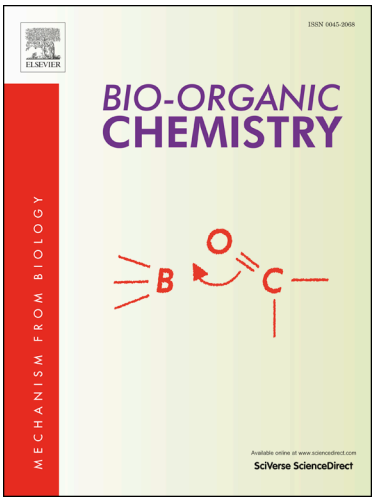


2-*S*-cysteinylhydroquinone is an intermediate for the firefly luciferin biosynthesis that occurs in the pupal stage of the Japanese firefly, *Luciola lateralis*

Shusei Kanie, Ryosuke Nakai, Makoto Ojika, Yuichi Oba

PII: S0045-2068(18)30484-X
DOI: https://doi.org/10.1016/j.bioorg.2018.06.028
Reference: YBIOO 2408

To appear in: *Bioorganic Chemistry*

Received Date: 16 May 2018
Revised Date: 18 June 2018
Accepted Date: 20 June 2018

Please cite this article as: S. Kanie, R. Nakai, M. Ojika, Y. Oba, 2-*S*-cysteinylhydroquinone is an intermediate for the firefly luciferin biosynthesis that occurs in the pupal stage of the Japanese firefly, *Luciola lateralis*, *Bioorganic Chemistry* (2018), doi: https://doi.org/10.1016/j.bioorg.2018.06.028

2-*S*-cysteinylhydroquinone is an intermediate for the firefly luciferin biosynthesis that occurs in the pupal stage of the Japanese firefly, *Luciola lateralis*

Shusei Kanie [a,†], Ryosuke Nakai [a], Makoto Ojika [a] & Yuichi Oba [a,b,*]

[a] *Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, 464-8601, Japan*
[b] *Department of Environmental Biology, Chubu University, Kasugai, 487-8501, Japan*

† Present address: Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Hokkaido Center, Sapporo, 062-8517, Japan.
* Correspondence and requests for materials should be addressed to Y.O. (yoba@isc.chubu.ac.jp)

Keywords: bioluminescence; biosynthesis; firefly; insect; luciferin; mass spectrometry; natural product

Abstract

Firefly luciferin is a natural product that is well-known to function as the substrate of the bioluminescence reaction in luminous beetles. However, the details of the biosynthetic system are still unclear. In this study, we showed by LC-MS/MS analysis that stable isotope-labeled 2-*S*-cysteinylhydroquinone was incorporated into firefly luciferin in living firefly specimens. Comparison of the incorporation efficiency among the developmental stages suggested that firefly luciferin is biosynthesized predominantly in the pupal stage. We also accomplished the *in vitro* biosynthesis of firefly luciferin using 2-*S*-cysteinylhydroquinone and the crude buffer extract of firefly pupae, suggesting the presence of a biosynthetic enzyme in the pupal extract.

1. Introduction

Luminous beetles, including fireflies, generate light by the biochemical reaction of firefly luciferin and luciferase in the presence of ATP, Mg^{2+}, and O_2 [1,2]. The chemical structure of firefly luciferin has been determined to be 2-(6'-hydroxy-2'-benzothiazolyl)-2-thiazoline-4-carboxylic acid, which has a benzothiazole moiety rarely found in natural products [3]. In fireflies, the (*S*)-enantiomer (D-firefly luciferin) functions as the substrate for the luminescence reaction, while the (*R*)-enantiomer (L-firefly luciferin) inhibits the reaction [4]. Currently, the firefly bioluminescence system is widely used to visualize biological processes in life science and technology, and organic chemists have developed

chemical synthetic methods for firefly luciferin and its analogs [5]. However, the biosynthetic system of firefly luciferin is still insufficiently understood.

Since the 1970s, several attempts have been made to clarify firefly luciferin biosynthesis [6,7,8]. In a previous report, our group revealed all the chemical components necessary for the biosynthesis of firefly luciferin and its partial biosynthetic mechanism by incorporation experiments using stable isotope-labeled compounds and the living Japanese firefly *Luciola lateralis* (Syn. *Aquatica lateralis*). Our results suggested that D-firefly luciferin is biosynthesized from one molecule of hydroquinone/*p*-benzoquinone and two molecules of L-cysteine, and that decarboxylation of L-cysteine occurs in the biosynthetic process to form the benzothiazole ring [9]. Recently, we reported an unexpected phenomenon that seems to occur in parallel with the biosynthesis of firefly luciferin [10]: *p*-benzoquinone reacts with cysteine in a neutral aqueous solution to form a small amount of firefly luciferin through the decarboxylation of cysteine. This reaction also gives 2-*S*-cysteinylhydroquinone (CysHQ) and 6-hydroxybenzothiazole-2-carbaldehyde (HBTC), further each of these products further gives small amount of firefly luciferin when mixed with cysteine in a neutral aqueous solution [10].

To understand the biosynthetic pathways of natural compounds, probing of the intermediates is sometimes crucial [11]. In this study, we focused on CysHQ and HBTC to examine the biosynthetic intermediate of firefly luciferin by incorporation studies using the living *L. lateralis* specimens.

