORBAX SB-C18 (Agilent Technologies, USA) coupled with the Nexera X2 (Shimadzu, Japan) was eluted with Solvent A (0.1 % aqueous trifluoroacetic acid) and solvent B (acetonitrile) by linear gradient from 10 to 35% (15 min) of solvent B at flow rate 3 ml/min. Fractions with bioluminescence activity and Pink were collected separately and lyophilized.

Conversion of luciferin into oxyluciferin, *in vitro* luminescent reaction experiment

For *in vitro* bioluminescence reaction TBS buffer (150 mM Tris-HCl, 1M NaCl, pH 7.5) was used. Each tube contained 60 µl of pure luciferin fraction, 40 µl of TBS buffer and 20 µl of highly-purified luciferase, obtained as described in (5), or TBS buffer in a case of negative control. Light emission was observed during the reaction, and there was no light from the control tube. The course of the reaction was monitored by taking 5 µl aliquots of the reaction and control mixes, quenching them after 0, 5, 15, 30, 60, 120, 180 and 300 min by addition of 20 µl of methanol, centrifugation, upon which the supernatants were analyzed by reverse-phase HPLC. Shim-Pack XR-ODS (3.0 x 75 mm, 2.2.µm) column coupled with the Nexera X2 (Shimadzu, Japan) was used. Solvent A was A - 0.1% formic acid in water, pH 4.9, solvent B was acetonitrile. The elution was done by linear gradient from 1 to 20% of solvent B (15 min) at flow rate 0.7 ml/min.

Separation of luciferin and *O*-desulfated luciferin, luminescence activity measurement of *O*-desulfated luciferin

Separation of luciferin and *O*-desulfated luciferin was performed using a Luna C-18 5 µm column (4.6 mm x 250 mm, Phenomenex, USA) with a Shimadzu chromatography system (Shimadzu corporation, Kyoto, Japan). Elution was performed with solvent A 0.1% trifluoroacetic acid in water and solvent B 0.08% trifluoroacetic acid in acetonitrile with a 20-37% B gradient over 20 min and a 1 ml/min flow rate. Absorbance was monitored at 220, 250, 330 and 410 nm. Two fractions containing predominantly luciferin and *O*-desulfated luciferin respectively were collected separately and lyophilized. Both samples were analyzed by reverse-phase chromatography, using the same column and conditions.

We were able to achieve only partial separation, obtaining two samples predominantly containing either luciferin or *O*-desulfated luciferin, each of which in turn included traces of the other (Fig. S12). Measurement of bioluminescence activity of partially separated compounds was carried out (Fig. S13). Low-level bioluminescence activity observed for *O*-desulfated luciferin sample is most likely due to the presence of trace amounts of luciferin in it as it is roughly proportional to the content of luciferin admixture. Thus the *O*-desulfated compound most likely does not possess bioluminescence activity.

Purification of native luciferase and recombinant luciferase samples preparation

Luciferase samples for luminescence measurements and *in vitro* conversion experiments were obtained as previously described in (5).

Purified samples of luciferase were obtained by consecutive purification steps of lyophilized worms water extract: ion exchange chromatography on DEAE Sepharose HiTrap Fast Flow column (GE Healthcare, Uppsala, Sweden), ultrafiltration with Amicon® Ultra centrifugal filter unit (Merck Millipore, Germany) and size exclusion chromatography on Superdex 200 column (Phenomenex, USA).

Recombinant luciferase was prepared from the luciferase cDNA, that was synthesized as linear dsDNA fragment (Twist Biosciences, USA) and cloning was performed by Golden Gate assembly. Eukaryotic expression plasmids were assembled into MoClo kit plasmid pICH47742 as a backbone, and the following parts were cloned in Level 0 vectors: CMV promoter, luciferase candidate gene, stop-codon containing DNA part and SV40poyA terminator. HEK293NT cells were transfected by the result plasmid with FuGene 6 reagent (Promega, Fitchburg, WI, USA) in accordance to the manufacturer's protocol. Transfected cells were cultivated under standard conditions.

X-ray crystallography, NMR spectroscopy and mass spectrometry procedures

NMR spectroscopy

High-resolution NMR spectra of Pink (7.2 absorbance units), Green (3.6 o.d.u at pH 3.3 and 10 o.d.u at pH 6.0) and Compound 476 (5.o.d.u at pH 4.2 and 5.3) were acquired at Bruker Avance III 600 MHz (pH 6.0) or 800 MHz (pH 3.3) NMR spectrometer equipped with 5mm PATXI ^{1}H-^{13}C/^{15}N/D Z-GRD probe. Samples were dissolved in H_2O/10%D_2O, pH values were measured and spectra acquired at 15°C to prevent degradation. The measured pH values in NMR samples were: Pink - 2.5, Green - 3.3 and 6.0 in two different preparations and 4.3 and 5.2 (two data sets) in Compound 476. The following spectra were acquired: ^{1}H with water suppression, ^{13}C, 2D ^{13}C-HSQC, 2D ^{13}C-HMBC (J_{long}= 6.0Hz), multiplicity-edited 2D ^{13}C-HSQC. Chemical shifts were referenced to the water signal (4.882 ppm) at 15°C (1).

The NMR spectra of luciferin (2.6 absorbance units) were acquired in methanol-d_4 at Bruker Avance III 800 MHz NMR spectrometer equipped with cryogenically cooled 5mm CPCTI ^{1}H-^{13}C/^{15}N/D Z-GRD cryoprobe, the spectra were acquired at 10°C to prevent degradation. The following spectra were acquired: ^{1}H, multiplicity-edited 2D ^{13}C-HSQC and three 2D ^{13}C-HMBC (J_{long}= 3.5, 6.0 and 9.0Hz). The raw NMR data of all compounds is available at https://goo.gl/HFYToB

Mass spectrometry

HPLC-MS analysis was performed using a Thermo Scientific LTQ Orbitrap hybrid instrument with Waters Alliance 2695 separation module equipped with a Phenomenex Aeris WIDEPORE C4 column (3.6 µm, 150 × 2.1 mm). Samples were eluted with a H_2O-MeCN gradient (from 5 to 55% of MeCN) with 0.1% formic and 0.02% trifluoroacetic acids as eluent additive. Acquisition was carried out by full scan MS in FT mode (15K resolution, small mass range) with electrospray ionisation and positive or negative ion detection. MS^n experiments were performed with the same instrument in continuous flow direct sample infusion. Spectra were collected in FT mode using CID or PQD fragmentation. Fragmentation energy was tuned manually at each fragmentation step to achieve maximum fragment ions intensity.

HRMS spectra of Compound 476 were obtained on an Agilent 6224 TOF LC/MS System (Agilent Technologies, Santa Clara, CA, USA) equipped with a YMC-Triart C18 column (3 µm, 12 nm, 75 × 4.6 mm) and dual-nebulizer ESI source. Samples were eluted with a H_2O-MeCN gradient (from 5 to 50% of MeCN) with 0.1% trifluoroacetic

acids as eluent additive. Data acquisition and analysis was performed by the MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA).

X-ray Crystallography of Pink

Crystals of the product of non-enzymatic oxidation of luciferin (Pink) grown from methanol in air contained three water molecules per two symmetry-independent species of Pink and two hydroxonium cations ($C_{26}H_{22}O_{23}S6$, M = 894.79). The crystals were red, triclinic, space group P-1. X-ray diffraction data were collected at protein station of Kurchatov Centre for Synchrotron radiation (λ = 0.9699 Å) at 100(2) K: a = 7.4300(15), b = 13.970(3), c = 16.760(3) Å; α = 94.64(3), β = 100.50(3), γ = 102.81(3)°. Intensities of 17466 reflections were measured, and 6282 independent reflections [R_{int} = 0.0693] were used in the further refinement. The structure was solved by the direct method and refined by the full-matrix least-squares against F^2 in the anisotropic approximation for non-hydrogen atoms with the SHELXTL PLUS software (2). Hydrogen atoms of OH groups were found in difference Fourier synthesis; the H(C) atom positions were calculated. All hydrogen atoms were refined in the isotropic approximation in the riding model. The refinement converged to wR_2 = 0.2421 and GOF = 1.086 for all the independent reflections (R_1 = 0.0808 was calculated against F for 4141 observed reflections with I > 2σ(I)). Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre, CCDC No. 1840215.

X-ray Crystallography of Green

Crystallization

Purified oxyluciferin (Green) was crystallized from a mixture of acetonitrile/methanol (1:3) containing 0.1% of formic acid by slow evaporation of the solvent. After 4 days, two large transparent reddish rod-shaped crystals appeared. The crystals were fragile and cracked upon retrieval from the mother liquor. A fragment of sufficient size (0.05×0.05×0.2 mm) was mounted in a cryoloop with a drop of parabar oil and flash frozen in cold nitrogen gas stream.

Data collection and processing

Two X-ray diffraction datasets were collected at 100 K on a Kappa Apex II diffractometer (Bruker) using CuKα radiation. After a complete dataset was obtained, the crystal was annealed by diverting the nitrogen gas stream for several seconds and then vitrified again. The annealing improved data quality as judged by reflection profiles and merging statistics, therefore the second dataset was used in a further analysis. The

data were integrated with *Saint* and scaled with *Sadabs* in *Apex2* software suit (Bruker. SAINT, SADABS, APEX2. Bruker AXS Inc., Madison, Wisconsin, USA, 2008–2012).

