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Supplementary Materials for

Mechanism and color modulation of fungal bioluminescence

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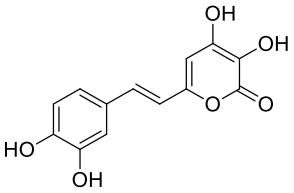
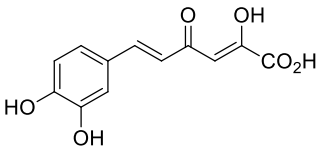
Synthetic procedures

Synthesis of target compounds 1-9: fungal luciferin, analogues and oxyluciferins

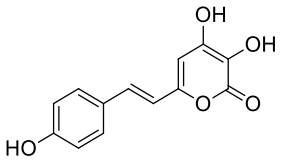
*General method **A** for the synthesis of compounds **1**-**9**: Protective groups cleavage.* To the solution of a protected substrate (1 eq.) in dry CH_2Cl_2 (0.1 mL) 1M BBr_3 in dry CH_2Cl_2 (0.2 mL, more than 30 eq.) is added in Argon atmosphere. The reaction mixture is left overnight, then diluted with EtOAc (2 × 0.5 mL), washed with phosphate buffer solution (pH 7.0, 0.5 mL) and dried over Na_2SO_4. After solvent removal, the residue is purified by HPLC.



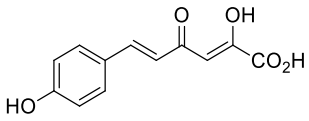
(*E*)-6-(3,4-dihydroxystyryl)-3,4-dihydroxy-2*H*-pyran-2-one (1). To a solution of pyrone **14** (30 mg, 0.176 mmol) and piperonyl aldehyde (98 mg, 0.588 mmol) in absolute MeOH (0.5 mL) a solution of $Mg(OMe)_2$ in MeOH (prepared by dissolving 50 mg of Mg in 1mL of MeOH) was added in argon atmosphere. The reaction was left stirring for 48 hours at room temperature and then diluted by chloroform (2 × 10 mL), washed with phosphate buffer solution (10 mL) and dried over Na_2SO_4. The solvent was evaporated and the residue purified by flash chromatography to give **17**, which was used in the next step without further purification. Compound **1** (12 mg, 26% for two steps) was obtained by general method **A** from **17** (fig. S12). NMR spectra matched those described for fungal luciferin in literature (*11*). ^{1}H-NMR (800 MHz, Acetone-d_6) δ 7.12 (d, J_1=2.0 Hz, 1H), 7.10 (d, J_2=16.0 Hz, 1H), 6.99 (dd, $J_{1,3}$=8.2, 2.0 Hz, 1H), 6.86 (d, J_3=8.2 Hz, 1H), 6.64 (d, J_2=16.0 Hz, 1H), 6.21 (s, 1H). HRMS (ESI) *m/z*: calcd for $C_{13}H_{11}O_6^+$ ($[M+H]^+$) 263.0550, found 263.0570.



(2*Z*,5*E*)-6-(3,4-dihydroxyphenyl)-2-hydroxy-4-oxohexa-2,5-dienoic acid (2) (*17*). Obtained by general method A from **26** (33 mg, 0.11 mmol), yielding 2 as a yellow solid (8 mg, 29%). HPLC R_t 10.7 min. ^{1}H-NMR (300 MHz, CD_3OD) δ 7.67 (brd, J_1=15.8 Hz, 1H), 7.13 (brs, 1H), 7.06 (brd, J_2=8.0 Hz, 1H), 6.82 (d, J_2=8.0 Hz, 1H), 6.62 (d, J_1=15.8 Hz, 1H), 6.55 (brs, 1H). HRMS (ESI) *m/z*: calcd for $C_{12}H_{10}NaO_6^+$ ($[M+Na]^+$) 273.0370, found 273.0364.

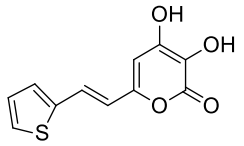


(*E*)-3,4-dihydroxy-6-(4-hydroxystyryl)-2*H*-pyran-2-one (3). Obtained by general method **A** from **18** (1 mg, 0.003 mmol), yielding **3** as a yellow solid (0.5 mg, 60%). HPLC R_t 19.5 min. ^{1}H-NMR (700 MHz, Acetone-d_6) δ 7.49 (d, J_1=8.5 Hz, 2H), 7.16 (d, J_2=16.0 Hz, 1H), 6.88 (d, J_1=8.5 Hz, 2H), 6.69 (d, J_2=16.0 Hz, 1H), 6.22 (s, 1H). ^{13}C NMR (176 MHz, acetone-d_6) δ 158.1, 151.6, 149.4, 131.5, 128.7, 127.8, 123.6, 116.6, 115.7, 101.6. HRMS (ESI) *m/z*: calcd for $C_{13}H_{11}O_5^+$ ($[M+H]^+$) 247.0601, found 247.0582.

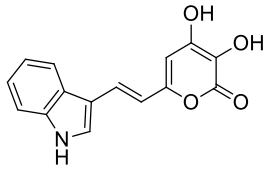


(2*Z*,5*E*)-2-hydroxy-6-(4-hydroxyphenyl)-4-oxohexa-2,5-dienoic acid (4). Obtained by general method **A** from **27** (100 mg, 0.38 mmol), yielding **4** as a yellow solid (62 mg, 70%). ^{1}H-NMR (700 MHz, CD_3OD) δ 7.75 (d, J = 15.8 Hz,

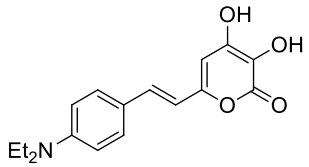
1H), 7.56 (d, *J* = 8.5 Hz, 2H), 6.86 (d, *J* = 8.6 Hz, 2H), 6.71 (d, *J* = 15.8 Hz, 1H). ^{13}C-NMR (75 MHz, CD_3OD) δ 185.7, 166.7, 160.6, 143.9, 143.7, 130.5, 126.0, 119.3, 115.7. HRMS (ESI) *m/z*: calcd for $C_{12}H_{10}NaO_5^+$ ([M+Na]$^+$) 257.0420, found 257.0419.



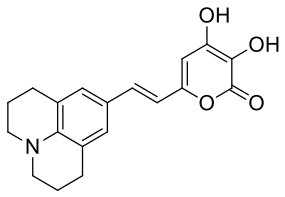
(*E*)-3,4-dihydroxy-6-(2-(thiophen-2-yl)vinyl)-2*H*-pyran-2-one (5). Obtained by general method **A** from **19** (3.4 mg, 0.001 mmol), yielding **5** as a yellow solid (2.5 mg, 82%). HPLC R_t 14.1 min. ^{1}H-NMR (600 MHz, Acetone-d_6) δ 7.48 (d, J_1=5.1 Hz, 1H), 7.37 (d, J_2=15.8 Hz, 1H), 7.34 (d, J_3=3.6 Hz, 1H), 7.10 (dd, $J_{1,3}$=5.1, 3.6 Hz, 1H), 6.62 (d, J_2=15.8 Hz, 1H), 6.31 (s, 1H). ^{13}C-NMR (150 MHz, Acetone-d_6) δ 150.4, 149.1, 141.2, 128.7, 128.1, 126.3, 124.6, 124.0, 118.4, 102.6. HRMS (ESI) *m/z*: calcd for $C_{11}H_9O_4S^+$ ([M+H]$^+$) 237.0216, found 237.0196.



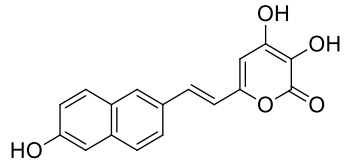
(*E*)-6-(2-(1*H*-indol-3-yl)vinyl)-3,4-dihydroxy-2*H*-pyran-2-one (6). Obtained by general method **A** from **20** (2.3 mg, 0.006 mmol), yielding **6** as a yellow solid (0.8 mg, 51%). HPLC R_t 13.7 min. ^{1}H-NMR (600 MHz, Acetone-d_6) δ 8.02 (d, J_1 = 7.8Hz, 1H), 7.76 (s, 1H), 7.50 (d, 1H), 7.48 (d, J_2=16.1 Hz, 1H), 7.25 – 7.16 (m, 2H), 6.84 (d, J_2= 16.1 Hz, 1H), 6.25 (s, 1H). ^{13}C-NMR (150 MHz, Acetone-d_6) δ 152.6, 149.9, 137.6, 127.8, 126.0, 125.3, 122.8, 122.4, 120.4, 119.9, 114.5, 113.7, 111.9, 99.8. HRMS (ESI) *m/z*: calcd for $C_{15}H_{12}NO_4^+$ ([M+H]$^+$) 270.0761, found 270.0758.



(*E*)-6-(4-(diethylamino)styryl)-3,4-dihydroxy-2*H*-pyran-2-one (7). Obtained by general method **A** from **21** (1.6 mg, 0.005 mmol), yielding **7** as a yellow-orange solid (0.9 mg, 62%). HPLC R_t 19.5 min. ^{1}H-NMR (700 MHz, Acetone-d_6) δ 7.44 (d, J_1=8.7 Hz, 2H), 7.13 (d, J_2=15.9 Hz, 1H), 6.73 (d, J_1=8.7 Hz, 2H), 6.58 (d, J_2=15.9 Hz, 1H), 6.14 (s, 1H), 3.45 (q, J_3=7.1 Hz, 4H), 1.18 (t, J_3=7.1 Hz, 6H). ^{13}C-NMR (176 MHz, Acetone-d_6) δ 152.3, 149.7, 148.4, 132.2, 128.7, 123.0, 122.9, 113.7, 111.5, 100.1, 44.0, 12.1. HRMS (ESI) *m/z*: calcd for $C_{17}H_{20}NO_4^+$ ([M+H]$^+$) 302.1387, found 302.1367.



