A novel and unusually long-lived chemiluminophore based on the 7-hydroxycoumarin scaffold

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Supporting Information

Abbreviations

The following abbreviations are used throughout the text of this ESI file: NBS, *N*-Bromosuccinimide; TEAA, triethylammonium acetate; PBS, phosphate buffered saline; PCC, pyridinium chlorochromate.

Experimental: Detailed synthetic procedures for compounds 1-10

General

Flash column chromatography purifications were performed on Geduran® Si 60 silica gel (40-63 µm) from Merck. TLC were carried out on Merck DC Kieselgel 60 F-254 aluminium sheets. The spots were visualised by illumination with UV lamp (λ = 254 nm or 365 nm) and/or staining with $KMnO_4$ solution. All solvents were dried following standard procedures (CH_3CN: distillation over CaH_2, CH_2Cl_2: distillation over P_2O_5, DMF: distillation over BaO, THF: distillation over Na°/benzophenone, toluene: distillation over Na°), pyridine: distillation over KOH, DMSO:distillation over KOH, N,N-diethylaniline: reflux with acetic anhydride for 4 h then fractionally distillation. Activated MnO_2 (tech., Mn 58% min) was obtained from Alfa Aesar. Phosphate Buffered Saline (PBS, 100 mM phosphate + 150 mM NaCl, pH 7.5) and aq. mobile phases for HPLC were prepared using deionised water purified with a Milli-Q system (purified to 18.2 MΩ.cm). Triethylammonium acetate (TEAA, 2.0 M) buffer was prepared from distilled triethylamine and glacial acetic acid. ^1H and ^{13}C NMR spectra were recorded on a Bruker DPX 300 spectrometer (Bruker, Wissembourg, France). Chemical shifts are expressed in parts per million (ppm) and relative to tetramethylsilane from $CDCl_3$ (δ_H = 7.26, δ_C = 77.16)[1]. *J* values are in hertz (Hz). Melting points were determined on a Kofler bench device (Wagner and Munz). Infrared (IR) spectra were recorded with a universal ATR sampling accessory on a Perkin Elmer FT-IR Spectrum 100 spectrometer. The elemental analyses were carried out with a Flash 2000 Organic Elemental Analyzer (Thermo Scientific). Low-resolution mass spectra were obtained with a Finnigan LCQ Advantage MAX (ion trap) apparatus equipped with an electrospray (ESI) probe. Analytical HPLC was performed on a Thermo Scientific Surveyor Plus instrument equipped with a PDA detector. The following chromatographic system was used: RP-HPLC (Thermo Hypersil GOLD C_{18} column, 5 µm, 4.6 × 100 mm) with CH_3CN and aq. triethylammonium acetate (TEAA 25 mM, pH 7.0) as eluents [100% TEAA (5 min), followed by linear gradient from 0 to 100% (33 min) of CH_3CN] at a flow rate of 1.0 mL min^{-1}. UV-vis detection was achieved at 220-450 nm (max-plot mode). UV-visible spectra were obtained on a Varian Cary 50 scan spectrophotometer. Fluorescence and chemiluminescence spectroscopic studies were performed with a Varian Cary Eclipse spectrophotometer. The absorption spectra of chemiluminophore **1** and its corresponding keto ester **11** were recorded (220-800 nm) in DMSO (BioUltra grade, Sigma-Aldrich) at 25 °C using a rectangular quartz cell (Varian, standard cell, Open Top, 10 × 10 mm, 3.5 mL). The emission spectra were recorded under the same conditions after excitation at 350 nm (excitation and emission filters: auto, excitation and emission slit: 5 nm) with a semi-micro quartz fluorescence cell (Hellma, 104F-QS, 10 × 4 mm, 1400 µL). Relative quantum yields were measured in DMSO and PBS at 25 °C by a relative method using 7-hydroxycoumarin (ϕ_F = 0.76 in PBS) as a standard[2]. The following equation was used to determine the relative fluorescence quantum yield:

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[1] H. E. Gottlieb, V. Kotlyar and A. Nudelman, *J. Org. Chem.*, 1997, **21**, 7512.

[2] K.-i. Setsukinai, Y. Urano, K. Kikuchi, T. Higuchi and T. Nagano, *J. Chem. Soc., Perkin. Trans. 2*, 2000, **12**, 2453.

$$\Phi_F(x) = \frac{(A_s)\,(F_x)}{(A_x)\,(F_s)}\left(\frac{n_x}{n_s}\right)^2\Phi_F(s)$$

Where A is the absorbance (in the range 0.01-0.1 A.U.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements (n = 1.337 for PBS, n = 1,477 for DMSO), and the subscripts s and x represent standard and unknown, respectively. Chemiluminescence spectra were recorded in Bio/Chemiluminescence data mode (gate time: 5 ms, em. slit: 5 nm, data interval: 1 nm, PMT voltage: high, no. of scans: 20, time: 0.02 min) with a spectral window of 400-660 nm. Chemiluminescence kinetics spectra were recorded in the same mode with the same parameters (except: em. slit: 10 nm).