Structure determination and refinement

All further structure determination and refinement procedures were performed in *Olex2* software suit (3). The structure of oxyluciferin in space group *P-1* was readily solved by intrinsic phasing with *ShelXT* program (4). Two nearly planar molecules related by an inversion were stacked in the unit cell and bonded through interactions with two metal ions. The metal ions were identified as potassium based on the total number of electrons in the electron density peak and average M-O distance of 2.82 Å, which corresponds well with the average value for K from CSD (2.81 Å) (5). Each potassium ion was bound by two carboxyl oxygen atoms and two sulfate oxygen atoms of oxyluciferin molecules within the same unit cell, and two sulfate oxygen atoms belonging to molecules from adjacent cells. Crystal packing features pores possibly occupied by solvent molecules. After all non-hydrogen atoms were refined in the anisotropic approximation with the *ShelXL* program (6), residual electron density peaks were modeled by water and methanol molecules coordinated with potassium ions, and an additional water molecule linked to oxyluciferin by a hydrogen bond. After resolving solvent molecules, the R-factor was 10.7%, and further refinement was done by solvent masking in *Olex2*. The residual void volume was 78.7 $Å^3$ with 22.8 e^- per unit cell and was probably occupied by a mixture of acetonitrile (86.9 $Å^3$, 22 e^-) and formic acid (62.8 $Å^3$, 24 e^-). This final refinement resulted in the oxyluciferin structure shown in Fig. 7B of the main text with the R-factor equal to 8.2%. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre, CCDC No. 1840216.

Supplementary Figures

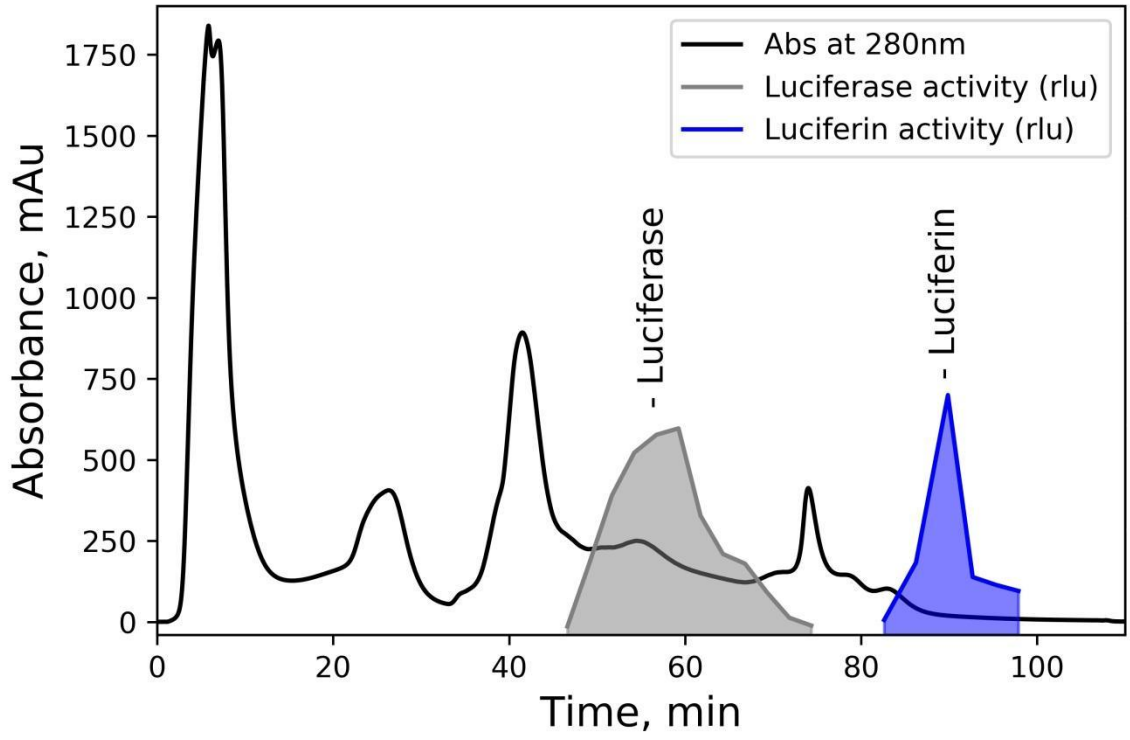

Figure S1. Chromatographic profile of water extract from lyophilized *Odontosyllis undecimdonta* worms anion-exchange chromatography on DEAE Sepharose HiTrap Fast Flow column. Solid line – 280 nm absorption signal. Activity profiles of *Odontosyllis* luciferase and luciferin (relative light units) are shown as gray and blue lines respectively. See “luminescence measurement assay section” for protocol details.

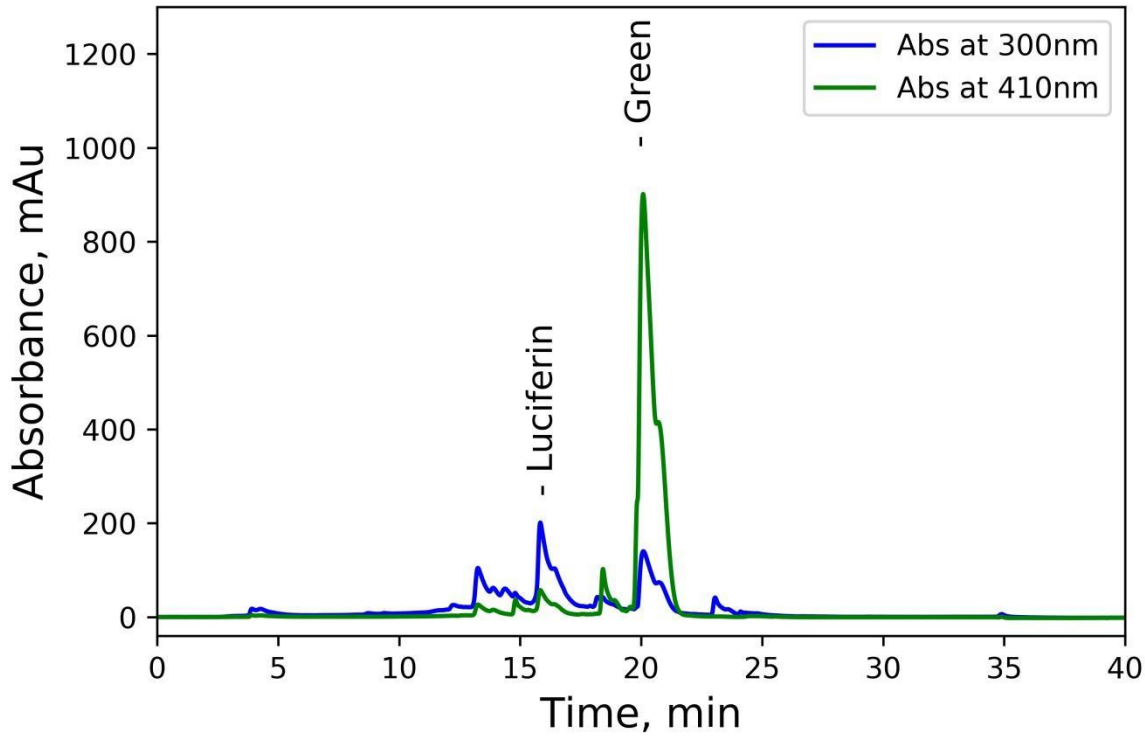

Figure S2. Reverse phase chromatographic profile of luciferin-containing fraction from anion-exchange chromatography on TSK ODS 120T 5µm column (monitored at two different wavelengths: 300 nm and 410 nm). Luciferin and *Odontosyllis* oxyluciferin (Green) peaks are assigned respectively.

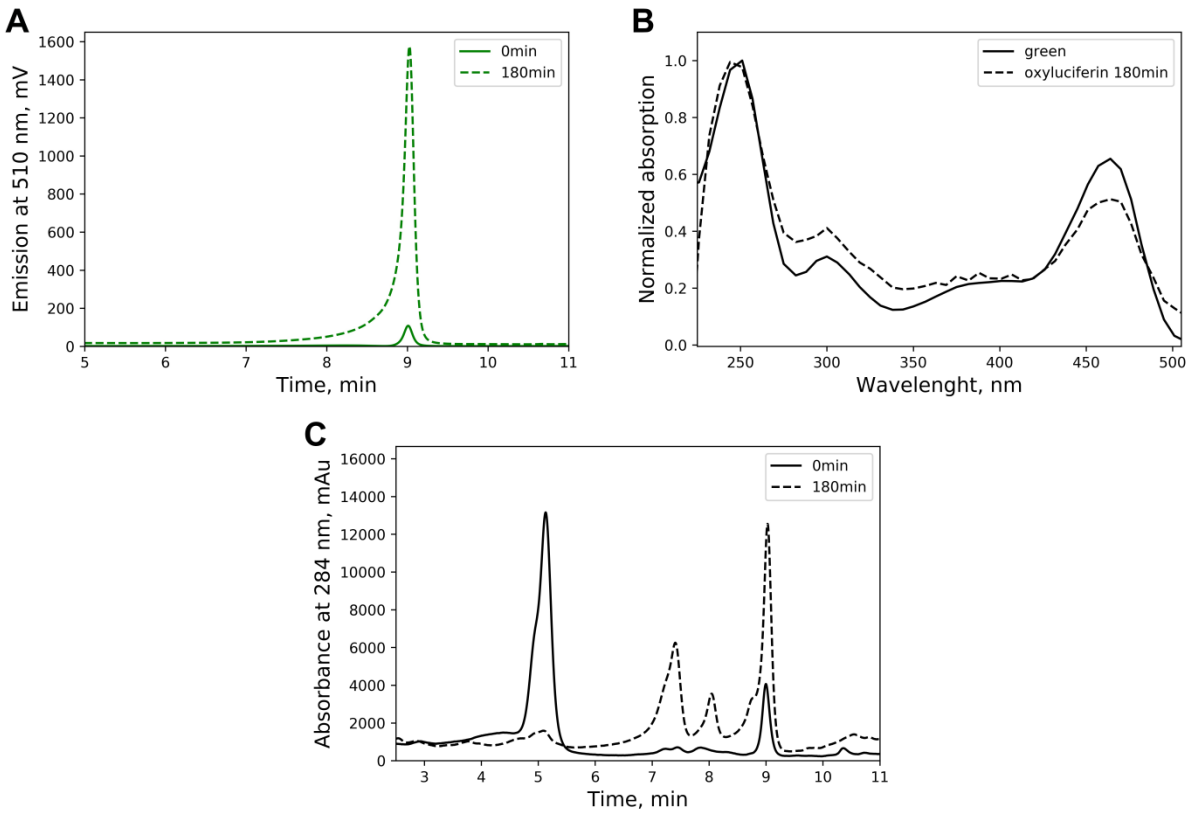

Figure S3. Conversion of luciferin into oxyluciferin, *in vitro* luminescent reaction experiment. **A** – Reverse phase chromatographic profile of reaction mix at start of experiment (solid line) and after 180 min (dashed line) on Shim-Pack XR-ODS (3.0 x 75 mm, 2.2.µm) column. Fluorescence detector was used, excitation – 465 nm and emission – 510 nm. **B** – UV-vis spectra of purified Green (solid line) and oxyluciferin (dashed line) from experiment after 180 min of *in vitro* luminescent reaction. **C**. Reverse phase chromatographic profile of reaction mix at start of experiment (solid line) and after 180 min (dashed line) on Shim-Pack XR-ODS (3.0 x 75 mm) column. Decrease of luciferin peak at 5 min and increase of oxyluciferin peak at 9 min are simultaneously observed.