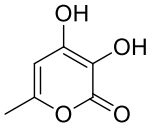
(*E*)-6-(2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-*ij*]quinolin-9-yl)vinyl)-3,4-dihydroxy-2*H*-pyran-2-one (8). Obtained by general method **A** from **22** (1.1 mg, 0.003 mmol), yielding **8** as an orange solid (0.5 mg, 50%). HPLC R_t 18.8 min. ^{1}H-NMR (800 MHz, Acetone-d_6) δ 7.03 (d, J_1=15.9 Hz, 1H), 6.99 (s, 2H), 6.52 (d, J_1=15.9 Hz, 1H), 6.12 (s, 1H), 3.24 (t, J_2 = 6.4 Hz, 4H), 2.74 (t, J_2 = 6.4 Hz, 4H), 1.95 (m, 4H). ^{13}C-NMR (200 MHz, Acetone-d_6) δ 152.4, 149.7, 143.6, 132z, 4.6, 126.1, 122.9 (2C), 121.1, 113.2, 99.9, 49.7, 27.3, 21.6. HRMS (ESI) *m/z*: calcd for $C_{19}H_{20}NO_4^+$ ([M+H]$^+$) 326.1387, found 326.1393.



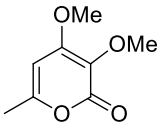
(*E*)-3,4-dihydroxy-6-(2-(6-hydroxynaphthalen-2-yl)vinyl)-2*H*-pyran-2-one (9). Obtained by general method **A** from **23** (1.9 mg, 0.005 mmol), yielding **9** as a light-yellow solid (0.6 mg, 43%). HPLC R_t 13.3 min. ^{1}H-NMR (700 MHz, Acetone-d_6) δ 7.96 (s, 1H), 7.82 (d, J_1=8.8 Hz, 1H), 7.73– 7.66 (m, 2H), 7.36 (d, J_1=16.0 Hz, 1H), 7.22 (d, J_3=2.4 Hz, 1H), 7.18 (dd, $J_{1,3}$=8.8, 2.4 Hz, 1H), 6.94 (d, J_1=16.0 Hz, 1H), 6.31 (s, 1H). ^{13}C-NMR (176 MHz, acetone-d_6) δ 156.1, 151.2, 149.3, 135.3, 131.7, 130.8, 129.9, 128.5, 127.9, 126.8, 124.0,

123.6, 118.7, 118.3, 109.1, 102.3. HRMS (ESI) *m/z*: calcd for $C_{17}H_{13}O_5^+$ ($[M+H]^+$) 297.0757, found 297.0724.

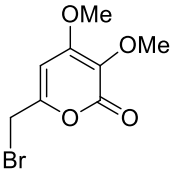
Synthesis of compounds 13-16: building blocks (figs. S13 and S14)



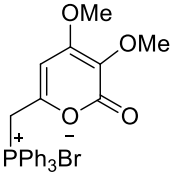
3,4-dihydroxy-6-methyl-2*H*-pyran-2-one (13). Compounds **11** and **12** were obtained from commercially available dehydroacetic acid **10** according to the procedures described in literature *(28,31)*. NaOH (5 M, 275 mL) was added to 6.7 g (18.8 mmol) of **12** and the mixture stirred for 2 hours at 25°C. The solution was then acidified to pH 3.0 with aqueous HCl and extracted with EtOAc (400 mL, 5 × 200 mL, 5 × 100 mL), dried over Na_2SO_4 and solvent evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel ($CHCl_3$:MeOH:AcOH, 94:5:1), R_f 0.42, yielding 2.2 g (82%) of **13** as a yellow crystalline solid. m.p. (decomp.) 199ºC; ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.51 (s, 1H), 8.34 (s, 1H), 5.96 (s, 1H), 2.10 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.8, 153.0, 151.0, 121.8, 101.0, 18.6. HRMS (ESI) *m/z*: calcd for $C_6H_7O_4^+$ ($[M+H]^+$) 143.0339, found 143.0333.



3,4-dimethoxy-6-methyl-2*H*-pyran-2-one (14). To a solution of a pyranone **13** (600 mg, 4.22 mmol) in acetone (20 mL) methyl sulfate (2.4 mL, 25.3 mmol), sodium carbonate (2.7 g, 25.3 mmol) and DIPEA (150 μL, 0.84 mmol) were added. The reaction mixture was heated at 60ºC for 6 hours, then left stirring overnight at room temperature. The solution was diluted with EtOAc (50 mL), washed with phosphate buffer solution (pH 7.0, 70 mL), concentrated NaCl (50 mL), dried over Na_2SO_4 and solvent evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel (EtOAc:hexane, gradient 0:1 to 1:1), R_f 0.45 ($CHCl_3$:EtOH 98:2), yielding 486 mg (68%) of **14** as a light-yellow crystalline solid. m.p. 103ºC; ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.92 (s, 1H), 3.94 (s, 3H), 3.80 (s, 3H), 2.22 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.1, 159.0, 158.0, 126.9, 97.0, 60.3, 57.5, 20.0. HRMS (ESI) *m/z*: calcd for $C_8H_{11}O_4^+$ ($[M+H]^+$) 171.0652, found 171.0652.



6-(bromomethyl)-3,4-dimethoxy-2*H*-pyran-2-one (15). Method was adopted from literature with modifications *(30)*. To the solution of **14** (100 mg, 0.59 mmol) in CCl_4 (12 mL) *N*-bromosuccinimide (105 mg, 0.59 mmol) and benzoyl peroxide (1 mg) were added. The reaction mixture was stirred under irradiation with daylight lamp and monitored by TLC, then diluted with CH_2Cl_2 (20 mL), washed with concentrated $Na_2S_2O_3$ (20 mL), dried over Na_2SO_4 and solvent evaporated under reduced pressure. The residue was purified by flash chromatography (EtOAc:hexane 1:1), R_f 0.47, yielding 50 mg (34%) of **15** as a white crystalline solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.25 (s, 1H), 4.14 (s, 2H), 3.99 (s, 3H), 3.85 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.9, 157.6, 154.1, 128.8, 99.8, 60.4, 58.0, 26.8. HRMS (ESI) *m/z*: calcd for $C_8H_{10}BrO_4^+$ ($[M+H]^+$) 248.9757, found 248.9752.

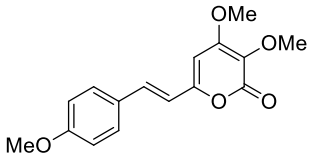


[(3,4-dimethoxy-2-oxo-2*H*-pyran-6-yl)methyl]triphenylphosphonium bromide (16). To PPh_3 (152 mg, 0.58 mmol) a solution of bromide **15** (120 mg, 0.48 mmol) in dry toluene (14 mL) was added under argon atmosphere. The reaction mixture was

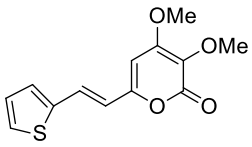
refluxed for 4 hours under argon, then left stirring overnight at room temperature. The solvent was evaporated and residue washed with Et_2O (4 × 10 mL), yielding 199 mg (81%) of **16** as a yellow-orange solid. ^{1}H NMR (300 MHz, $CDCl_3$) 7.96-7.37 (m, 15H), 5.45 (d, *J*=14.7 Hz, 2H), 5.27 (s, 1H), 3.83 (s, 3H), 3.69 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 160.4, 158.8, 147.7, 135.5, 134.3, 132.1, 130.4, 117.2, 104.4, 60.2, 58.3, 29.2.

Synthesis of compounds 18-23, precursors of luciferin analogues 3, 5-9 (fig. S15)

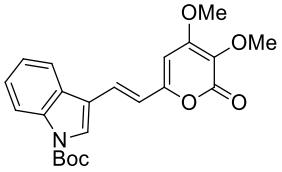
General method B for the synthesis of compounds 18-23: Wittig reaction. To a stirred mixture of 2 M NaOH (1mL) and a solution of the phosphonium salt 16 (0.029 mmol) in CH_2Cl_2 (1 mL) an appropriate aldehyde (0.044 mmol, 1.5 eq) is added. After 3 hours the reaction mixture is diluted with CH_2Cl_2 (2 × 1mL), washed with water (2 × 1mL), dried over anhydrous Na_2SO_4 and the solvent evaporated under reduced pressure. The residue is subjected to flash chromatography on silica gel to afford compounds 18-23.



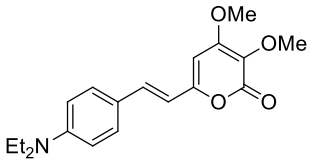
(*E*)-3,4-dimethoxy-6-(4-methoxystyryl)-2*H*-pyran-2-one (18). Obtained by general method **B** from **16** (15 mg, 0.03 mmol) and 4-methoxybenzaldehyde (6 mg, 0.04 mmol). Purification by flash chromatography (EtOAc:hexane gradient from 0:1 to 3:7), R_f 0.45 (EtOAc:hexane 1:1), yielded **18** as a light-yellow solid (1 mg, 12%). ^{1}H-NMR (700 MHz, $CDCl_3$) δ 7.51 (d, J_1=8.8 Hz, 2H), 7.47 (d, J_2=15.8 Hz, 1H), 6.98 (d, J_1=8.8 Hz, 2H), 6.53 (d, J_2=15.8 Hz, 1H), 6.13 (s, 1H), 4.06 (s, 3H), 3.95 (s, 3H), 3.91 (s, 3H). ^{13}C-NMR (176 MHz, $CDCl_3$) δ 161.0, 160.8, 159.0, 155.4, 135.9, 128.9, 128.2, 127.8, 116.2, 114.5, 97.0, 60.4, 57.3, 55.5. HRMS (ESI) *m/z*: calcd for $C_{16}H_{17}O_5^+$ ([M+H]$^+$) 289.1071, found 289.1067.