2. Results and Discussion

2.1. In vivo formation of [$^2H_3$]firefly luciferin from [$^2H_3$]CysHQ

Chiral HPLC analyses of the firefly extracts showed a rapid increase in the content of both D- and L-firefly luciferin during development from the early pupal stage to early adult stage in both males and females (Fig. S1). Similar results were also reported in the American firefly *Photuris pennsylvanica* [12] and *L. lateralis* [7]. Hence, in this study, we used three different stages of the pupae (early, middle, and late; Fig. S2) and the adult for studying the biosynthesis of firefly luciferin. Synthetic [$^2H_3$]CysHQ in water was injected into the living *L. lateralis*. After treatment for 24 h, the whole body of the specimen was minced in ice-cold methanol and the extract was subjected to chiral HPLC to fractionate D- and L-firefly luciferin. LC-MS/MS analyses of both the D- and L-firefly luciferin fraction showed a significant increase in the *m/z* 284 (+3) signal (Figs. 1, S3, S4), suggesting that [$^2H_3$]firefly luciferin was produced from L-[$^2H_3$]CysHQ in the living firefly specimen. The

incorporation efficiency in the adult stage was much lower than that in the early and middle stages of pupae (Fig. 1, S3, S4). These results indicated that fireflies biosynthesize firefly luciferin using CysHQ as an intermediate and that the biosynthesis occurs mainly in the middle stages of the pupa (Scheme 1). It has been reported that the benzothiazole moieties of some natural products, such as rifamycin Q, pheomelanin, and pleurothiazole, are also biosynthesized through the cysteine addition to quinone followed by condensation [13]. Of note, we could not detect CysHQ in the pupal extract by HPLC (data not shown). This is probably explained by the fact that the biosynthetic intermediate is usually not detected in the organisms except when the biosynthetic process is experimentally blocked [11].

2.2. [$^2H_3$]HBTC was not incorporated into firefly luciferin

Synthetic [$^2H_3$]HBTC in 50% DMSO aq. was injected into the middle stage of the pupal body. After treatment for 24 h, the whole body of the specimen was minced in ice-cold methanol and the extract was subjected to chiral HPLC to fractionate D- and L-firefly luciferin. LC-MS/MS analyses of both the D- and L-firefly luciferin fraction showed no significant incorporation of [$^2H_3$]HBTC into firefly luciferin (n = 3; Fig. 2). On the other hand, the efficient incorporation of [$^2H_3$]CysHQ was observed even under the same DMSO-containing condition (Fig. 2). Thus, the results indicate that HBTC is unlikely to be a biosynthetic intermediate of firefly luciferin, although firefly luciferin was generated from HBTC and Cys in very low yield under a neutral buffer condition [10].

2.3. In vitro formation of [$^2H_3$]firefly luciferin from [$^2H_3$]CysHQ

Synthetic [$^2H_3$]CysHQ in water was mixed with a “crude buffer extract” of the middle stage of pupae. After incubation for 3 h, the ethyl acetate extract was analyzed by LC-MS/MS. The results showed that isotopic incorporation was detected in L-firefly luciferin, while no significant incorporation was observed in D-firefly luciferin (Scheme 1; Fig. 3). When [$^2H_3$]CysHQ was mixed with the “heat denatured extract”, the amount of L-[$^2H_3$]firefly luciferin produced was decreased (Fig. 3). When [$^2H_3$]CysHQ was mixed with the “high MW extract” prepared using a molecular weight-cutoff filter membrane of both 10K and 50K, the amount of L-[$^2H_3$]firefly luciferin produced was larger than that in the mixture of [$^2H_3$]CysHQ with the “low MW extract” (Fig. S5). These results suggested that a biosynthetic enzyme(s) was involved in the *in vitro* formation of firefly luciferin. That D-firefly luciferin was not generated *in vitro* might be due to the shortage of cofactors for racemization

of firefly luciferin [7] in the reaction. We speculate that a candidate enzyme responsible for the firefly luciferin biosynthesis might be a *p*-phenoloxidase, such as the laccase expressed at the metamorphosis stage of some insects, which is involved in the oxidative polymerization of cysteinyldopa to produce the benzothiazole-ring of pheomelanin [14].

3. Conclusion

We demonstrated using firefly specimens that L-[$^{2}H_3$]CysHQ was incorporated into firefly luciferin *in vivo* and *in vitro*, suggesting that L-CysHQ is an intermediate for the biosynthesis of firefly luciferin, as we proposed previously (Scheme 1) [9]. Comparison of the *in vivo* incorporation efficiency among the developmental stages suggested that the biosynthesis occurs predominantly in the early and middle stages of the pupa

4. Experimental section

4.1. General

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on an AVANCE-400 (400 MHz) NMR spectrometer (Bruker, Billerica, MA, USA). The NMR chemical shifts (ppm) were referenced to the tetramethylsilane (TMS) peak, or the 3-(trimethylsilyl)propionic acid-d_4 sodium salt (TSP-d_4) peak. Data were reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet), coupling constant, and integration. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on an AVANCE-400 (100 MHz) NMR spectrometer (Bruker). The NMR chemical shifts (ppm) were referenced to the solvent peaks (acetone-d_6 as δ = 29.8) and the 3-(trimethylsilyl)propionic acid-d_4 sodium salt (TSP-d_4) peak. High-resolution mass spectra (HRMS) were recorded on a Mariner Biospectrometry Workstation (Applied Biosystems, Foster City, CA, USA) in the positive ESI mode, and were reported in *m/z*. Reactions were monitored by using thin layer chromatography (TLC) on 0.25 mm silica gel-coated glass plates (60 F_{254}; Merck, Darmstadt, Germany). In TLC analysis, UV light (254 nm or 366 nm) and 5% ethanolic phosphomolybdic acid solution were used as visualizing agents.

4.2. Chemicals

The materials used in the present study were obtained from the following commercial suppliers. L-cysteine was from Kanto Chemical (Tokyo) and D-firefly luciferin was from Wako Pure Chemical Industries). *p*-benzoquinone-d_4 (isotopic purity, 98.2%) was purchased from C/D/N Isotopes Inc. (Quebec, Canada). Hydroquinone-d_6 (isotopic purity, 98%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). All of the materials were used without further purification. L-firefly luciferin was kindly provided by Dr. Yoshiaki Toya (Aichi Univ. of Education, Aichi, Japan).