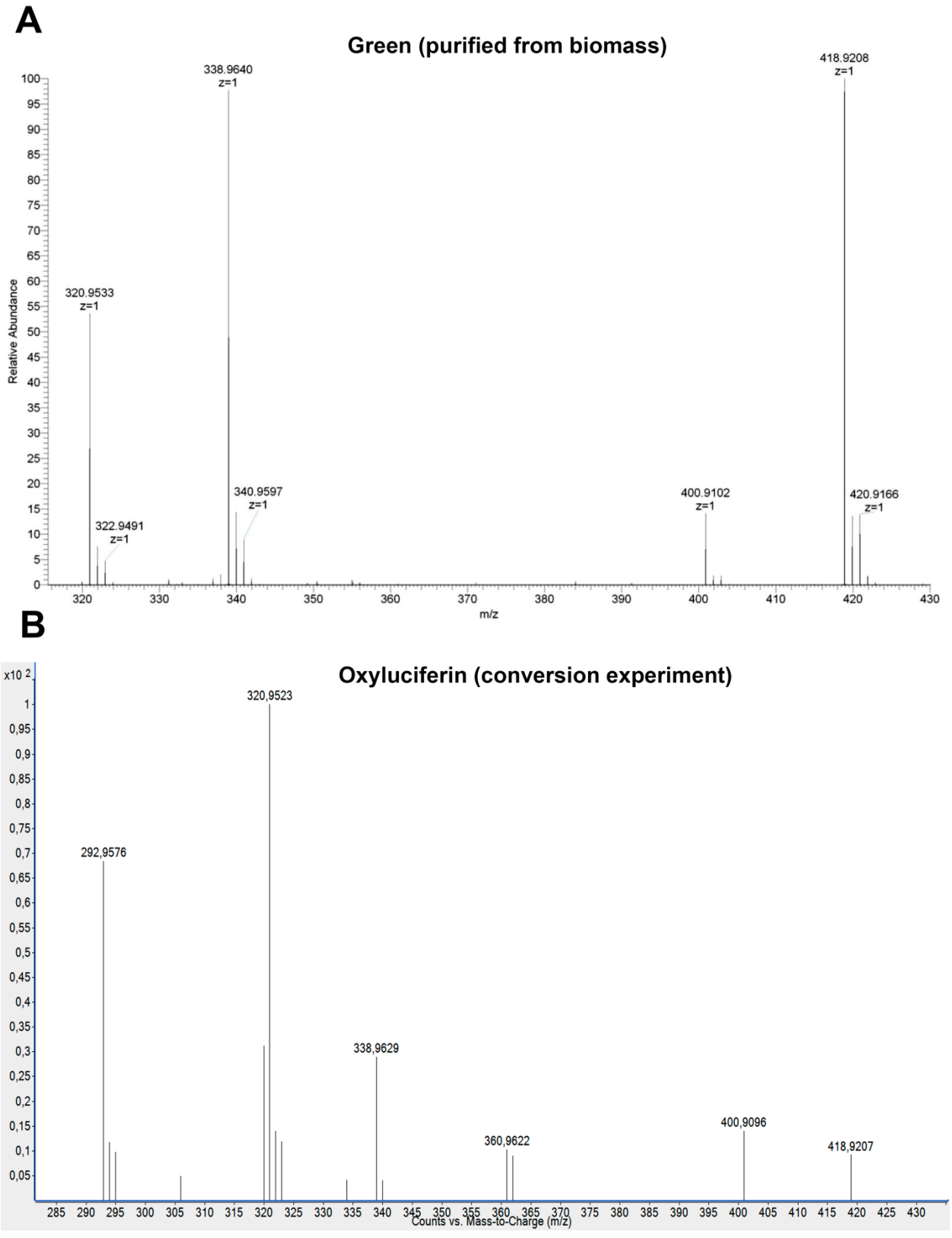

Figure S4. Mass spectra of Green (**A**) and oxyluciferin –product of *in vitro* bioluminescence reaction (**B**) displaying identical molecular ions and similar fragmentation patterns.

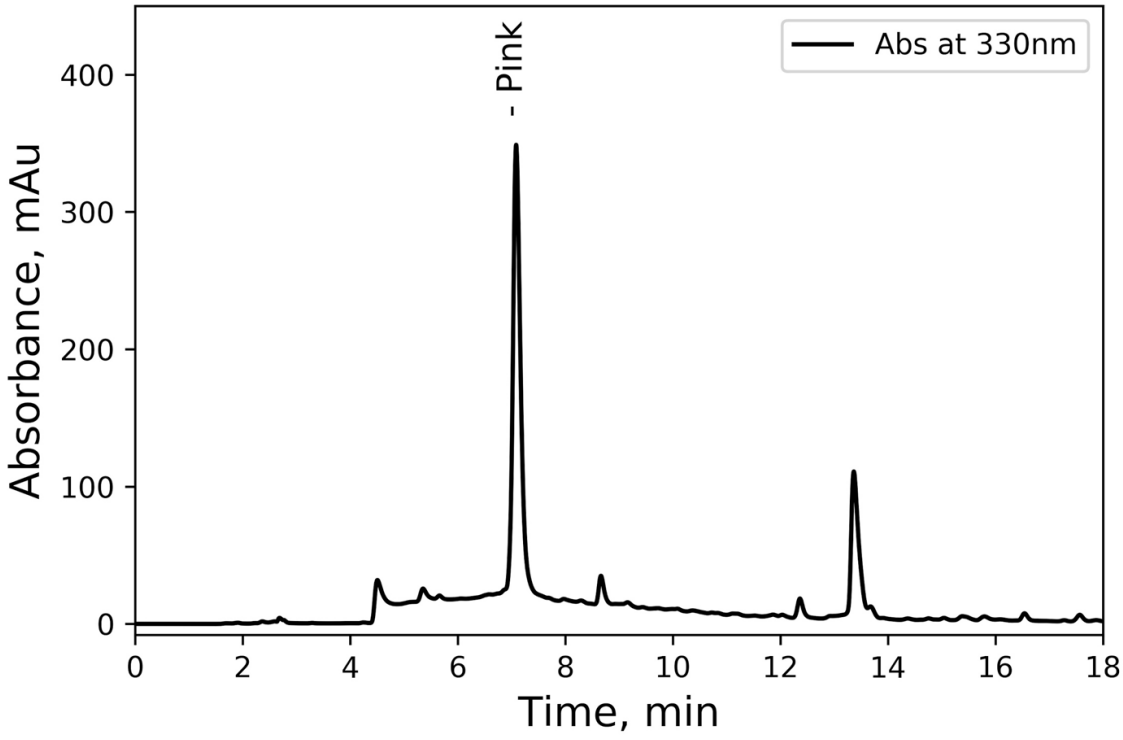

Figure S5. Reverse phase chromatographic profile (λ_{abs} = 330nm) of samples collected from Prof. Inoue on ZORBAX SB-C18 column. The product of non-enzymatic oxidation of luciferin peak assigned as Pink.