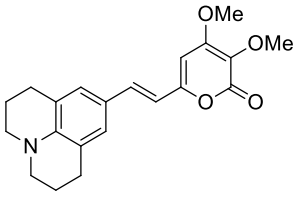
(*E*)-3,4-dimethoxy-6-(2-(thiophen-2-yl)vinyl)-2*H*-pyran-2-one (19). Obtained by general method **B** from **16** (15 mg, 0.03 mmol) and thiophene-2-carbaldehyde (4 μL, 0.04 mmol). Purification by flash chromatography (EtOAc:hexane gradient from 0:1 to 3:7), R_f 0.71 (EtOAc:hexane 1:1), yielded **19** as a white-yellow solid (3.4 mg, 44%). ^{1}H-NMR (700 MHz, $CDCl_3$) δ 7.62 (d, J_1=15.6 Hz, 1H), 7.38 (d, J_2=5.0 Hz, 1H), 7.24 (d, J_3=3.5 Hz, 1H), 7.11 (m, 1H), 6.46 (d, J_1=15.6 Hz, 1H), 6.14 (s, 1H), 4.05 (s, 3H), 3.95 (s, 3H). ^{13}C-NMR (176 MHz, $CDCl_3$) δ 160.9, 158.9, 154.5, 140.8, 129.5, 128.2 (2C), 128.1, 127.0, 117.5, 97.6, 60.3, 57.3. HRMS (ESI) *m/z*: calcd for $C_{13}H_{13}O_4S^+$ ([M+H]$^+$) 265.0529, found 265.0511.



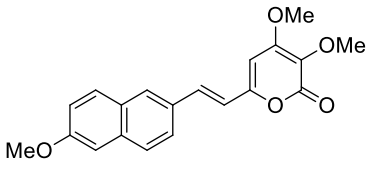
(*E*)-tert-butyl 3-(2-(3,4-dimethoxy-2-oxo-2*H*-pyran-6-yl)vinyl)-1*H*-indole-1-carboxylate (20). Obtained by general method **B** from **16** (15 mg, 0.03 mmol) and *tert*-butyl 3-formyl-1*H*-indole-1-carboxylate (11 mg, 0.04 mmol). Purification by flash chromatography (EtOAc:hexane gradient from 0:1 to 3:7), R_f 0.65 (EtOAc:hexane 1:1), yielded **20** as a yellow-orange solid (4.5 mg, 39%). ^{1}H-NMR (700 MHz, $CDCl_3$) δ 8.27 (brd, J_1=7.9 Hz, 1H), 7.92 (d, J_2=8.1 Hz, 1H), 7.88 (s, 1H), 7.64 (d, J_3=16.0 Hz, 1H), 7.46 (m, 1H), 7.41 (m, 1H), 6.77 (d, J_3=16.0 Hz, 1H), 6.19 (s, 1H), 4.08 (s, 3H), 3.96 (s, 3H), 1.76 (s, 9H). ^{13}C-NMR (176 MHz, $CDCl_3$) δ 167.9, 161.1, 158.9, 155.2, 136.3, 128.0 (2C), 127.1, 127.0, 125.3, 123.5, 120.0, 118.0, 117.6, 115.7, 97.0, 84.6, 60.4, 57.3, 28.2. HRMS (ESI) *m/z*: calcd for $C_{22}H_{24}NO_6^+$ ([M+H]$^+$) 398.1598, found 398.1580.



(*E*)-6-(4-(diethylamino)styryl)-3,4-dimethoxy-2*H*-pyran-2-one (21). Obtained by general method **B** from **16** (15 mg, 0.03 mmol) and 4-(diethylamino)benzaldehyde (8 mg, 0.04 mmol). Purification by flash chromatography (EtOAc:hexane gradient from 0:1 to 3:7), R_f 0.62 (EtOAc:hexane 1:1), yielded **21** as an orange solid (3.2 mg, 32%). ^{1}H-NMR (700 MHz, acetone-d_6) δ 7.47 (d, J_1=8.9 Hz, 2H), 7.23 (d, J_2=15.9 Hz, 1H), 6.75 (d, J_1=8.9 Hz, 2H), 6.62 (d, J_2=15.9 Hz, 1H), 6.40 (s, 1H), 4.00 (s, 3H), 3.75 (s, 3H), 3.47 (q, J_3=7.0 Hz, 4H), 1.19 (t, J_3=7.0 Hz, 6H). ^{13}C-NMR (176 MHz, Acetone-d_6) δ 160.0, 159.5, 156.1, 148.7, 134.4, 129.0, 126.8, 122.5, 113.5, 111.5, 95.8, 58.9, 43.9, 56.7, 12.0. HRMS (ESI) *m/z*: calcd for $C_{19}H_{24}NO_4^+$ ([M+H]$^+$) 330.1700, found 330.1677.

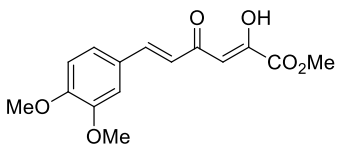


(*E*)-6-(2-(1,2,3,5,6,7-hexahydropyrido[3,2,1-*ij*]quinolin-9-yl)vinyl)-3,4-dimethoxy-2*H*-pyran-2-one (22). Obtained by general method **B** from **16** (15 mg, 0.03 mmol) and 1,2,3,5,6,7-hexahydropyrido[3,2,1-*ij*]quinoline-9-carbaldehyde (9 mg, 0.04 mmol). Purification by flash chromatography (EtOAc:hexane gradient from 0:1 to 3:7), R_f 0.38 ($CHCl_3$:methanol 98:2), yielded **22** as an orange solid (2.2 mg, 21%). ^{1}H-NMR (700 MHz, Acetone-d_6) δ 7.01 (s, 2H), 7.13 (d, J_1=15.9 Hz, 1H), 6.56 (d, J_1=15.9 Hz,1H), 6.38 (s, 1H), 4.00 (s, 3H), 3.75 (s, 3H), 3.25 (t, J_2=5.9 Hz, 4H), 2.74 (t, J_3=6.4 Hz, 4H), 1.95 (m, 4H). ^{13}C-NMR (176 MHz, Acetone-d_6) δ 160.1, 159.6, 156.3, 144.1, 134.9, 126.5 (2C), 122.5, 121.1, 113.0, 95.5, 59.0, 56.6, 49.6, 27.5, 21.6. HRMS (ESI) *m/z*: calcd for $C_{21}H_{24}NO_4^+$ ([M+H]$^+$) 354.1700, found 354.1669.



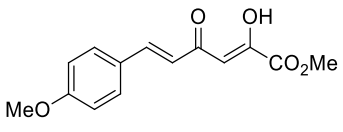
(*E*)-3,4-dimethoxy-6-(2-(6-methoxynaphthalen-2-yl)vinyl)-2*H*-pyran-2-one (23). Obtained by general method **B** from **16** (15 mg, 0.03 mmol) and 6-methoxy-2-naphthaldehyde (8 mg, 0.04 mmol). Purification by flash chromatography (EtOAc:hexane gradient from 0:1 to 3:7), R_f 0.53 (EtOAc:hexane 1:1), yielded **23** as a white-yellow solid (1.5 mg, 15%). ^{1}H-NMR (700 MHz, $CDCl_3$) δ 7.89 (s, 1H), 7.82 (d, J_1=9.0 Hz, 1H), 7.79 (d, J_2=8.6 Hz, 1H), 7.68 (dd, J_2=8.6 Hz, J_3<1.5 Hz, 1H), 7.64 (d, J_4=15.8 Hz, 1H), 7.24 (dd, J_1=9.0 Hz, J_5=2.4 Hz, 1H), 7.20 (d, J_5=2.4 Hz, 1H), 6.73 (d, 1H, J_4=15.8 Hz), 6.19 (s, 1H), 4.08 (s, 3H), 4.01 (s, 3H), 3.96 (s, 3H). ^{13}C-NMR (176 MHz, $CDCl_3$) δ 161.1, 158.9, 158.7, 155.2, 135.5, 135.2, 130.8, 130.0, 128.9, 128.6, 128.1, 127.5, 123.8, 119.5, 117.5, 106.2, 97.5, 60.5, 57.5, 55.4. HRMS (ESI) *m/z*: calcd for $C_{20}H_{19}O_5^+$ ([M+H]$^+$) 337.1227, found 337.1214.

Synthesis of compounds 26 and 27 - precursors for oxyluciferins (fig. S16)



(2*Z*,5*E*)-methyl 6-(3,4-dimethoxyphenyl)-2-hydroxy-4-oxohexa-2,5-dienoate (26). Compound **24** was obtained from commercially available 3,4-dimethoxybenzaldehyde according to the procedure described in literature (*29*). To the solution of **24** (100 mg, 0.485 mmol) in dry THF (10 mL) under argon 1 M LiHMDS in THF (1.1 mL) was added at -78^{o}C. The reaction was left stirring and warming up to r.t. slowly overnight and then diluted with EtOAc (2 × 50 mL), washed with 1 M HCl (50 mL). After solvent removal the crude product was

purified using column chromatography (EtOAc:hexane gradient 2:8 to 1:0, then EtOAc:EtOH gradient 9:1 to 1:1), yielding **26** as an orange-brown solid (113 mg, 80%). ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.69 (d, J_1=15.8 Hz, 1H), 7.16 (dd, $J_{2,3}$=8.3, 1.6 Hz, 1H), 7.07 (d, J_3=1.6 Hz, 1H), 6.88 (d, J_2=8.3 Hz, 1H), 6.53 (m, 2H), 3.92 (brs, 6H), 3.90 (s, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 185.7, 172.7, 162.9, 152.0, 149.5, 143.8, 143.8, 127.5, 123.7, 121.1, 111.3, 110.0, 100.7, 56.2, 56.1, 53.2. HRMS (ESI) *m/z*: calcd for $C_{15}H_{17}O_6^+$ ($[M+H]^+$) 293.1020, found 293.0999.