4.3. Specimens of firefly, Luciola lateralis

The larval specimens of *L. lateralis* were reared in an aerated aquarium with feeding of the freshwater snail *Physa acuta* at 23 ± 2 °C. Last instar larvae were pupated in moistened soil (vermiculite: palm peat: river sand, 2:2:1-v/v/v) [15]. The developmental stages of pupae (early, middle, and late) were judged by the days after pupation and pigmentation (see Fig. S2). The sex of the pupal and adult specimens was determined by the morphology of the abdominal segments. The specimens for measuring the content of firefly luciferin were weighed after lyophilization and stored at -80 °C until use.

4.4. Measurement of firefly luciferin content

The lyophilized whole body of a single specimen was homogenized in 1 mL of ice-cold methanol containing 0.1% (v/v) formic acid using a refrigerated beads cell disrupter Micro Smash (model MS-100R; Tomy, Tokyo). The homogenate was stirred for 10 min at 4 °C using a rotary mixer (model NRC-20D; Nissin, Tokyo) and centrifuged at 17,400 × g for 3 min at 4 °C. The supernatant (800 μL) was filtered through a centrifugal filter Ultrafree-MC (0.45 μm; Millipore Billerica, MA, USA). The racemization of firefly luciferin was negligible under these extraction processes (data not shown). The filtrates from adult specimens were diluted with water to be 50% (v/v) aqueous solution, and 30 μL of the solution was subjected to chiral HPLC analysis. The filtrates from larval, pre-pupal, and pupal specimens were diluted with water to be 30% (v/v) aqueous solution, and 40 μL of the solution was subjected to chiral HPLC analysis.

4.5. Chemical synthesis of deuterium-labeled compounds

4.5.1. [$^2H_3$]CysHQ

A solution of *p*-benzoquinone-d_4 (15.8 mg, 0.14 mmol) in water (8 mL) was added dropwise over 15 min to a stirred solution of L-cysteine (17.1 mg, 0.14 mmol) in water (1 mL) under a nitrogen atmosphere (Scheme 1). The mixture was stirred for 70 min at room temperature under a nitrogen atmosphere with monitoring of the reaction by using TLC (ethyl acetate-ethanol-*n*-butanol-water, 3:2:2:3-v/v/v/v), and lyophilized to give a quantitative yield of [$^2H_3$]CysHQ (32.1 mg, 0.14 mmol, white solid) (Fig. S6). TLC, HPLC, and NMR analyses confirmed the completion of the reaction and no starting materials remained. ^{1}H NMR (400 MHz, D_2O): δ 3.79 (dd, *J* = 4.0, 9.0 Hz, 1H), 3.48 (dd, *J* = 4.0, 14.6 Hz, 1H), 3.22 (dd, *J* = 9.2, 14.6 Hz, 1H); ^{13}C NMR (100 MHz, D_2O): δ 175.3, 152.4, 152.0, 123.2 (t, *J* = 23.0 Hz), 121.5, 120.0 (t, *J* = 23.8 Hz), 119.4 (t, *J* = 25.0 Hz), 56.6, 37.9; HRMS (*m/z*): $[M+H]^+$ calcd. for $C_9H_9D_3NO_4S$, 233.06699; found, 233.06678. Chiral HPLC analysis using CROWNPAK CR-I (+) (ϕ 3.0 × 150 mm; Daicel Chemical Industry, Tokyo) showed that the synthesized [$^2H_3$]CysHQ is L-form with > 99% e.e. (data not shown). Isotopic unlabeled CysHQ was prepared in the same manner.

4.5.2. [$^2H_3$]HBTC

[$^2H_3$]HBTC was synthesized as a light yellow-green solid, according to the methods previously reported [10,16] using *p*-benzoquinone-d_4 (Fig. S7). ^{1}H NMR (400 MHz, Acetone-d_6): δ 10.06 (s, 1H); ^{13}C NMR (100 MHz, Acetone-d_6): δ 186.2, 163.3, 159.3, 148.5, 139.1, 127.0 (t, *J* = 25.5 Hz), 118.6 (t, *J* = 25.0 Hz), 107.4 (t, *J* = 25.0 Hz); HRMS (*m/z*): $[M+H]^+$ calcd. for $C_8H_3D_3NO_2S$, 183.03021; found, 183.03054. Isotopic unlabeled HBTC was prepared in the same manner.

4.6. In vivo incorporation of labeled compounds

A 200 mM solution of [$^2H_3$]CysHQ in water (1 μL), 25 mM solution of [$^2H_3$]CysHQ in 50% (v/v) DMSO aqueous solution (2 μL), 25 mM solution of [$^2H_3$]HBTC in 50% (v/v) DMSO aqueous solution (2 μL), or water (1 μL) was injected into the body cavity of living *L. lateralis* using a micro syringe (model 1701N 10 μL SYR; Hamilton, Reno, NV, USA) from the ventral side of the abdominal segment near the photophores. After keeping at 24 ± 2 °C for 24 h, the still-living specimens were frozen in liquid nitrogen and stored at -80 °C until use.

The whole body of a single specimen was homogenized in 70 μL of ice-cold methanol on ice, and centrifuged at 17,400 × g for 3 min at 4 °C. The supernatant was filtered by an Ultrafree-MC centrifugal filter. The filtrate was washed twice with 60 μL of *n*-hexane, and diluted with the same volume of water. The entire solution was then subjected to chiral HPLC to separate D- and L-firefly

luciferin. The eluted fractions containing D- or L-firefly luciferin (ca. 600-1000 μL) were acidified with 1 M HCl (20-40 μL) to below pH 3. Each acidified fraction was extracted twice with 500 μL of ethyl acetate. The combined organic layer was concentrated to dryness under a nitrogen stream at room temperature, and the residue was dissolved in water so that the concentration of firefly luciferin was below 0.5 μM. The 10 μL aliquot was subjected to LC-MS/MS analyses.