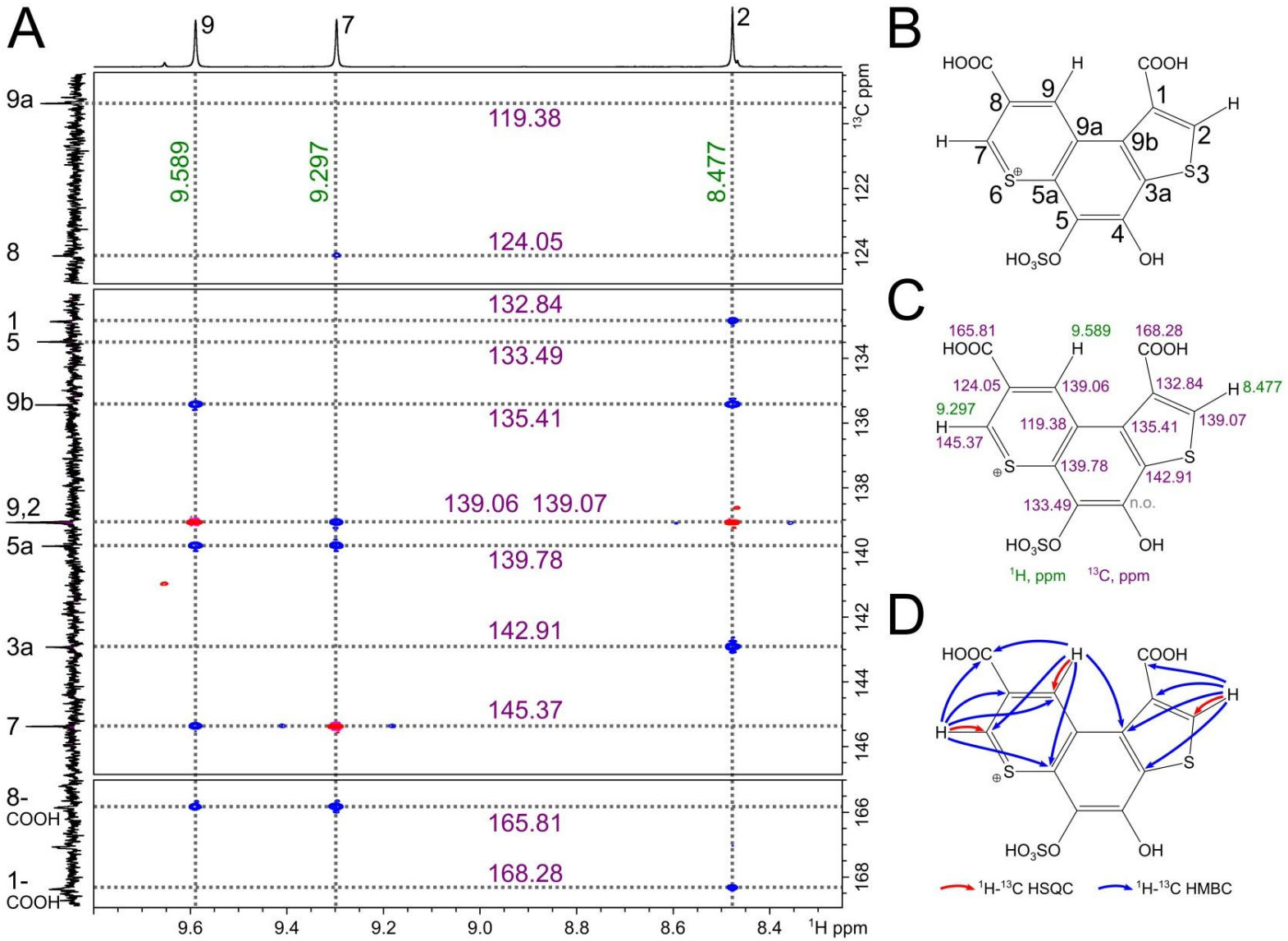

Figure S6. NMR spectra of Pink from *Odontosyllis*. **A** 1D and 2D NMR spectra of Pink (D_2O, 15°C 800MHz). ^{1}H NMR is overlaid on the top and ^{13}C NMR spectrum is at left corner of 2D correlations ^{13}C-HSQC (red) and ^{13}C-HMBC (blue) NMR spectra. Atom numbers and NMR chemical shifts are outlined. **B.** Chemical formula of Pink with heavy atom numbering. **C.** ^{1}H and ^{13}C chemical shifts of Pink, n.o. - not observed. **D.** ^{1}H-^{13}C correlations in 2D NMR spectra of Pink used for chemical shift assignment.

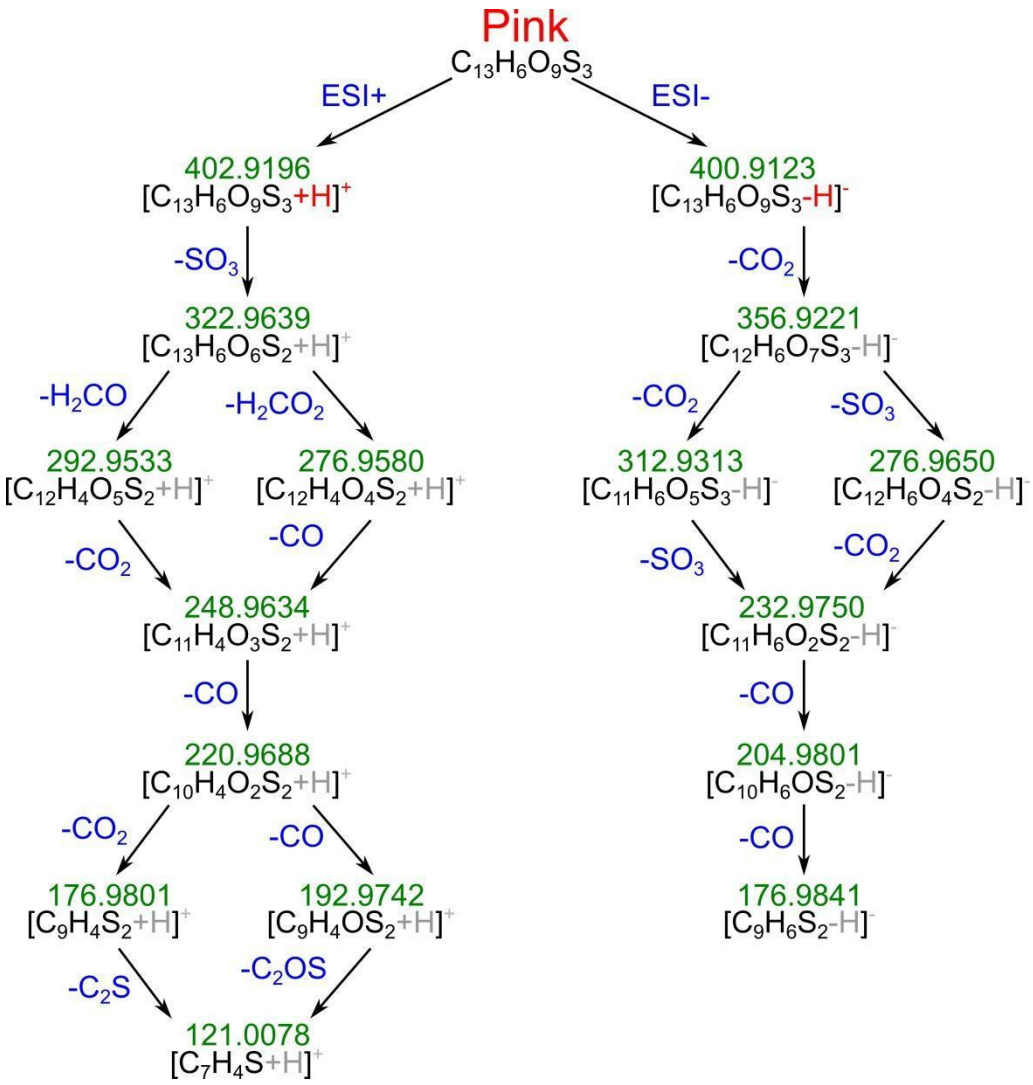

Figure S7. MS fragmentation of Pink in positive and negative modes. Ionization/neutral fragments losses are outlined in blue, observed m/z peaks are green, proposed formula for each fragmentation product is black.

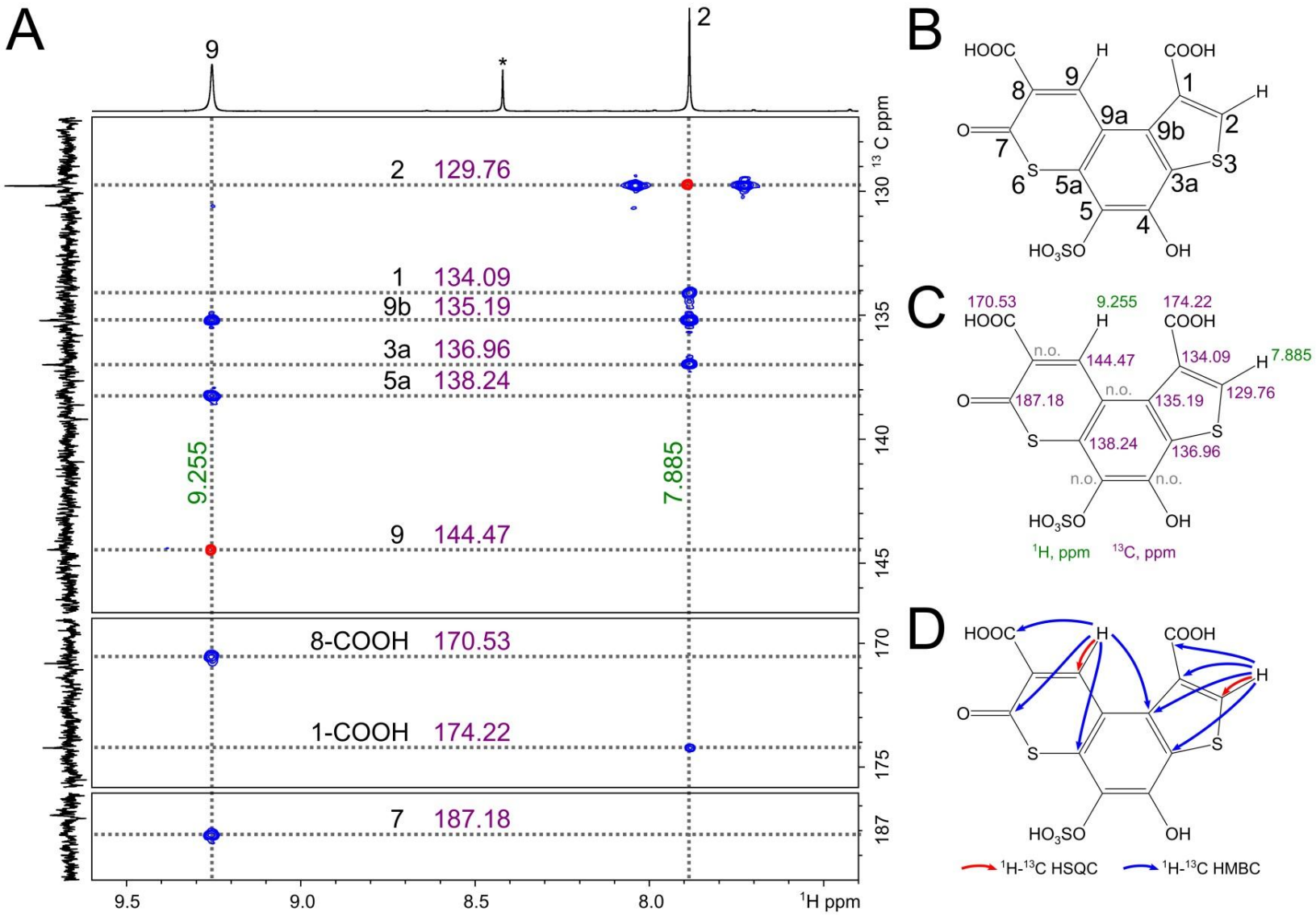

Figure S8. NMR spectra of Green from *Odontosyllis*. **A** 1D and 2D NMR spectra of Green (D_2O, 15^{o}C 600MHz). ^{1}H NMR is overlaid on the top and ^{13}C NMR spectrum is at the left corner of 2D correlations ^{13}C-HSQC (red) and ^{13}C-HMBC (blue) NMR spectra. Atom numbers and NMR chemical shifts are outlined. **B.** Chemical formula of Green with heavy atom numbering. **C.** ^{1}H and ^{13}C chemical shifts of Green, n.o. - not observed. **D.** ^{1}H-^{13}C correlations in 2D NMR spectra of Green used for chemical shift assignment.