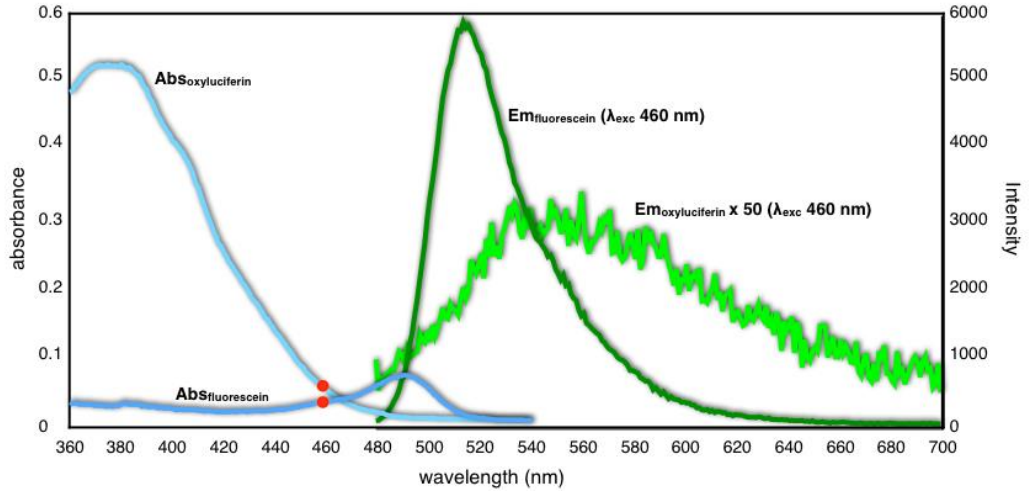


(2*Z*,5*E*)-methyl-2-hydroxy-6-(4-methoxyphenyl)-4-oxohexa-2,5-dienoate (27). Compound **25** was obtained from commercially available 4-methoxybenzaldehyde according to the procedure, described in literature *(29)*. Synthesis of **27** from compound **25** (500 mg, 2.84 mmol) was performed according to the same procedure as for **26**. Column chromatography purification ($CHCl_3$: EtOH gradient from 100:0 to 99.5:0.5) yielded **27** as a yellow-orange solid (273 mg, 37%). ^{1}H-NMR (700 MHz, $CDCl_3$) δ 7.79 (d, *J* = 15.8 Hz, 1H), 7.61 (d, *J* = 8.7 Hz, 2H), 7.01 (d, *J* = 8.7 Hz, 2H), 6.62 (d, *J* = 16.0 Hz, 1H), 6.60 (s, 1H), 3.99 (s, 3H), 3.93 (s, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 186.1, 162.8, 162.1, 161.8, 143.5, 130.4, 127.1, 120.8, 114.6, 100.7, 55.5, 53.1. HRMS (ESI) *m/z*: calcd for $C_{14}H_{14}NaO_5^+$ ($[M+Na]^+$) 285.0733, found 285.0731.

HPLC purification of the compounds 1-9

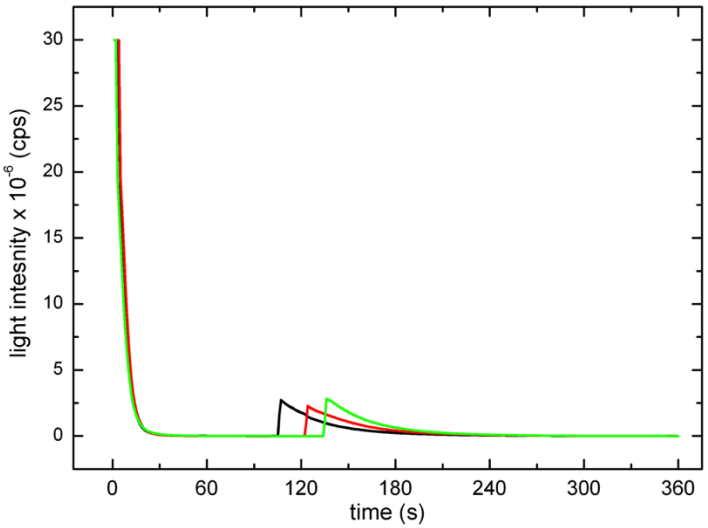
Compounds **1-3, 5-9** were purified using a semi-preparative column ZORBAX Eclipse XDB-C18 (250 × 9.4 mm, 5 μm; Agilent Technologies, USA) in a 1260 Infinity LC chromatograph equipped with a diode array detector (Agilent Technologies, USA). Compounds were dissolved in DMSO and separated at room temperature using (A) 0.1% aqueous formic acid and (B) acetonitrile as mobile phase at 3 $mL \cdot min^{-1}$. Different linear gradient programs were employed: 5 to 40% B during 25 min for compounds **1**, **3** and **7** and 5 to 100% B during 24 min for compounds **2, 5, 6, 8, 9**. Detection was performed at 210, 230, 250, 270, 290, 310, 330 and 360 nm.

Compound **4** was purified using a Discovery C18 column (150 x 4.6 mm, 5 μm; Sigma-Aldrich, USA) in a Nexera X2 liquid chromatograph equipped with a diode array detector (SPD-M20A; Shimadzu, Japan). The reaction residue was dissolved in DMSO and separated at room temperature using (A) 0.1% aqueous trifluoroacetic acid and (B) acetonitrile as mobile phase at 1 $mL.min^{-1}$. A concave gradient from 10 to 100% B in 11 min, with a curvature coefficient of 4, was employed. Detection was performed at 390 nm.



$$\Phi_{FL,OxyLn} = \frac{area_{FL,oxyluciferin}}{area_{FL,fluorescein}} \times \frac{Abs_{460nm,fluorescein}}{Área_{460nm,oxyluciferin}} \times \Phi_{FL,fluorescein} \times \left(\frac{n_{water}}{n_{acetone}}\right)^2 \quad \text{eq.1}$$

fig. S1. Determination of fluorescence quantum yield of the oxyluciferin. Absorption and emission spectra of aqueous fluorescein solution (0.1 M NaOH) and oxyluciferin in acetone used in the determination of oxyluciferin fluorescence quantum yield.



$$\mathbf{f_{luminol}} = \frac{\mathbf{\Phi_{CL,luminol}} \times \boldsymbol{n_{luminol}}}{\boldsymbol{Q_{luminol}}} \qquad \text{eq. 2}$$

fig. S2. Determination of the chemiluminescence and singlet quantum yields of the luciferin/luciferase reaction. Chemiluminescence of luminol reaction used for calibration of Berthold Sirius L luminometer. The reaction was performed in triplicates. Equation 2 was used to determine the luminometer calibration factor ($\mathbf{f_{luminol}}$) based on luminol chemiluminescence quantum yield ($\Phi_{CL,luminol}$ = 0.0114 ± 0.0006 $Emol^{-1}$). $\mathbf{f_{luminol}}$ is the luminol calibration factor; $\mathbf{\Phi_{CL,luminol}}$ is luminol chemiluminescence quantum yield; $\boldsymbol{n}_{\mathbf{luminol}}$ is the number of mols of luminol; $\mathbf{Q_{luminol}}$ is the integral of the light intensity (in counts) for luminol reaction..