4.7. In vitro incorporation of [$^2H_3$]CysHQ

Three frozen specimens were homogenized in 300 μL of ice-cold Tris-HCl (pH 7.5) on ice, and centrifuged at 17,400 × g for 3 min at 4 °C. Then, 90 μL (approximately equivalent to the extract from a single specimen) of the supernatant ("crude buffer extract") was mixed with 5 μL of 25 mM aqueous solution of [$^2H_3$]CysHQ, then incubated at 30 °C for 3 h. The resultant mixture was centrifuged at 17,400 × g for 3 min at 4 °C, and the supernatant was acidified with 30 μL of 1M HCl to below pH 3. The acidified solution was extracted twice with 120 μL of ethyl acetate, and the combined organic layer was concentrated to dryness under a nitrogen stream at room temperature, then dissolved in 100 μL of water. The solution was filtered by Ultrafree-MC, and the filtrate was subjected to chiral HPLC to separate D- and L-firefly luciferin. The eluted fractions containing D- or L-firefly luciferin (ca. 500-900 μL) were acidified with 1 M HCl (20 μL) to below pH 3, and extracted twice with 500 μL of ethyl acetate. The combined organic layer was concentrated to dryness under a nitrogen stream at room temperature, and the residue was dissolved in water so that the concentration of firefly luciferin was below 0.5 μM. The 10 μL aliquot was subjected to LC-MS/MS analyses.

Heat denaturation of the crude buffer extract ("heat denatured extract") was performed at 95 °C for 30 min using a block incubator WSC-2620 PowerBLOCK (ATTO Corporation, Tokyo). To prepare the "molecular weight-cutoff extracts", the crude buffer extracts were centrifuged using an Amicon Ultra centrifugal filter (10K and 50K, Millipore) at 14,000 × g for 15 min at 4 °C, and the retentates and filtrates were used as the "high MW extract" and "low MW extract", respectively. Using these treated extracts, *in vitro* incorporation and the analysis were performed in the same manner.

4.8. Chiral HPLC analysis and separation

Chiral HPLC analysis and separation were performed on a PU-1580 HPLC system (Jasco, Tokyo) equipped with a chiral column CHIRALCEL OD-RH (ϕ 4.6 × 150 mm; Daicel Chemical Industry), a multiwavelength detector (MD-2018 Plus; Jasco) and a fluorescence detector (FP-1520; Jasco). The

HPLC conditions were as follows: mobile phase, 27% (v/v) acetonitrile in H_2O containing 0.1% (v/v) formic acid; flow rate 1.0 mL/min; fluorescence detection, excitation/emission, 330/530 nm.

4.9. LC-MS/MS analysis

LC-MS/MS analysis was performed by electrospray ionization ion trap-mass spectrometry (ESI-IT-MS) on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) with an HCTplus mass spectrometer (Bruker). The MS conditions were as follows: ionization and detection, electrospray ionization-ion trap (ESI-IT) positive MRM (multiple reaction monitoring) mode; Target Mass, *m/z* 280; Fragmentation, ON (amplitude, 1.00 V). The HPLC conditions were as follows: column, Unison UK-C8 (ϕ 2.0 × 75 mm; Imtakt, Kyoto, Japan); mobile phase, 25% (v/v) acetonitrile in H_2O containing 0.1% (v/v) formic acid; flow rate 0.2 mL/min.

Acknowledgements

We thank Mr. Haruyoshi Ikeya (Toin Gakuen High School, Japan) for kindly providing *L. lateralis* specimens, Dr. Masaki Honda (Meijo Univ., Aichi, Japan), Mrs. Yukari Kawai, and Mr. Shigeyuki Kitamura (Nagoya Univ., Aichi, Japan) for MS analysis, and Prof. Hitoshi Mori (Nagoya Univ., Aichi, Japan) for the instrument used to homogenize fireflies. This work was supported by a Grant-in-Aid for JSPS Fellows (no. 16J03104), by the Program for Leading Graduate Schools "Integrative Graduate Education and Research in Green Natural Sciences" of MEXT, Japan, and by a grant from Chubu University (no. 29IM07A).

6. References

[1] O. Shimomura, Bioluminescence–Chemical Principles and Methods, Revised ed., World Scientific Publishing, Singapore, 2012.

[2] Y. Oba, Insect Bioluminescence in the Post-Molecular Biology Era, in: K.H. Hoffmann (Ed.), Insect Molecular Biology and Ecology, CRC Press, Florida, 2014, pp. 94-120.

[3] L.L. Bozec, C.J. Moody, Naturally occurring nitrogen-sulfur compounds. The benzothiazole alkaloids, Aust. J. Chem. 62 (2009) 639-647.

[4] S. Inouye, Firefly luciferase: an adenylate-forming enzyme for multicatalytic functions, Cell. Mol. Life Sci. 67 (2010) 387-404.