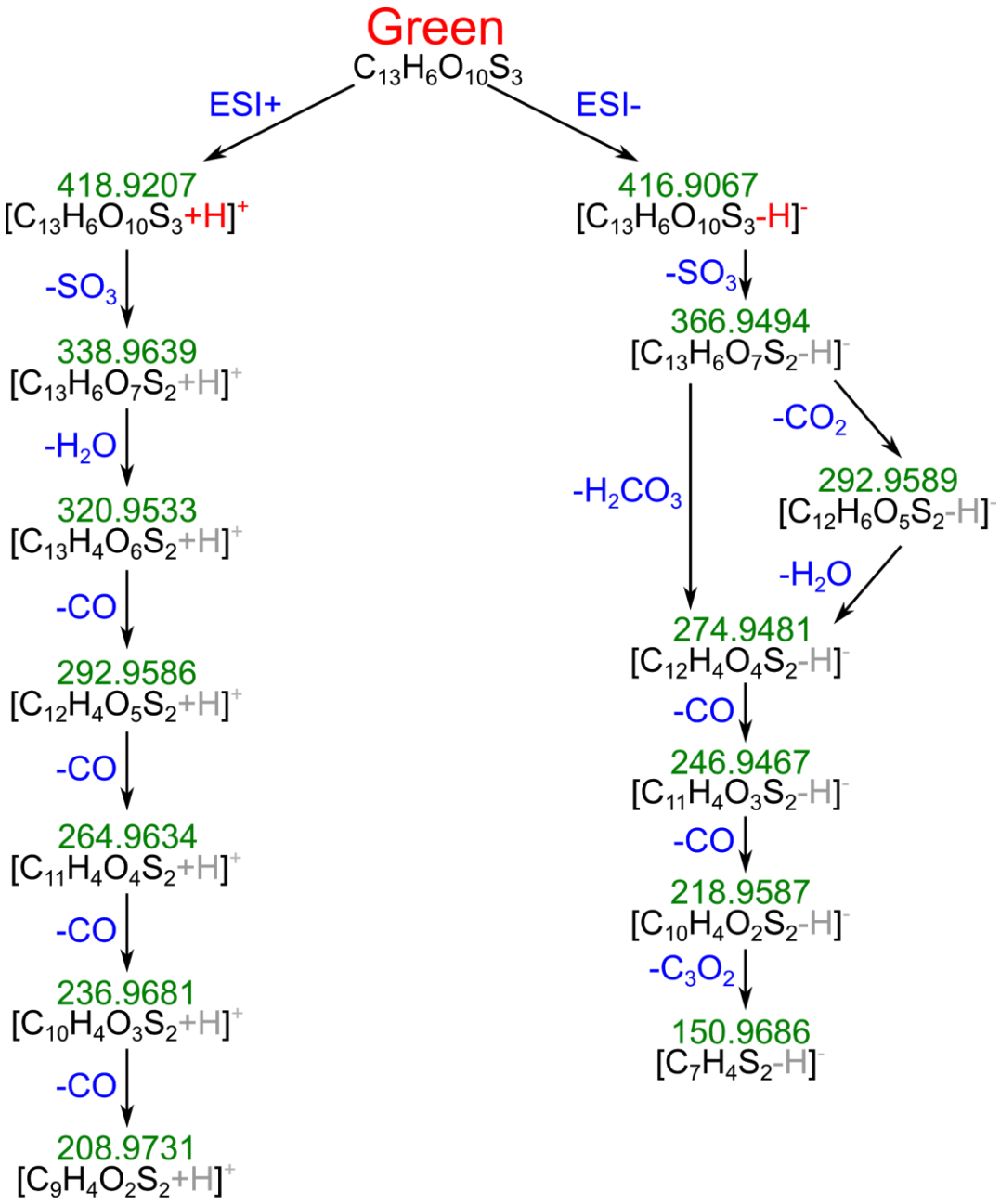

Figure S9. MS fragmentation of Green in positive and negative modes. Ionization/neutral fragments losses are outlined in blue, observed *m/z* peaks are green, proposed formula for each fragmentation product is black.

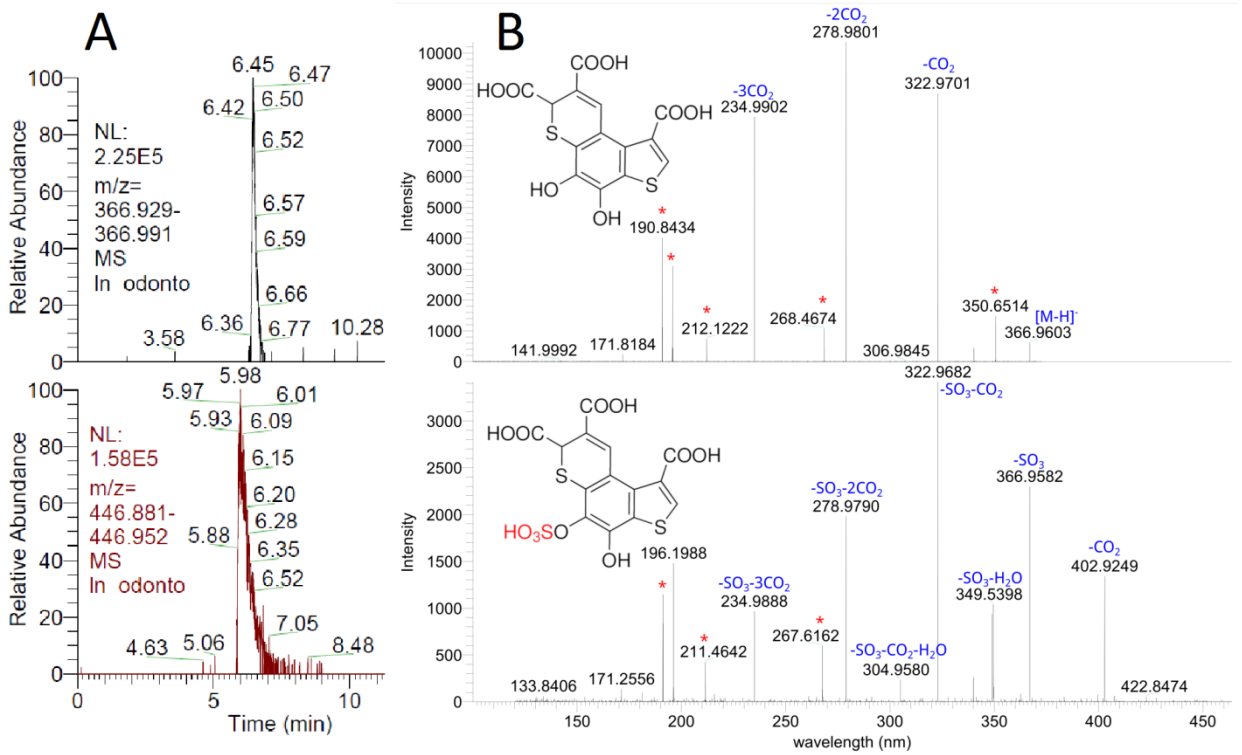

Figure S10. **A.** Extracted ion current chromatograms and **B**. MS^2 spectra of *O*-desulfated *Odontosyllis* luciferin (top) and luciferin (bottom). Noise peaks are marked with red asterisks.

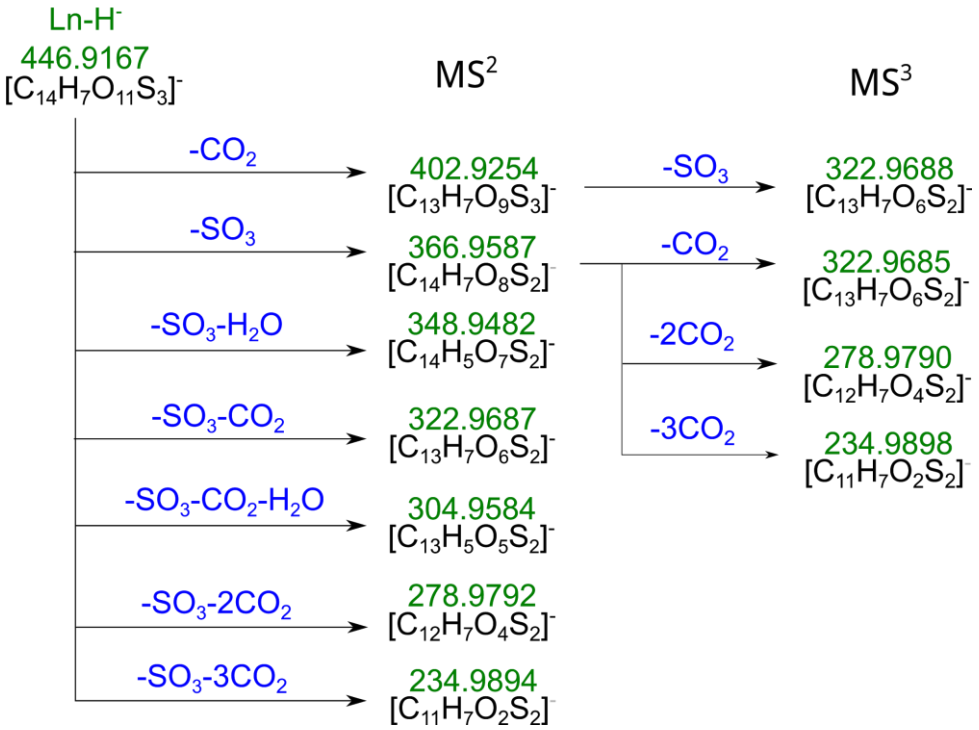

Figure S11. MS fragmentation of *Odontosyllis* luciferin in a negative-ion mode. Ionization/neutral fragments losses are outlined in blue, observed *m/z* peaks are green, proposed formula for each fragmentation product is black.