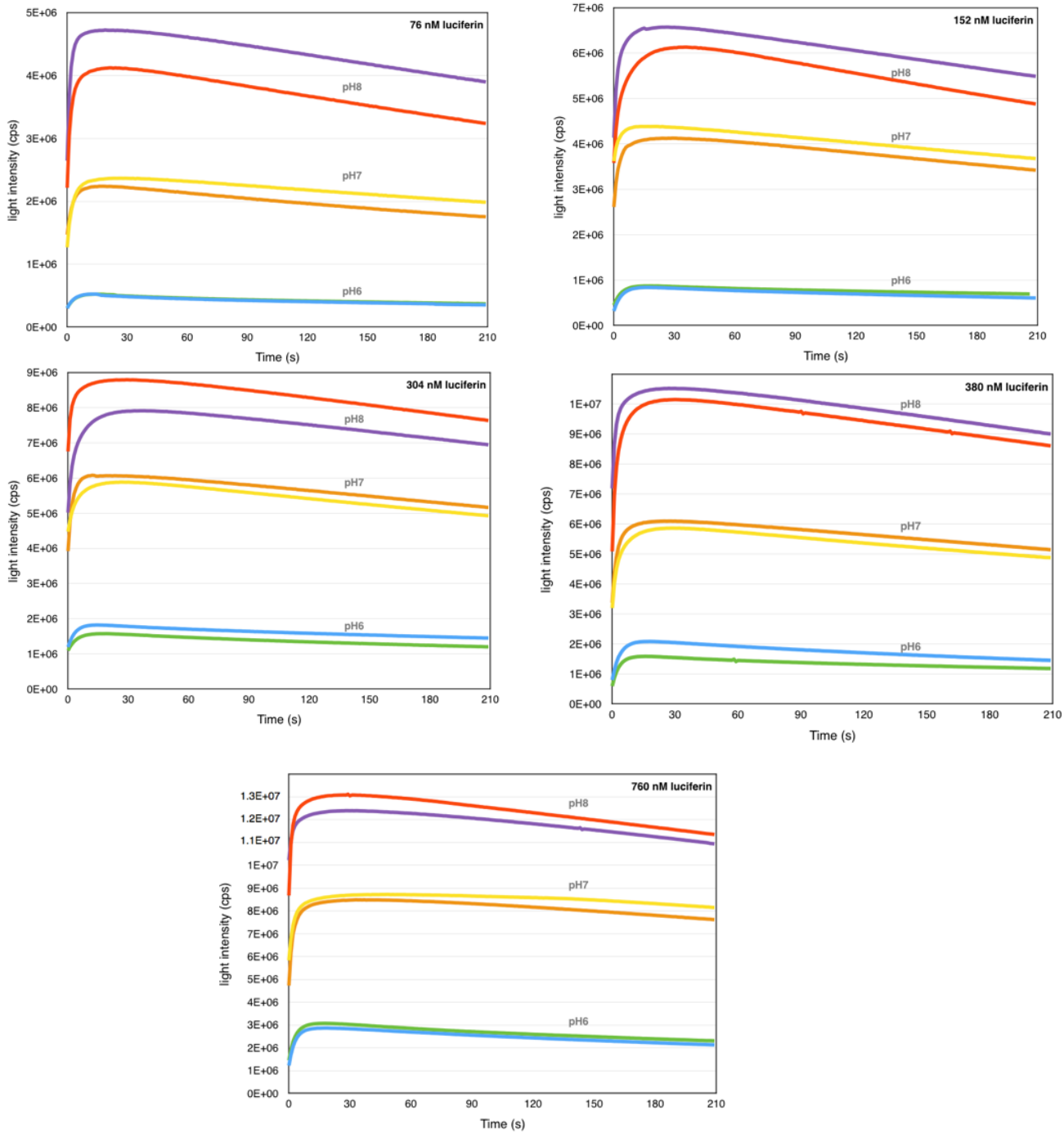
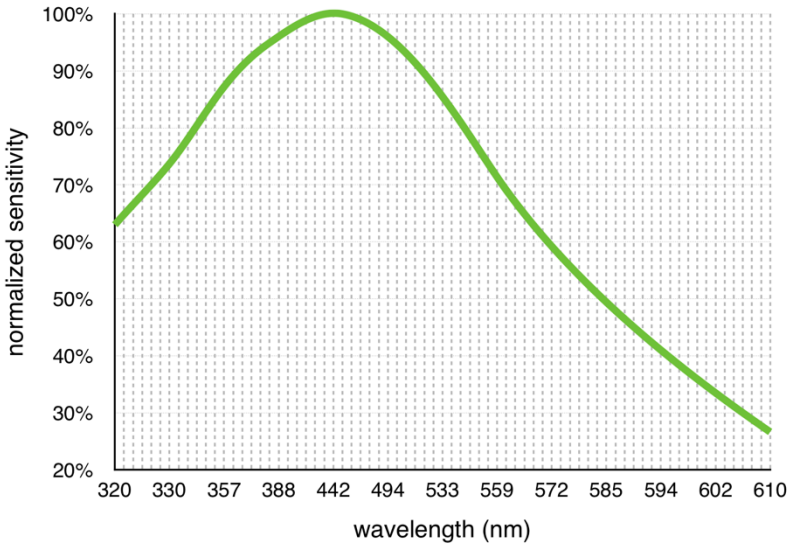


fig. S3. Dependence of the chemiluminescence intensity from luciferin/luciferase reaction on the concentration of luciferin (76 to 760 nM) and phosphate buffer (pH 6 to 8). Experiments were performed in duplicates at 25ºC.



$$\Phi_{CL} = \frac{Q \times f_{luminol} \times f_{PMT}}{n_{substrate}} \qquad \text{eq. 3}$$

fig. S4. Photocathode spectral response curve provided by Berthold Detection Systems (PMT type 9107). The total light emitted (Q), obtained initially in arbitrary units (cps) for a particular reaction can be thus converted to Einstein (E) using $f_{luminol}$ = (4.4 ± 0.3) × 10^{-23} E/counts and equation 3. Where: $\mathbf{\Phi_{CL}}$ is chemiluminescence quantum yield of the reaction; $\mathbf{f_{PMT}}$ is the photomultiplier correction factor; $\boldsymbol{n}_{\mathbf{substrate}}$ is the number of mols of luciferin or analogue; **Q** is the integral of the light intensity (in counts) for the reaction.

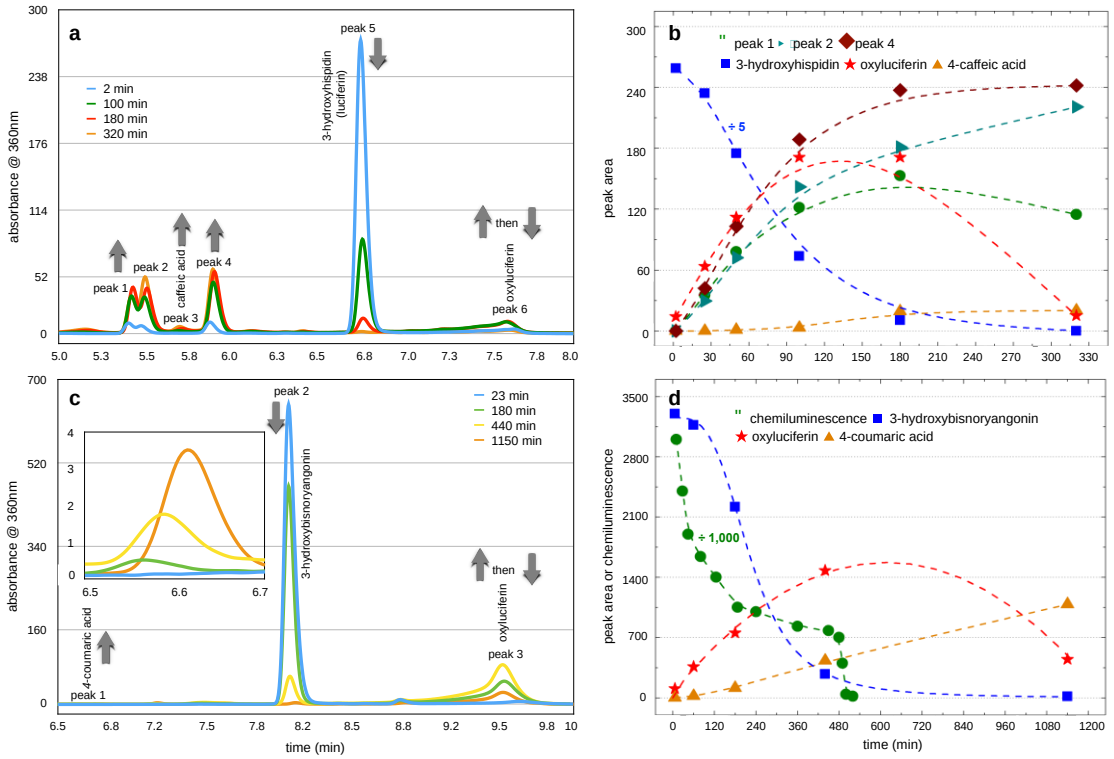


fig. S5. Dynamics of the reaction between substrate and luciferase microsomal preparation of *N. nambi*. (**a**) Chromatogram of fungal luciferin (3-hydroxyhispidin) and products formation followed for 320 min by UV at 360 nm and MS. Peaks 1, 2 and 4 were decomposition products from either luciferin **1** or oxyluciferin **2** (fig. S1 and S2). peak 3 was identified as caffeic acid which was originated from the decomposition of oxyluciferin **2** (peak 6), and peak 5 is the luciferin **1**. (**b**) Time courses of the luciferin decay and products formation. **(c**) Chromatogram of 3-hydroxybisnoryangonin **3** (used as substrate) and products formation (its oxyluciferin **4** and *p*-coumaric acid) followed for 1150 min by UV at 360 nm and MS. Peak 1 was identified as *p*-coumaric acid, which was originated from the decomposition of the respective oxyluciferin **4** (Peak 3). (**d**) Time courses of substrate decay, products formation and light emission indicate their correlation.

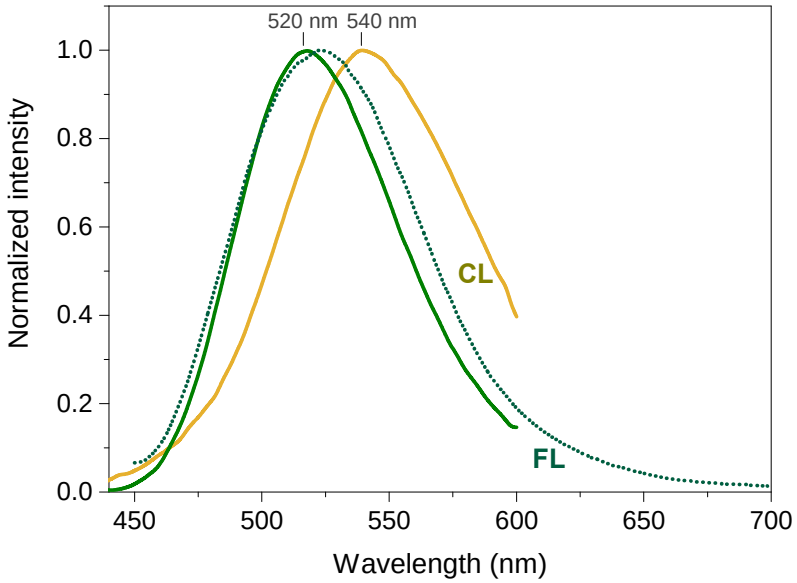


fig. S6. Chemiluminescence spectra of 1 (orange curve) and 3 (green curve) and the fluorescence spectrum of 4 in acetone (dotted curve). The chemiluminescence spectrum maximum (520 nm) from the reaction of 3-hydroxybisnoryangonin (**3**) and the luciferase-enriched fraction of *N. gardneri* is *ca.* 20 nm shifted to blue than the one obtained with the native fungal luciferin (**1**, 540 nm). Fluorescence spectrum of oxyluciferin **4** matches the chemiluminescence spectrum of **3**.