[5] (a) G. Meroni, P. Ciana, A. Maggi, E. Santaniello, A new synthesis of

2-cyano-6-hydroxybenzothiazole, the key intermediate of D-luciferin, starting from 1,4-benzoquinone, Synlett 16 (2009) 2682-2684; (b) C.C. Woodroofe, P.L. Meisenheimer, D.H. Klaubert, Y. Kovic, J.C. Rosenberg, C.E. Behney, T.L. Southworth, B.R. Branchini, Novel heterocyclic analogues of firefly luciferin, Biochemistry 51 (2012) 9807-9813; (c) P. Ciuffreda, S. Casati, G. Meroni, E. Santaniello, A new synthesis of dehydroluciferin [2-(6'-hydroxy-2'-benzothiazolyl)-thiazole-4-carboxylic acid] from 1,4-benzoquinone, Tetrahedron 69 (2013) 5893-5897; (d) N.P. Prajapati, R.H. Vekariya, H.D. Patel, Microwave induced facile one-pot access to diverse 2-cyanobenzothiazole -A key intermediate for the synthesis of firefly luciferin, Int. Lett. Chem. Phys. Astron. 44 (2015) 81-89; (e) D.C. McCutcheon, W.B. Porterfield, J.A. Prescher, Rapid and scalable assembly of firefly luciferase substrates, Org. Biomol. Chem. 13 (2015) 2117-2121; (f) S.T. Adams Jr., S.C. Miller, Beyond D-luciferin: expanding the scope of bioluminescence imaging *in vivo*, Current Opinion in Chemical Biology 21 (2014) 112-120; (g) M. Kiyama, R. Saito, S. Iwano, R. Obata, H. Niwa, S.A. Maki, Multicolor bioluminescence obtained using firefly luciferin, Current Topics in Medicinal Chemistry 16 (2016) 2648-2655; (h) D.K. Sharma, S.T. Adams Jr., K.L. Liebmann, S.C. Miller, Rapid access to a broad range of 6'-substituted firefly luciferin analogues reveals surprising emitters and inhibitors, Org. Lett. 19 (2017) 5836-5839.

[6] (a) F. McCapra, Z. Razavi, A model for firefly luciferin biosynthesis, J. Chem. Soc. Chem. Commun. (1975) 42-43; (b) F. McCapra, Z. Razavi, Biosynthesis of luciferin in *Pyrophorus pellucens*, J. Chem. Soc. Chem. Comm. (1976) 153-154; (c) K. Okada, H. Iio, T. Goto, Biosynthesis of firefly luciferin. Probable formation of benzothiazole from *p*-benzoquinone and cysteine, J. Chem. Soc. Chem. Comm. (1976) 32; (d) P. Colepicolo, D. Pagni, E.J.H. Bechara, Luciferin biosynthesis in larval *Pyrearinus termitilluminans* (Coleoptera: Elateridae), Comp. Biochem. Physiol. 91B (1988) 143-147.

[7] K. Niwa, M. Nakamura, Y. Ohmiya, Stereoisomeric bio-inversion key to biosynthesis of firefly D-luciferin, FEBS Lett. 580 (2006) 5283-5287.

[8] T.R. Fallon, F.-S. Li, M.A. Vicent, J.-K. Weng, Sulfoluciferin is biosynthesized by a specialized luciferin sulfotransferase in fireflies, Biochemistry, 55 (2016) 3341-3344.

[9] Y. Oba, N. Yoshida, S. Kanie, M. Ojika, S. Inouye, Biosynthesis of firefly luciferin in adult lantern: decarboxylation of L-cysteine is a key step for benzothiazole ring formation in firefly luciferin synthesis, PLoS ONE 8 (2013) e84023.

[10] S. Kanie, T. Nishikawa, M. Ojika, and Y. Oba, One-pot non-enzymatic formation of firefly luciferin in a neutral buffer from *p*-benzoquinone and cysteine, Sci. Rep. 6 (2016) 24794.

[11] H.–C. Lin, G. Chiou, Y.–H. Chooi, T.C. McMahon, W. Xu, N.K. Garg, Y. Tang, Elucidation of the concise biosynthetic pathway of the communesin indole alkaloids, Angew. Chem. Int. Ed. 54 (2015) 3004-3007.

[12] L.G. Strause, M. DeLuca, J.E. Case, Biochemical and morphological changes accompanying light organ development in the firefly, *Photuris pennsylvanica*, J. Insect Physiol. 25 (1979) 339-347.

[13] (a) R. Cricchio, P. Antonini, G.C. Lancini, G. Tamborini, R.J. White, E. Martinelli, Thiazorifamycins-I. Structure and synthesis of rifamycins P, Q and Verde, novel metabolites from mutants of *Nocardia mediterranea*, Tetrahedron 36 (1980) 1415-1421; (b) R. Cricchio, Thiazorifamycins-II. Mechanism of the reaction between rifamycin S and 2-amino ethanethiol derivatives, Tetrahedron 36 (1980) 2009-2013; (c) R. Cricchio, P. Antonini, G. Sartori, Thiazorifamycins. III. Biosynthesis of rifamycins P, Q and Verde, novel metabolites from a mutant of *Nocardia mediterranea*, J. Antibiot. 33 (1980) 842-846; (d) S. Ito, K. Wakamatsu, Chemistry of mixed melanogenesis-pivotal roles of dopaquinone, Photochem. Photobiol. 84 (2008) 582-592; (e) B. Sandargo, B. Thongbai, M. Stadler, F. Surup, Cysteine-derived pleurotin congeners from the nematode-trapping basidiomycete *Hohenbuehelia grisea*, J. Nat. Prod. 81 (2018) 286-291.

[14] M. Sugumaran, H. Barek, Critical analysis of the melanogenic pathway in insects and higher animals, Int. J. Mol. Sci. 17 (2016) 1753.

[15] Y. Oba, M. Furuhashi, M. Bessho, S. Sagawa, H. Ikeya, S. Inouye, Bioluminescence of a firefly pupa: involvement of a luciferase isotype in the dim glow of pupae and eggs in the Japanese firefly, *Luciola lateralis*, Photochem. Photobiol. Sci. 12 (2013) 854-863.