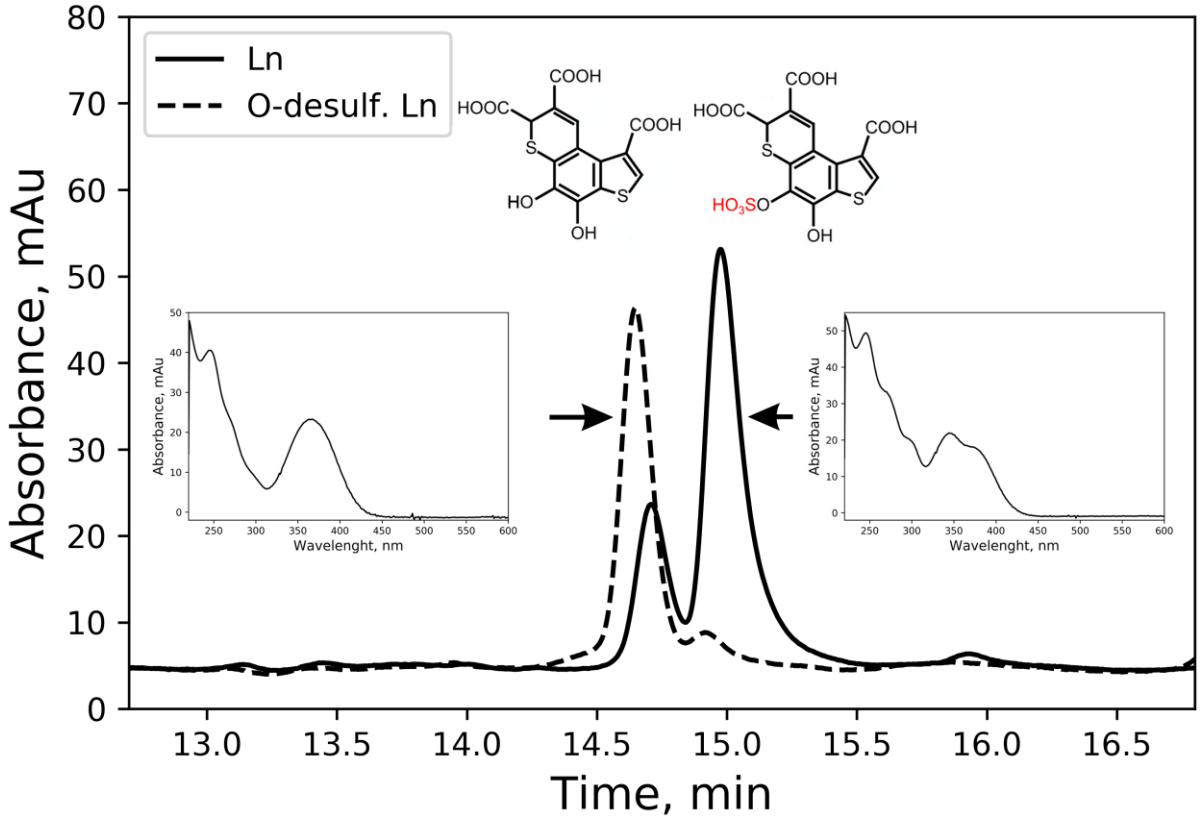

Figure S12. Reverse phase chromatographic profiles of samples containing luciferin (solid line) and *O*-desulfated luciferin (dashed line) after separation. Presence of cross-contamination in each sample is evident. The UV-vis spectra for each compound are shown in the insets.

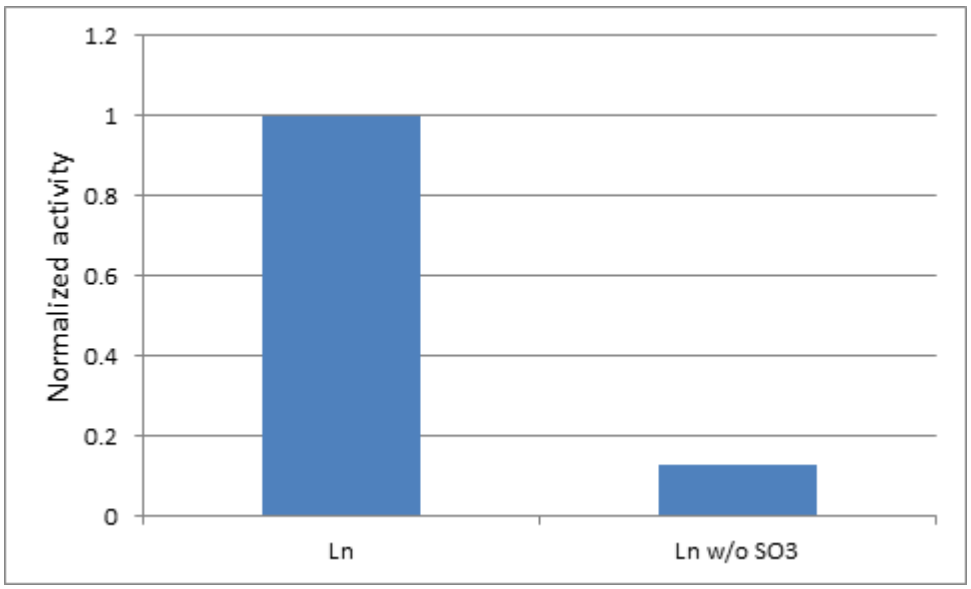

Figure S13. Normalized bioluminescence activity of partially separated luciferin and *O*-desulfated luciferin samples. Ln – luciferin, Ln w/o SO_3 - *O*-desulfated luciferin.

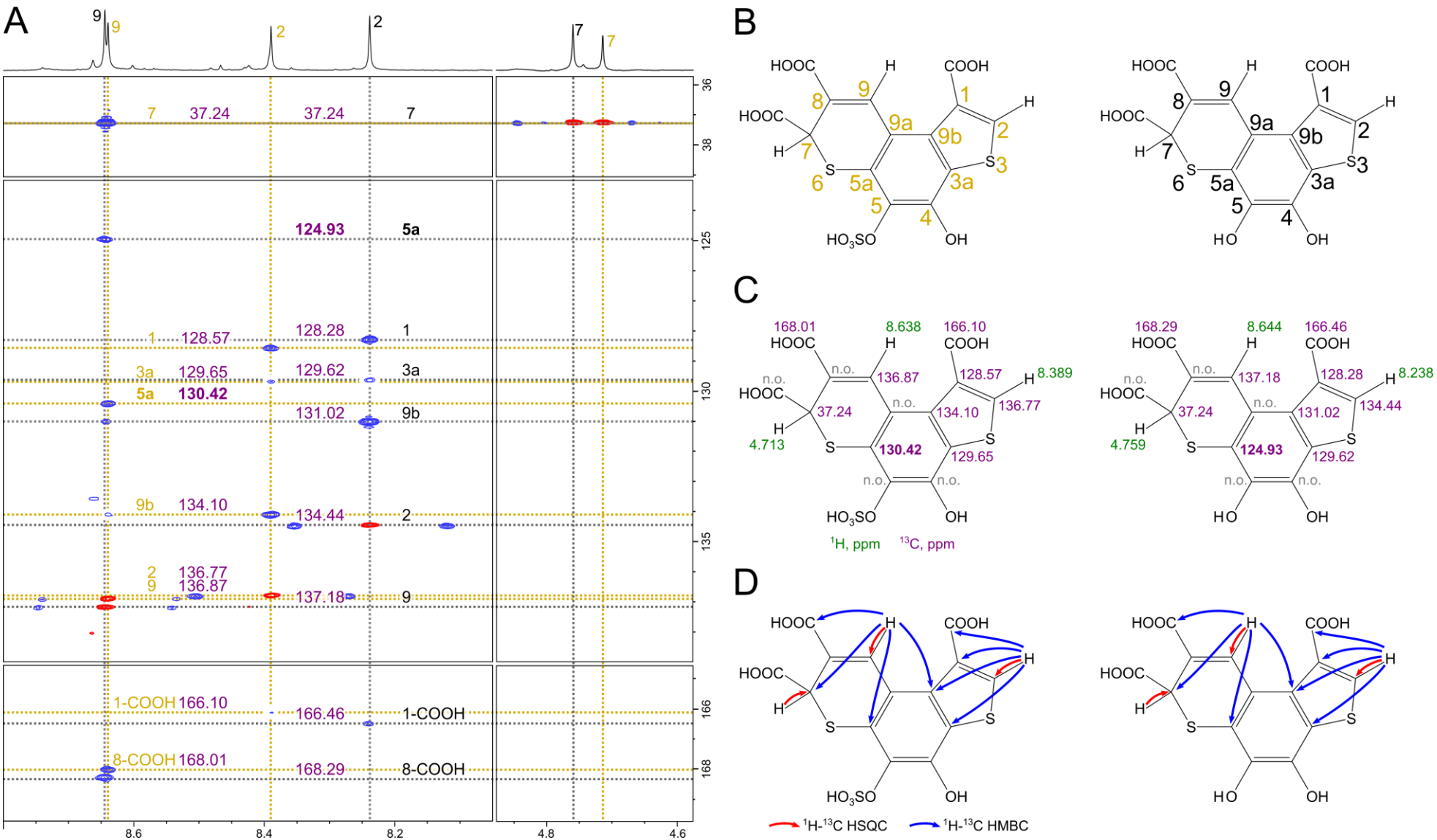

Figure S14. NMR spectra of the 1:1.25 mixture of luciferin and *O*-desulfated luciferin analog from *Odontosyllis*. **A** 1D and 2D NMR spectra of the luciferin in mixture (d_4-MeOD, 10°C 800 MHz). ^{1}H NMR is overlaid on the top of 2D correlations ^{13}C-HSQC (red) and ^{13}C-HMBC (blue) NMR spectra. Atom numbers and NMR chemical shifts are outlined, the atom numbers and dotted lines are colored to pick out the signals from luciferin. Chemical shift of most dissimilar of carbon atom 5a (outlined in bold) was used to distinguish the compounds in the NMR spectra. **B.** Chemical formula of luciferin and *O*-desulfated luciferin with heavy atom numbering. **C.** ^{1}H and ^{13}C chemical shifts of compounds, n.o. - not observed. **D.** ^{1}H-^{13}C correlations in 2D NMR spectra used for chemical shift assignment of the compounds.