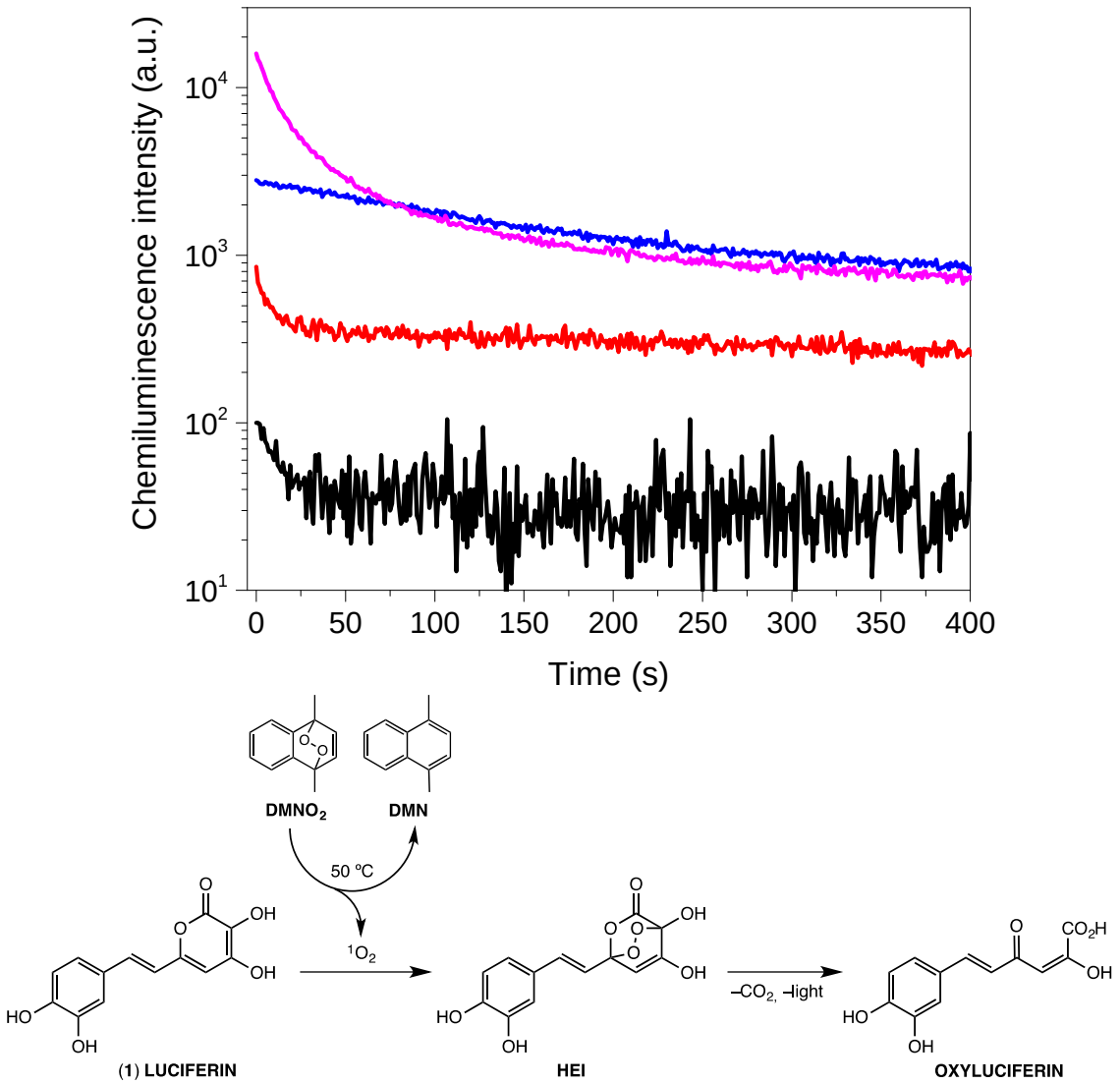


fig. S7. Chemiluminescence from the reaction of 1,4-dimethylnaphthalene endoperoxide ($DMNO_2$) with 3-hydroxyhispidin (luciferin). the baseline of equipment (–), emission of 290 mM $DMNO_2$ in acetone at 25ºC (–), 145 mM $DMNO_2$ and 0.45 mM luciferin in acetone at 25ºC (–) and 145 mM $DMNO_2$ and 0.45 mM luciferin in acetone heated at 55ºC for 15 s (–). A proposal to explain the observed light emission is also depicted below the graph.

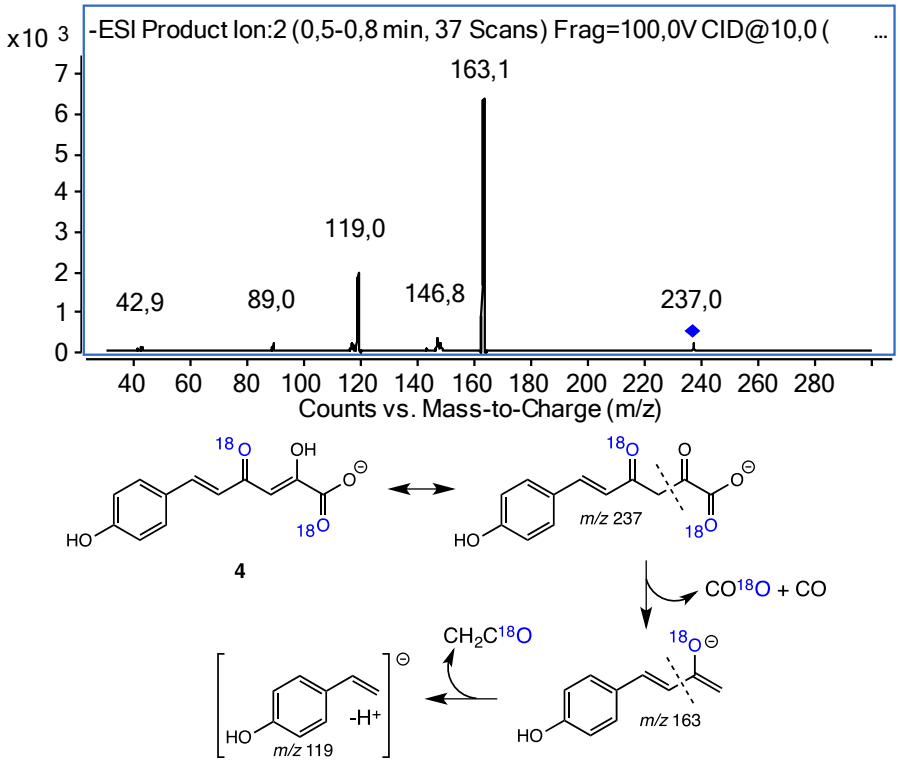


fig. S8. Collision-induced dissociation spectrum (ESI-MS/MS) of the labeled oxyluciferin—compound 4. The neutral loss of 74 Da (237 → 163) can be rationalized by the elimination of labeled $CO^{18}O$ and CO, leading to a resonance-stabilized enolate. For the corresponding unlabeled oxyluciferin this neutral loss is 72 Da (CO_2 + CO).

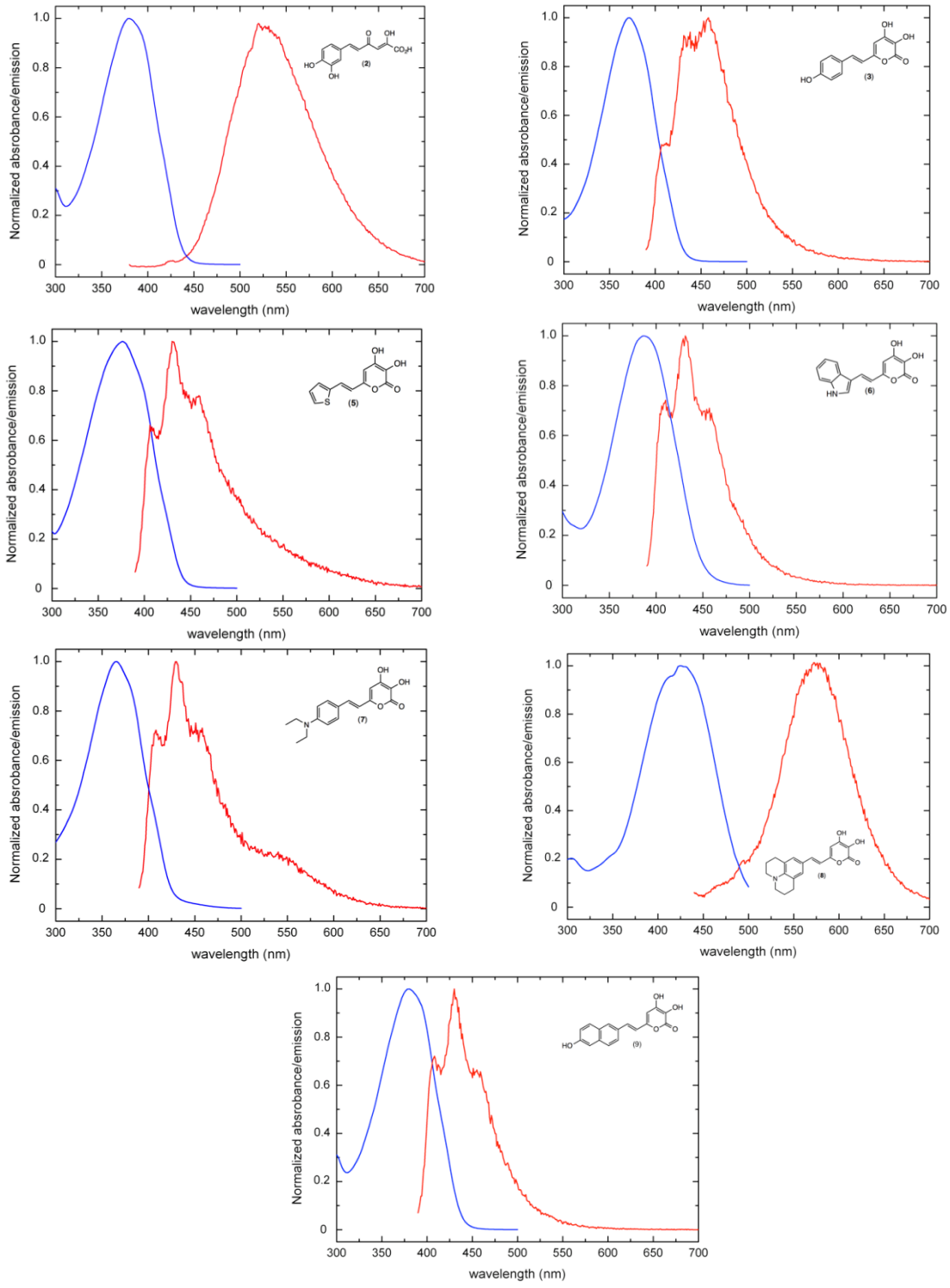


fig. S9. Absorption (blue) and fluorescence emission (red) spectra of compounds 2, 3, and 5 to 9.