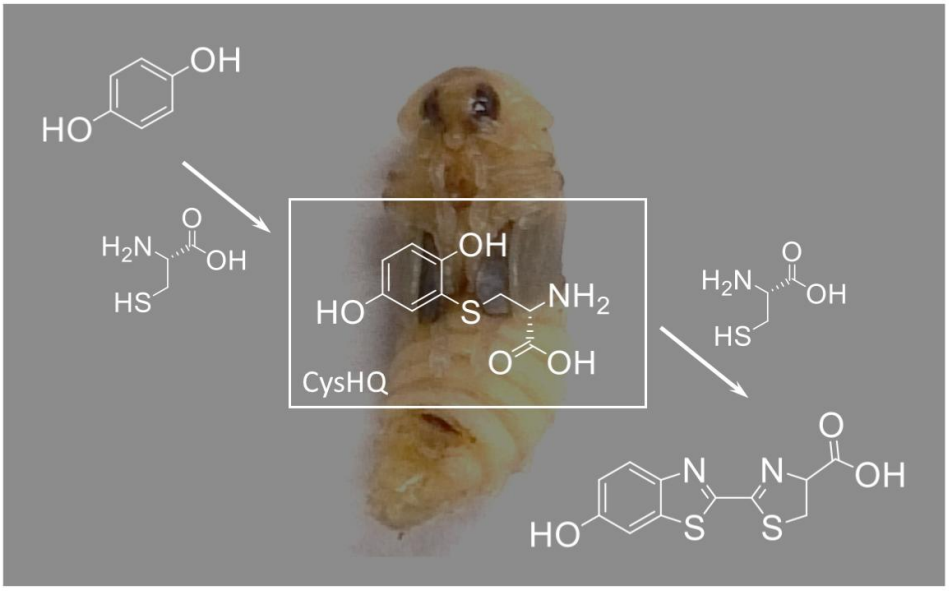
[16] D.W.P.M. Löwik, L.C. Tisi, J.A.H. Murray, C.R. Lowe, Synthesis of 6-hydroxybenzothiazole-2-carboxylic acid, Synthesis 12 (2001) 1780-1783.

Fig. 1. *In vivo* incorporation of [$^2H_3$]CysHQ into firefly luciferin using the middle stage of pupae. (A) Chemical structures of [$^2H_3$]CysHQ (left) and unlabeled/labeled firefly luciferins (middle/right) with the predicted mass fragment ions $[M+H]^+$. (B) Multiple reaction monitoring (MRM) for the chiral-HPLC separated D-firefly luciferins. (C) MRM for the chiral-HPLC separated L-firefly luciferins. Injection of (a) water, and (b) [$^2H_3$]CysHQ. XICs (left), MS/MS spectra for the fragment ions of *m/z* 281 (middle) and 284 (right). (D) Comparison of the incorporation efficiencies of [$^2H_3$]CysHQ. Empty and gray bars represent D- and L-[$^2H_3$]firefly luciferin (*m/z* 284), respectively. Each value (mean ± SEM) was calculated based on the peak area of chiral HPLC analysis and XIC peak area ratio of *m/z* 284/281 with correction for the natural isotopic abundance.

Fig. 2. *In vivo* incorporation of [$^2H_3$]HBTC into firefly luciferin using the middle stage of pupae. (A) Overview. (B) MRM for the chiral-HPLC separated D-firefly luciferins. (C) MRM for the chiral-HPLC separated L-firefly luciferins. Injection of (a) 50% DMSO aq., (b) [$^2H_3$]CysHQ in 50% DMSO aq., and (c) [$^2H_3$]HBTC in 50% DMSO aq. XICs (left), MS/MS spectra for the fragment ions of *m/z* 281 (middle) and 284 (right). (D) Empty and gray bars represent D- and L-[$^2H_3$]firefly luciferin (*m/z* 284), respectively. Each value (mean ± SEM) was calculated based on the peak area of chiral HPLC analysis and XIC peak area ratio of *m/z* 284/281 with correction for the natural isotopic abundance.

Fig. 3. *In vitro* incorporation of [$^2H_3$]CysHQ into firefly luciferin using crude buffer extract of the middle stage of pupae. (A) MRM for the chiral-HPLC separated D-firefly luciferins. (B) MRM for the chiral-HPLC separated L-firefly luciferins. (a) crude extract + H_2O, (b) crude extract + [$^2H_3$]CysHQ, (c) crude extract (heat) + [$^2H_3$]CysHQ. XICs (left), MS/MS spectra for the fragment ions of *m/z* 281 (middle) and 284 (right). (C) Empty and gray bars represent D- and L-[$^2H_3$]firefly luciferin (*m/z* 284), respectively. Each value was calculated based on the peak area of chiral HPLC analysis and XIC peak area ratio of *m/z* 284/281 with correction for the natural isotopic abundance. ND, not detected.

Scheme 1. Chemical synthesis of L-[$^2H_3$]CysHQ, and its incorporation into L-/D-firefly luciferin *in vivo* and *in vitro*. [$^2H_4$]BQ, *p*-benzoquinone-d_4; Cys, cysteine.



Highlights

- CysHQ is incorporated into firefly luciferin in *Luciola lateralis*.
- The incorporation efficiency of CysHQ in the pupa is higher than that in the adult.
- Firefly luciferin is formed from CysHQ in the crude pupal buffer extract.