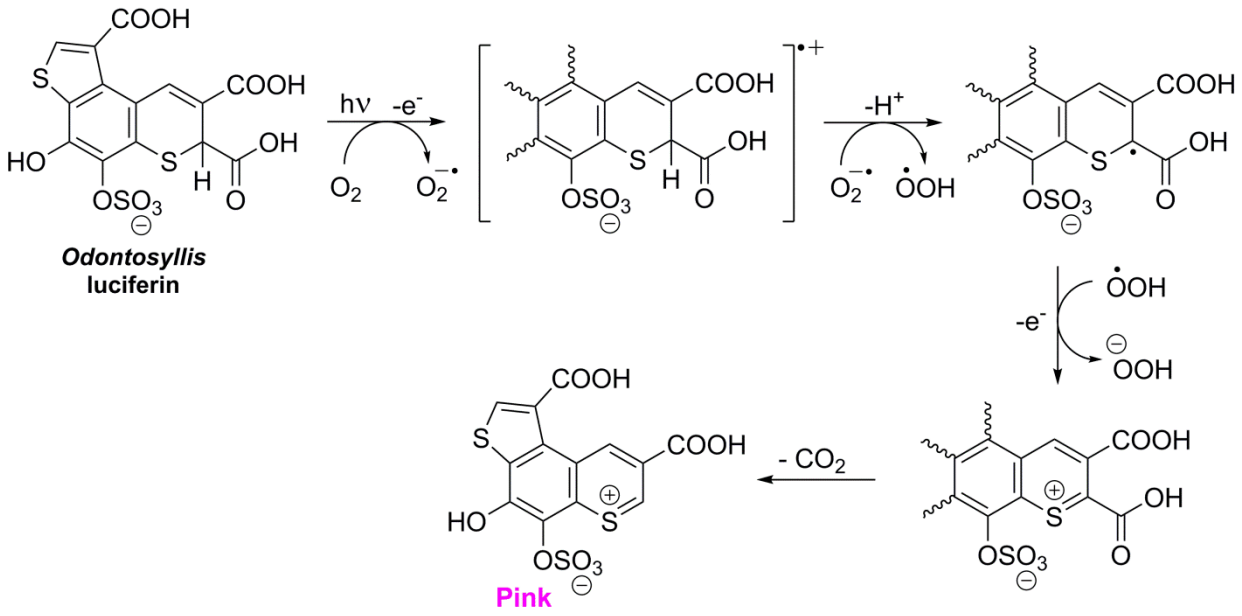

Figure S15. Possible pathway of photoinduced oxidative decarboxylation of *Odontosyllis* luciferin in the presence of oxygen.

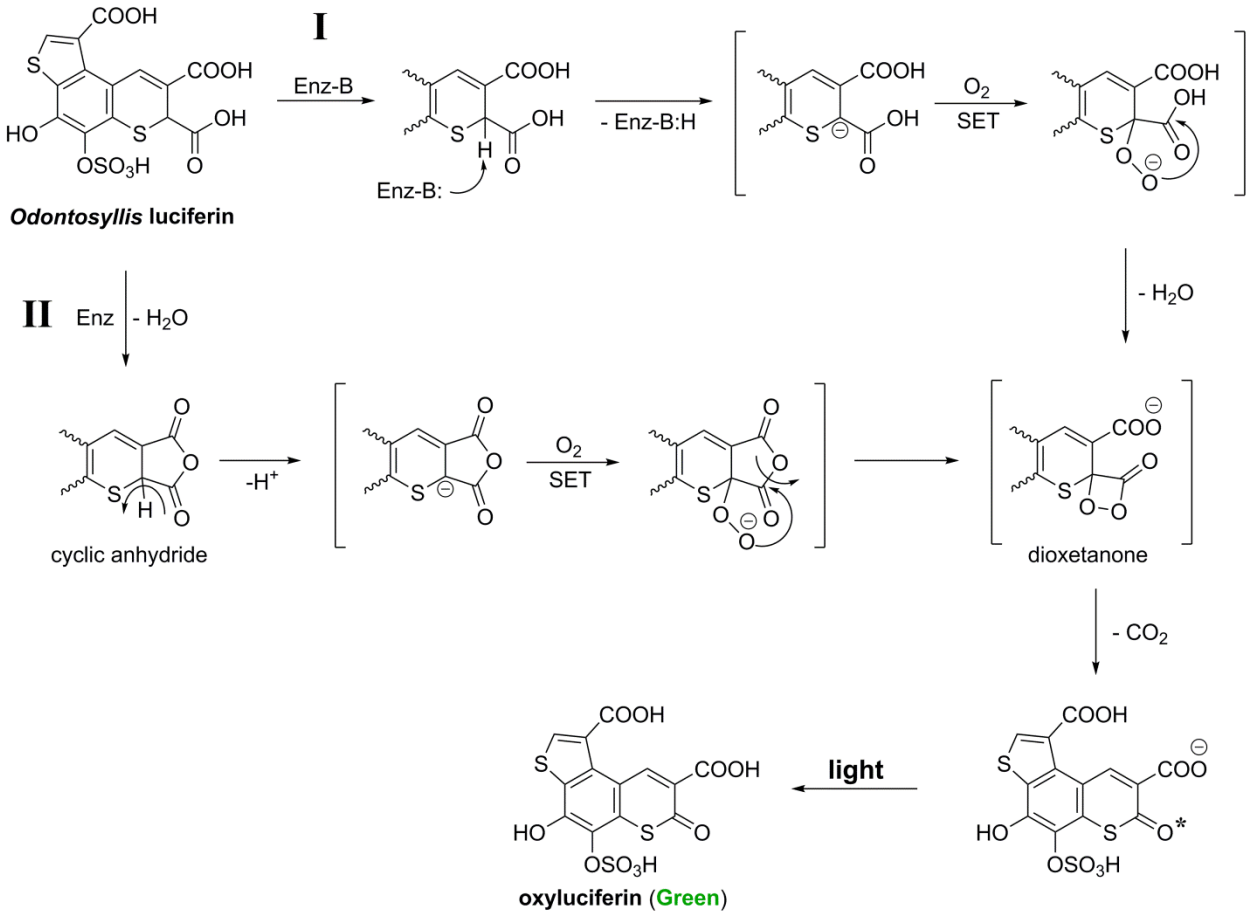

Figure S16. Two plausible pathways of the proposed *Odontosyllis undecimdonta* bioluminescence. **I.** The luciferase-catalyzed deprotonation at C7-position of *Odontosyllis* luciferin. **II.** Alternative possible pathway of *O. undecimdonta* bioluminescence proceeding via the formation of activated cyclic anhydride.

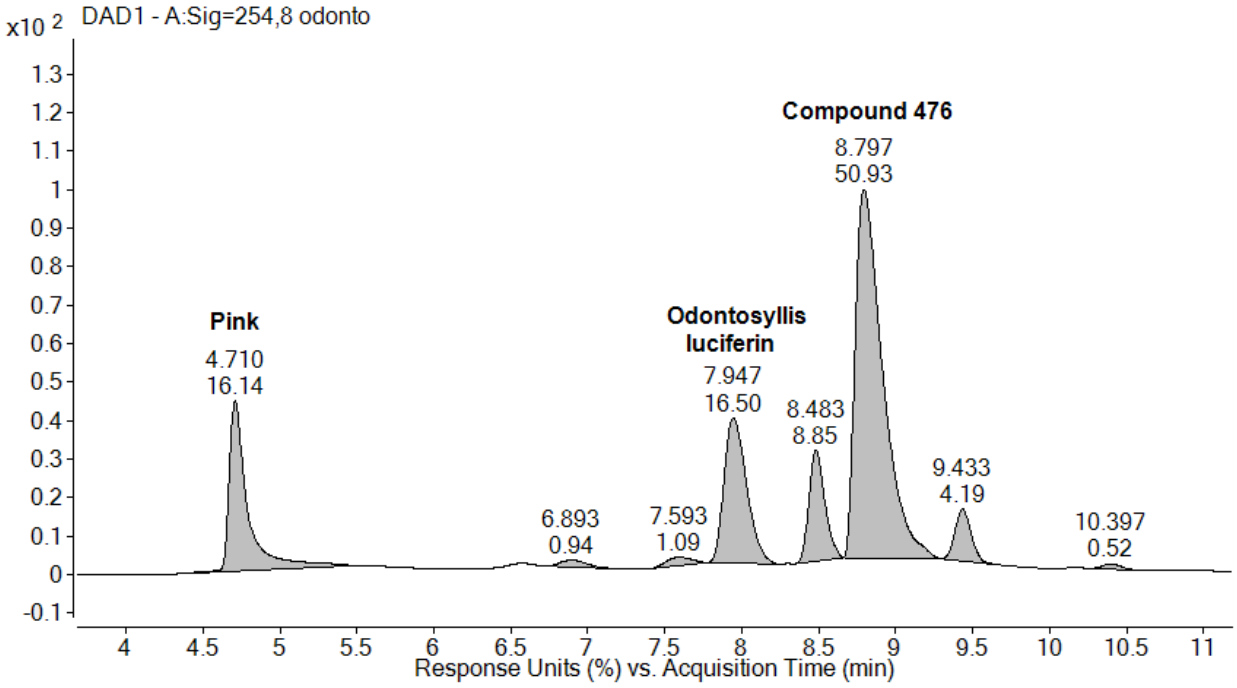

Figure S17. Reverse phase chromatographic profile of luciferin-containing fraction from anion-exchange chromatography of water extract of lyophilized *Odontosyllis undecimdonta* worms on YMC-Triart C18 column (3 µm, 12 nm, 75 × 4.6 mm) column (monitored at λ_{abs} = 254nm). *Odontosyllis* luciferin, Pink and Compound 476 peaks are assigned respectively.