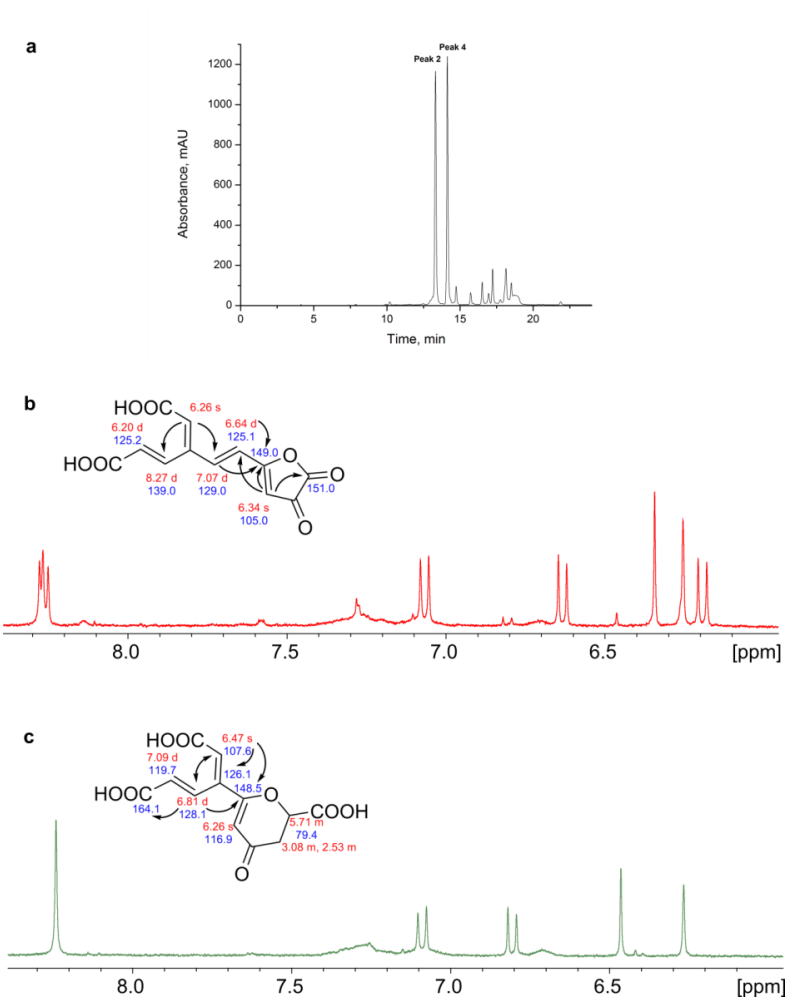


fig. S10. Isolation and identification of compounds in peaks 2 and 4 of the luciferin 1/luciferase reaction. (**a**) HPLC chromatogram for compound isolation. (**b**) (**c**) Chemical structures and ^{1}H-NMR spectra of the compounds in peaks 2 and 4. Chemical shifts of protons are shown in red, chemical shifts of carbons are shown in blue. Arrows show observed H-C interactions in [^{1}H-^{13}C]-HMBC spectra.

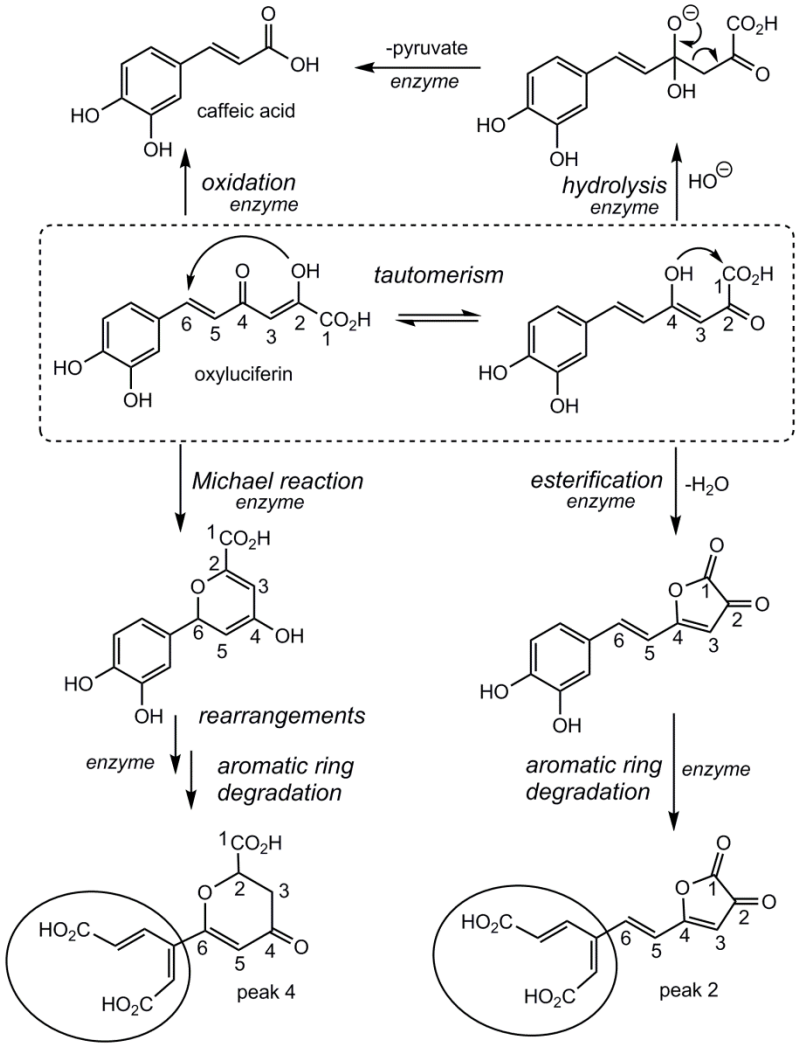


fig. S11. Proposed mechanisms for oxyluciferin degradation. Oxyluciferin (**2**) undergoes enzymatic degradation in the presence of partially purified microsomal luciferase fraction yielding caffeic acid (peak 3) and the substances corresponding to peaks 2 and 4.

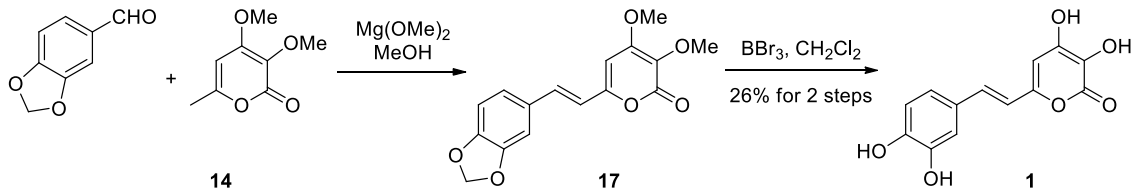


fig. S12. Synthesis of the fungal luciferin 1.

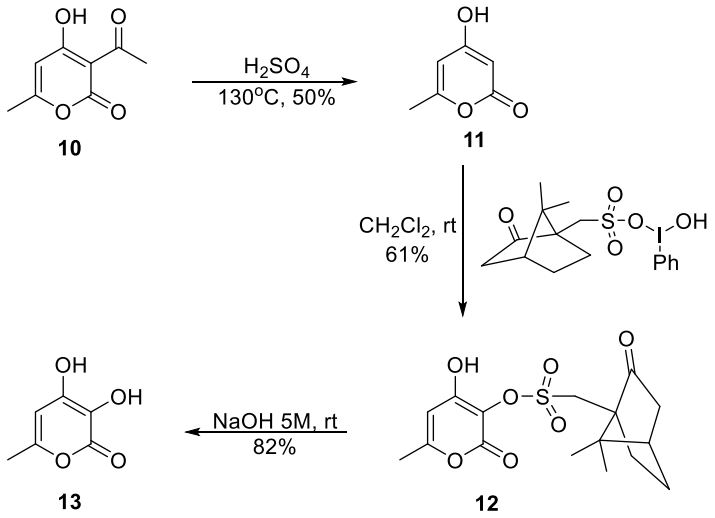


fig. S13. Synthesis of 3,4-dihydroxy-6-methyl-2*H*-pyran-2-one 13.

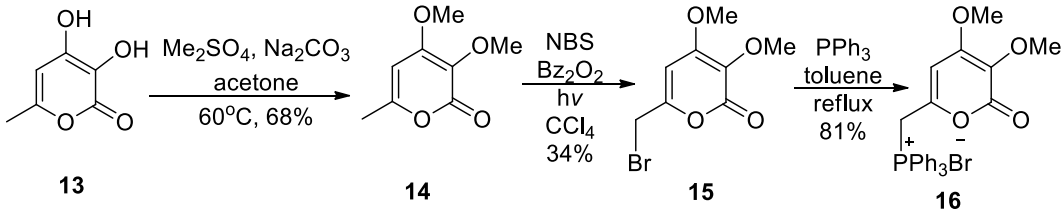


fig. S14. Synthesis of compounds 14 to 16.