Fig. 1

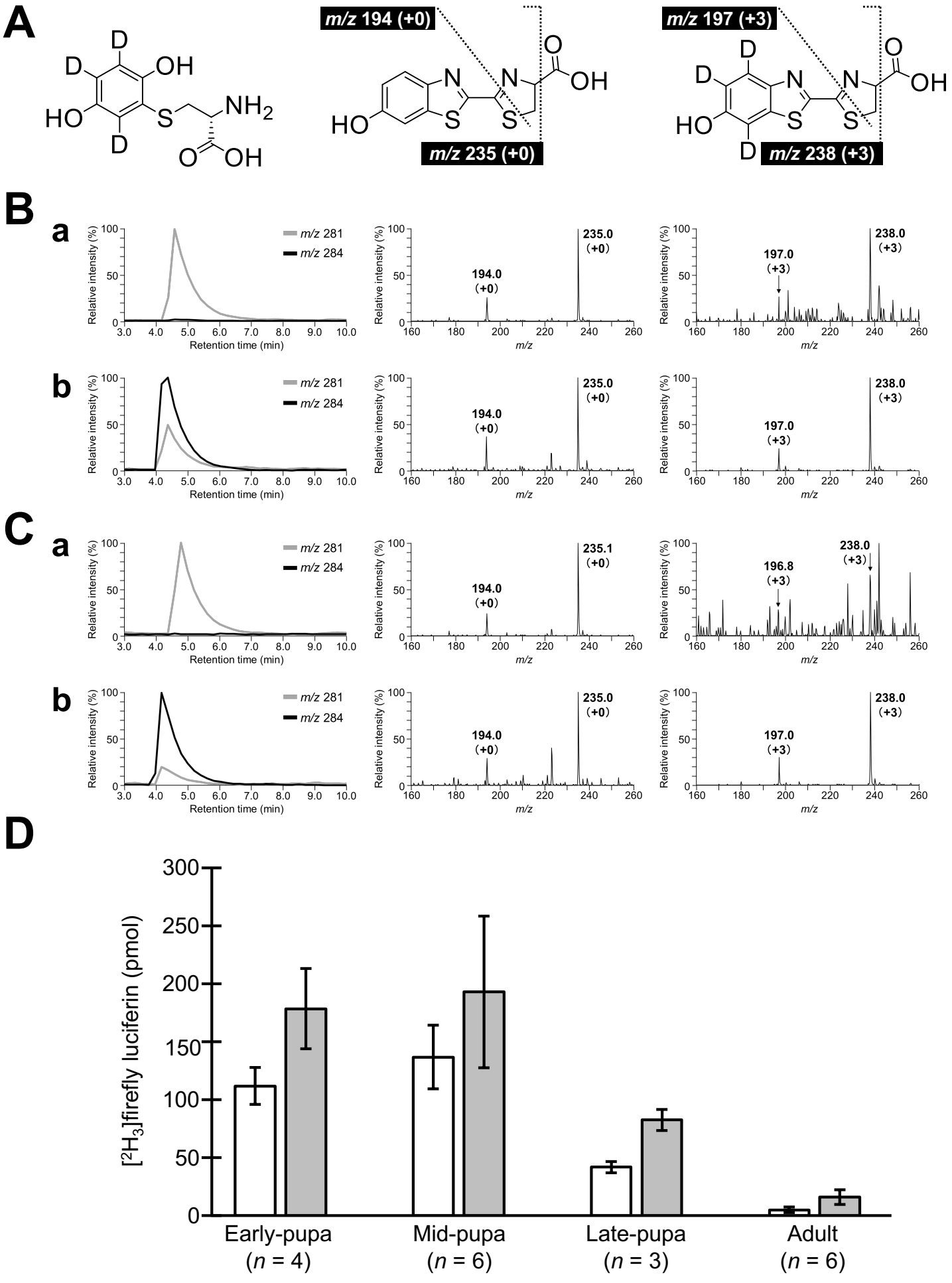


Fig. 2

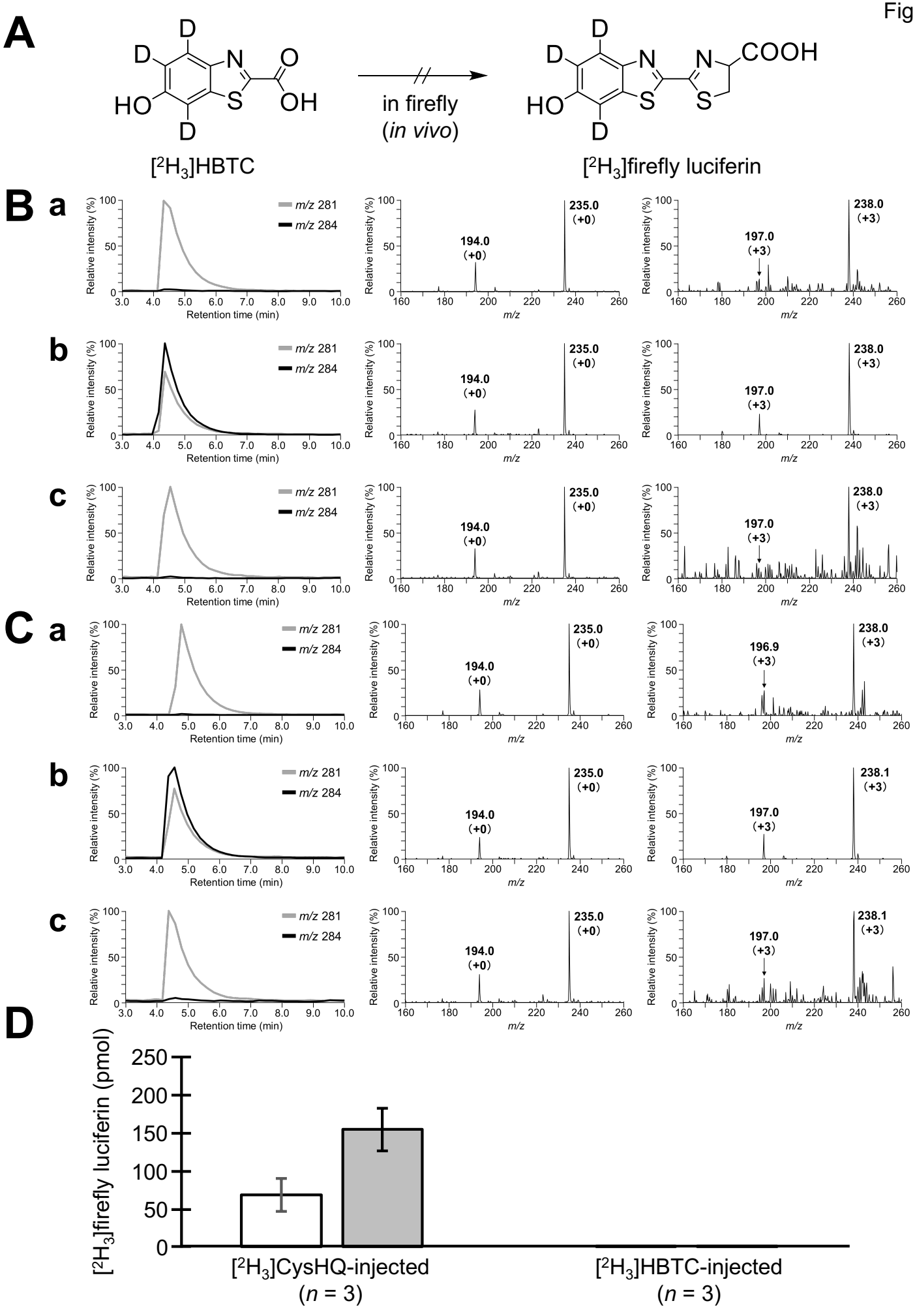