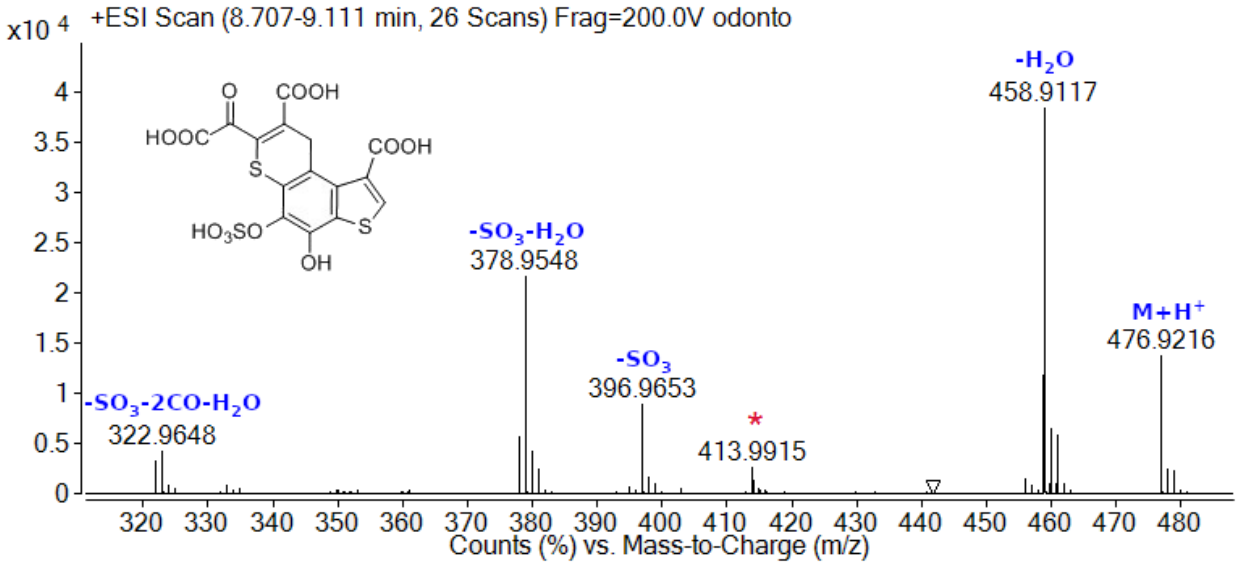

Figure S18. HRMS spectrum of Compound 476 displaying similar to *Odontosyllis* luciferin fragmentation patterns (noise peaks are marked with red asterisks).

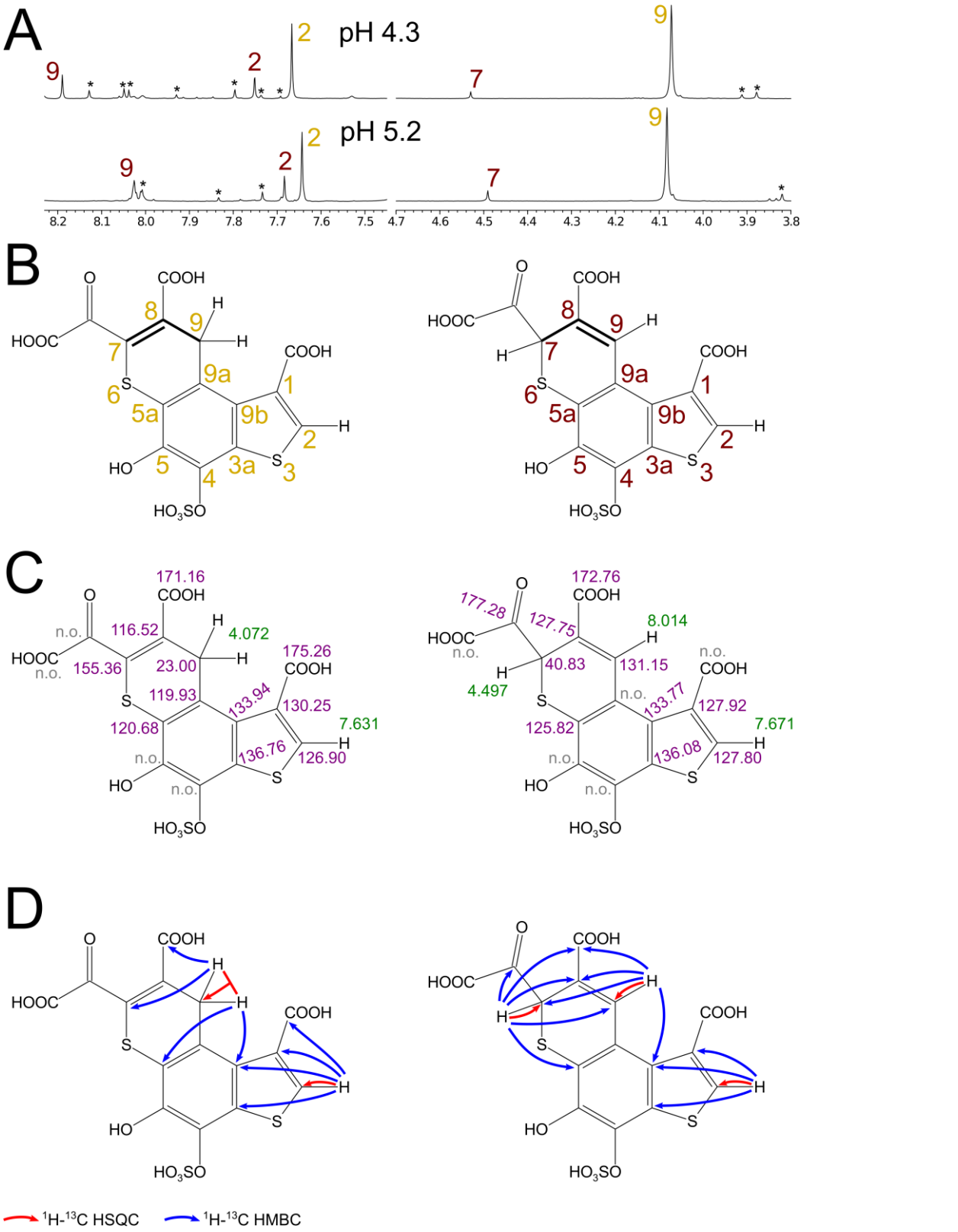

Figure S19. NMR spectra of Compound 476 tautomers (3:1 ratio) from *Odontosyllis* **A.** ^{1}H NMR spectra of Compound 476 in (D_2O, 15^{o}C 800MHz, pH 4.3 and 5.2). Atom numbers are outlined, signal 7 is attenuated due to water signal suppression. **B.** Chemical formulas of two isomers (3:1 ratio) of the Compound 476 with heavy atom numbering. **C.** ^{1}H and ^{13}C chemical shifts of the isomers of Compound 476 at pH 5.2, n.o. - not observed. **D.** ^{13}C-HSQC (red) and ^{13}C-HMBC (blue) ^{1}H-^{13}C correlations in 2D NMR spectra of the isomers of Compound 476 used for chemical shift assignment.

Table S1. NMR Chemical shifts (ppm) of Pink, oxyluciferin (Green), luciferin and *O*-desulfated luciferin.

Heavy atom number[a])	Pink 15°C H_2O+D_2O pH 2.5		Green 15°C H_2O+D_2O pH 3.3		Green 15°C H_2O+D_2O pH 6.0		Luciferin 10°C d_4-MeOD		*O*-desulfated luciferin 10°C d_4-MeOD	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	–[b)]	132.84	–	132.48	–	134.09	–	128.57	–	128.28
1-COOH	–	168.28	–	n.o.	–	174.22	–	166.10	–	166.46
2	8.477	139.07	8.202	134.76	7.885	127.76	8.389	136.77	8.238	134.44
3a	–	142.91	–	133.92	–	136.96	–	129.65	–	129.62
4	–	n.o.[c)]	–	n.o.	–	n.o.	–	n.o.	–	n.o.
5	–	133.49	–	n.o.	–	n.o.	–	n.o.	–	n.o.
5a	–	139.78	–	138.73	–	138.24	–	130.42	–	124.93
7	9.297	145.37	–	187.17	–	187.18	4.713	37.24	4.759	37.24
7-COOH	–	–	–	–	–	–	–	n.o.	–	n.o.
8	–	124.05	–	n.o.	–	n.o.	–	n.o.	–	n.o.
8-COOH	–	165.81	–	168.14	–	170.53	–	168.01	–	168.29
9	9.589	139.06	9.622	147.92	9.255	144.47	8.638	136.87	8.644	137.19
9a	–	119.38	–	n.o.	–	n.o.	–	n.o.	–	n.o.
9b	–	135.41	–	135.56	–	135.19	–	134.10	–	131.02

a) Heavy atom numbering is illustrated on figures S6B,S8B,S14B
b) "–" not applicable
c) n.o. - not observed

References

1. Gottlieb HE, Kotlyar V, Nudelman A (1997) NMR Chemical Shifts of Common Laboratory Solvents as Trace Impurities. *J Org Chem* 62(21):7512–7515.
2. Sheldrick GM, IUCr (2008) A short history of *SHELX*. *Acta Crystallogr Sect A Found Crystallogr* 64(1):112–122.
3. Dolomanov O V., Bourhis LJ, Gildea RJ, Howard JAK, Puschmann H (2009) *OLEX2* : a complete structure solution, refinement and analysis program. *J Appl Crystallogr* 42(2):339–341.
4. Sheldrick GM (2015) *SHELXT* – Integrated space-group and crystal-structure determination. *Acta Crystallogr Sect A Found Adv* 71(1):3–8.
5. Zheng H, Chruszcz M, Lasota P, Lebioda L, Minor W (2008) Data mining of metal ion environments present in protein structures. *J Inorg Biochem* 102(9):1765–1776.
6. Sheldrick GM (2015) Crystal structure refinement with *SHELXL*. *Acta Crystallogr Sect C Struct Chem* 71(1):3–8.