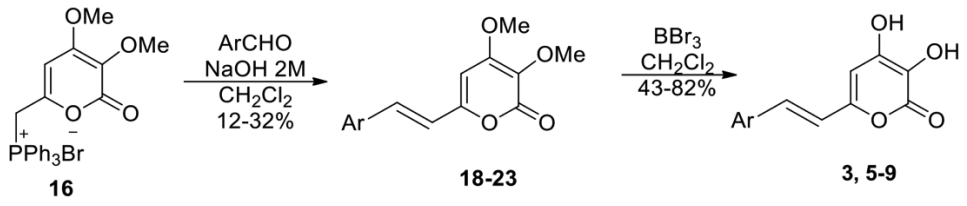


fig. S15. Synthesis of fungal luciferin analogs 3 and 5 to 9.

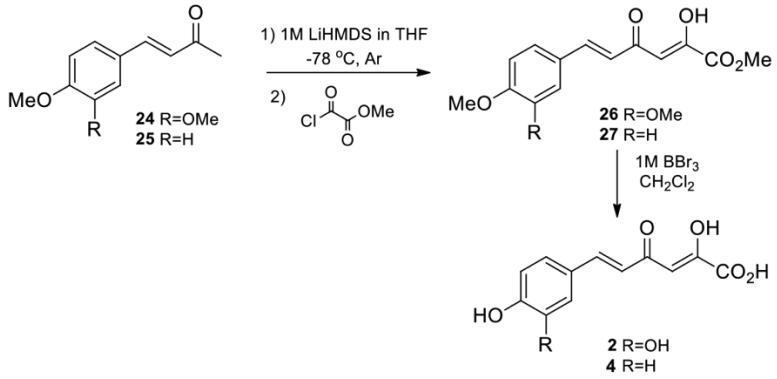


fig. S16. Synthesis of fungal oxyluciferins 2 and 4.

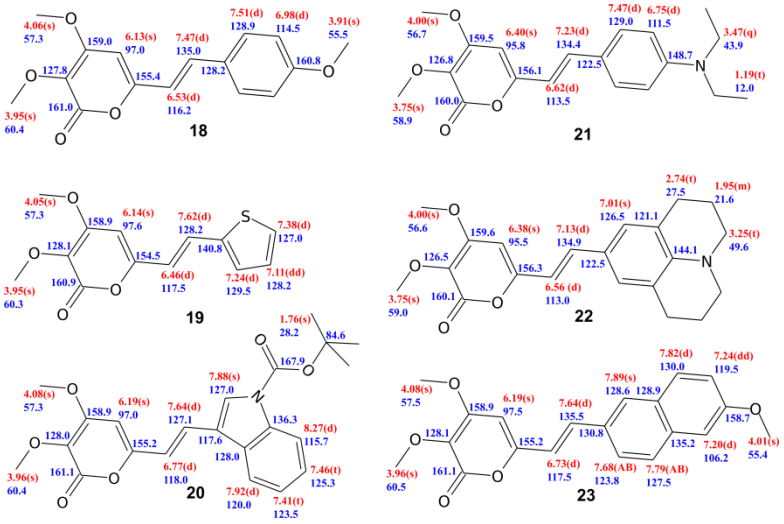


fig. S17. NMR spectra chemical shifts of compounds 18 to 23. Proton (red) and carbon (blue) chemical shifts are shown.

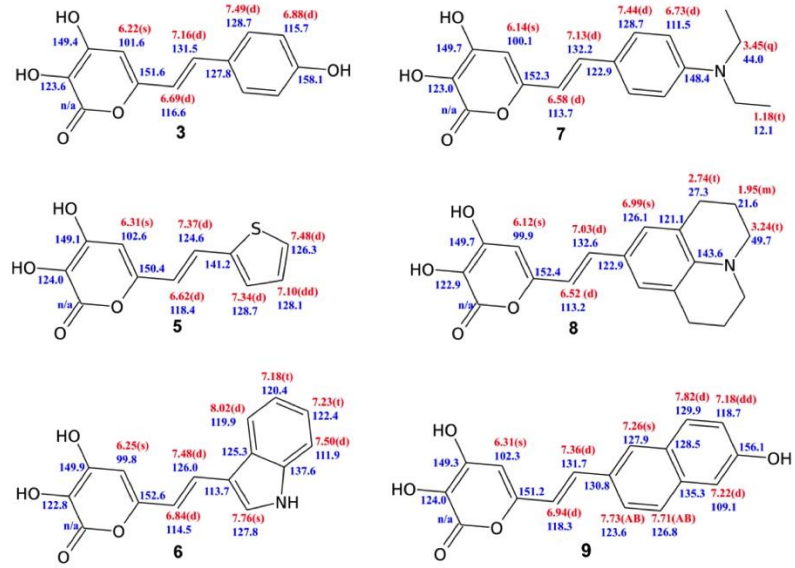


fig. S18. NMR spectra chemical shifts of luciferin analogs 3 and 5 to 9. Proton (red) and carbon (blue) chemical shifts are shown.

table S1. Observed decay rate constants (k_{obs}), the total light emitted (Q) by the reaction of luciferin/luciferase reaction, and chemiluminescence and singlet quantum yields (Φ_{CL} and Φ_S) at different pH and substrate concentrations.

[luciferin] (nM)	luciferin (pmol)	pH 6 $k_{obs} = (1.5 \pm 0.2)\times10^{-3}$ s^{-1}			pH 7 $k_{obs} = (1.0 \pm 0.2)\times10^{-3}$ s^{-1}			pH 8 $k_{obs} = (1.0 \pm 0.2)\times10^{-3}$ s^{-1}		
		Q ($\times 10^8$ counts)	Φ_{CL} (%)	Φ_S (%)	Q ($\times 10^9$ counts)	Φ_{CL} (%)	Φ_S (%)	Q ($\times 10^9$ counts)	Φ_{CL} (%)	Φ_S (%)
76	15.2	3.1 ± 0.2	0.11 ± 0.01	10 ± 1	2.0 ± 0.4	0.7 ± 0.2	63 ± 14	3.8 ± 0.9	1.3 ± 0.3	117 ± 30
152	30.4	7 ± 2	0.11 ± 0.03	10 ± 3	4.2 ± 0.5	0.7 ± 0.1	65 ± 9	6 ± 1	0.9 ± 0.2	86 ± 21
304	60.8	14 ± 3	0.12 ± 0.03	11 ± 3	6.3 ± 0.5	0.5 ± 0.1	48 ± 5	10 ± 1	0.9 ± 0.1	79 ± 5
380	76	11 ± 1	0.077 ± 0.004	7.0 ± 0.4	6.0 ± 0.5	0.41 ± 0.04	37 ± 4	11 ± 1	0.8 ± 0.1	69 ± 5
760	152	20 ± 2	0.07 ± 0.01	6 ± 1	14 ± 1	0.5 ± 0.1	42 ± 5	17 ± 1	0.56 ± 0.04	51 ± 4
	average	-	0.10 ± 0.02	9 ± 2	-	0.6 ± 0.1	51 ± 10	-	0.9 ± 0.2	80 ± 17

table S2. Dependence of chemiluminescence quantum yield (Φ_{CL}) and singlet quantum yield (Φ_S) on the pH of luciferin-luciferase reaction.

pH[a]	Φ_{CL} (%)	Φ_S (%)
6	0.10 ± 0.02	9 ± 2
7	0.6 ± 0.1	51 ± 10
8	0.9 ± 0.2	80 ± 17

[a] Phosphate buffer (20 mM), containing 1 mg/mL BSA.

table S3. Spectral characteristics of compounds 1 to 3 and 5 to 9.

Compound	CL, λ_{max} (nm)	Abs, λ_{max} (nm)	Exc, λ_{max} (nm)	FL, λ_{max} (nm)	relative rates	relative Φ_{CL}
1	538	-	-	-	1	1
2	-	380	401	520	-	-
3	520	372	367	458	0.25	0.6
5	-	376	350	430	-	-
6	480	386	351	431	0.68	0.003
7	504	366	370	430	0.64	3
8	534	424	450	574	2.3	0.004
9	564	380	350	430	0.32	0.01

table S4. Observed decay rate constant (k_{obs}), the total of light emission (Q), photomultiplier sensitivity correction factor (f_{PMT}), and the chemiluminescence quantum yield (Φ_{CL}) obtained from the reaction between the crude extract of *N. gardneri* and compounds 1, 3, and 5 to 9.

Compound	k_{obs} ($\times 10^{-3}$ s^{-1})	Relative k_{obs} (%)	Q (counts)	f_{PMT} (λ, nm)	Φ_{CL} ($Emol^{-1}$)	Relative Φ_{CL} (%)
1	4.4 ± 0.4	100	$(1.7 \pm 0.1) \times 10^8$	1.17 (534)	$(8.4 \pm 0.7) \times 10^{-3}$	100
3	1.1 ± 0.2	25	$(1.1 \pm 0.2) \times 10^8$	1.11 (516)	$(5 \pm 1) \times 10^{-3}$	60
5	no reaction	-	-	-	-	-
6	3.0 ± 0.1	68	$(5.7 \pm 0.7) \times 10^5$	1.01 (480)	$(2.4 \pm 0.3) \times 10^{-5}$	0.3
7	2.8 ± 0.3	64	$(6 \pm 1) \times 10^8$	1.08 (504)	$(2.7 \pm 0.5) \times 10^{-2}$	320
8	10.0 ± 0.1	227	$(7.0 \pm 0.9) \times 10^5$	1.17 (534)	$(3.4 \pm 0.5) \times 10^{-5}$	0.4
9	1.4 ± 0.5	32	$(1.7 \pm 0.1) \times 10^6$	1.49 (564)	$(1.1 \pm 0.3) \times 10^{-4}$	1.3