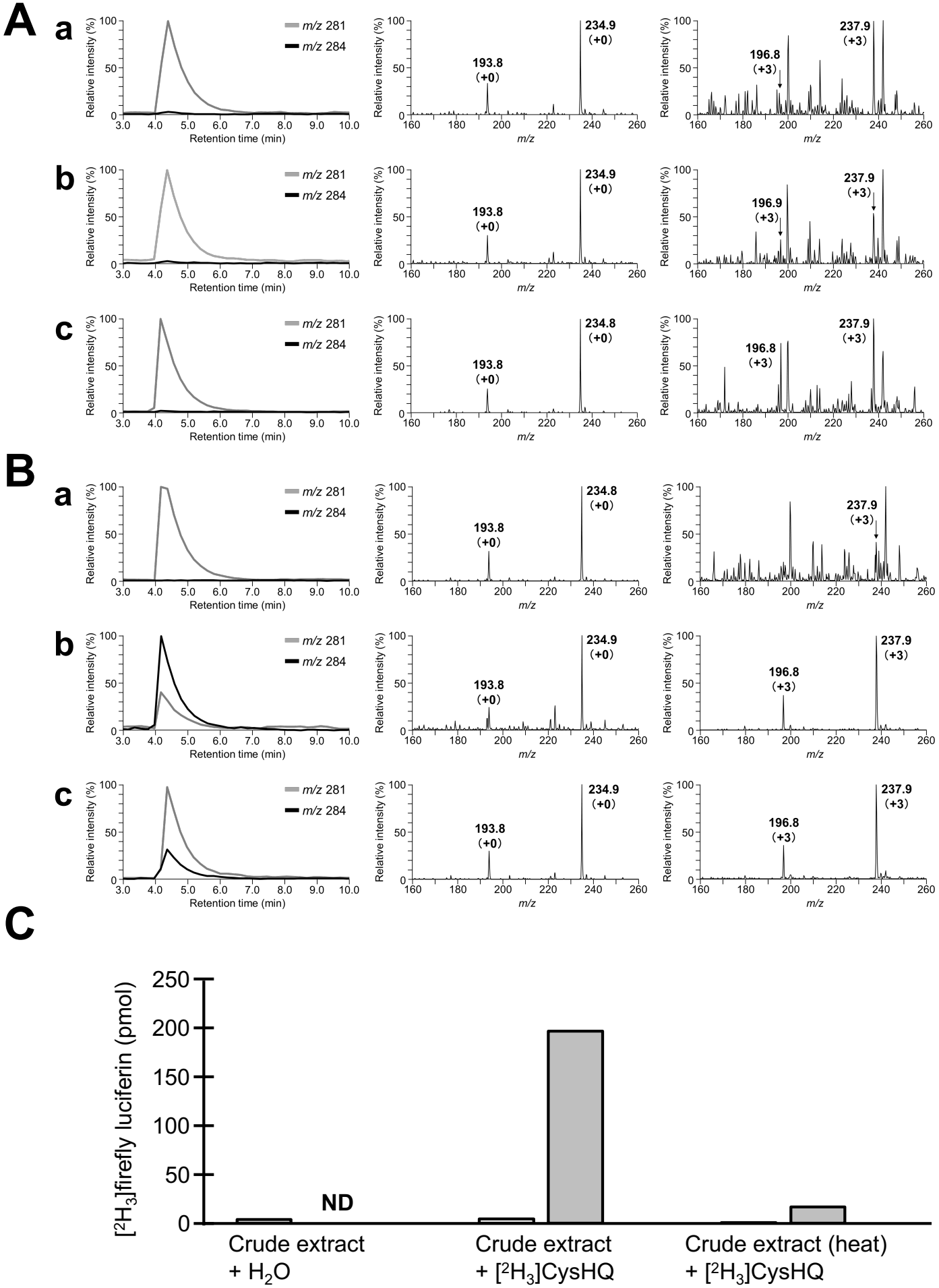


Fig. 3



Scheme 1