3,5-Dimethoxybenzyl bromide (3)

To an ice-cooled solution of 3,5-dimethoxybenzyl alcohol (18.71 g, 112 mmol, 1 equiv.) in dry dichloromethane (250 mL), PBr_3 (11.1 mL, 115 mmol, 1.05 equiv.) was added dropwise and the resulting reaction mixture was stirred under an Ar atmosphere. After stirring for 2 h at 0 °C, then 1 h at rt, the reaction mixture was poured into ice-cooled water (600 mL). Dichloromethane layer was separated and aq. phase was extracted with Et_2O (3 × 100 mL). The combined organic layers were washed with saturated aq. $NaHCO_3$ (200 mL), dried over anhydrous $MgSO_4$, filtered and the solvent was removed under reduced pressure. The residue was purified by flash-chromatography on a silica gel column with a step gradient of EtOAc (0-16%) in petroleum ether as the mobile phase to yield the benzyl bromide derivative **3** as white crystals (17.82 g, yield 69 %). R_f (Petroleum ether-EtOAc, 3 : 1, v/v) 0.7; mp 76 ± 2 °C; ν_{max}/cm^{-1} 1615 and 1592, 1474, 1456, 1431, 1327, 1206, 1154, 1069; δ_H(300 MHz, $CDCl_3$) 3.80 (s, 6H, OCH_3), 4.42 (s, 2H, CH_2Br), 6.40 (t, *J* 2.3 Hz, 1H, Ar-4-H), 6.54 (d, *J* 2.3 Hz, 2H, Ar-2-H, Ar-6-H); δ_C (75 MHz, $CDCl_3$) 33.8 (CH_2Br), 55.5 (OCH_3), 100.7 (C-4), 107.1 (C-2, C-6), 139.9 (C-1), 161.0 (C-3, C-5); MS (APCI+): *m/z* 231.00 and 233.00 $[M + H]^+$, calcd for $C_9H_{11}BrO_2$: 229.99 and 231.99; elemental analysis (%) calcd: C, 46.78; H, 4.80; found: C, 46.22; H, 4.75.

3-*tert*-Butyl-2-(3,5-dimethoxyphenyl)-4,4-dimethyltetrahydrofuran-3-ol (4)

(a) Alkylation: A solution of 2,2,4,4-tetramethylpentane-1,3-diol (12.27 g, 76.6 mmol, 1.1 equiv.) in a mixture of dry THF-DMF (6 : 4, v/v, 47 mL) was added dropwise to a suspension of NaH (60% dispersion in oil, 3.20 g, 80 mmol, 1.15 equiv.) in a mixture of dry THF-DMF (1 : 1, v/v, 80 mL) at 0 °C under an Ar atmosphere and the resulting reaction mixture was stirred for 45 min. To this suspension, a solution of benzyl bromide **3** (16.09 g, 69.2 mmol, 1.0 equiv.) in a mixture of dry THF-DMF (6 : 4, v/v, 60 mL) was added dropwise and the resulting reaction mixture was stirred at rt for 5 h. Thereafter, the reaction mixture was poured into a solution of saturated aq. NH_4Cl (200 mL) and then extracted with EtOAc (3 × 100 mL). The combined organic layers were washed with brine (2 × 100 mL), dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The resulting residue was purified by flash-chromatography on a silica gel column with a mixture of petroleum ether-EtOAc (7 : 1, v/v) as the mobile phase to afford 1-(3,5-dimethoxybenzyloxy)-2,2,4,4-tetramethylpentan-3-ol as a clear oil (20.97 g, yield 97 %). R_f (Petroleum ether-EtOAc, 5 : 1, v/v) 0.7; δ_H(300 MHz, $CDCl_3$) 1.02 (s, 9H, $C(CH_3)_3$), 1.07 (s, 6H, $(CH_3)_2$), 3.22 (d, *J* 0.8 Hz, 1H, HOCH), 3.24 (d, *J* 2.6 Hz, 1H, Ar-CH_2OCH_2), 3.38 (d, *J* 1.1 Hz, 1H, HOCH), 3.41 (d, *J* 2.6 Hz, 1H, Ar-CH_2OCH_2), 4.44 (s, 2H, Ar-CH_2OCH_2), 6.38 (t, *J* 2.3 Hz, 1H, Ar-4-H), 6.47 (d, *J* 2.3 Hz, 2H, Ar-2-H, Ar-6-H); δ_c(75 MHz, $CDCl_3$) 22.0, 25.9 ($(C\text{-}CH_3)_2$), 28.8 ($C(CH_3)_3$), 37.4 ($(C(CH_3)_3$), 40.7 ($(C\text{-}CH_3)_2$), 55.4 ($(OCH_3)_2$), 73.4 (Ar-CH_2OCH_2), 82.0 (Ar-CH_2OCH_2), 84.7 (OHCH), 99.7 (C-4), 105.3 (C-2, C-6), 140.4 (C-1), 161.0 (C-3, C-5); MS (ESI+): *m/z* 311.13 $[M + H]^+$, calcd for $C_{18}H_{30}O_4$: 310.21.

(b) Oxidation: A solution of 1-(3,5-dimethoxybenzyloxy)-2,2,4,4-tetramethylpentan-3-ol (20.97 g, 67.5 mmol, 1.0 equiv.) in dry CH_2Cl_2 (60 mL) was added dropwise to a suspension of Celite® 545 (43 g) and PCC (21.84 g, 101 mmol, 1.5 equiv.) in dry dichloromethane (200 mL) at rt and under an Ar atmosphere. The resulting reaction mixture was stirred overnight. Then, 2-propanol (50 mL) was added and the mixture was stirred for further 30 min. Thereafter, Et_2O (300 mL) was added to the reaction mixture which was filtered through a Celite® 545 pad. The filtrate was concentrated under reduced pressure and the resulting residue was purified by flash-chromatography on a silica gel column with a mixture of petroleum ether-EtOAc (7 : 1, v/v) as the mobile phase to afford 1-(3,5-dimethoxybenzyloxy)-2,2,4,4-tetramethylpentan-3-one as a clear oil (19.15 g, yield 92%). R_f (Petroleum ether-EtOAc, 5 : 1, v/v) 0.6; δ_H(300 MHz, $CDCl_3$) 1.23 (s, 9H, $C(CH_3)_3$), 1.28 (s, 6H, $(CH_3)_2$), 3.49 (s, 2H, Ar-CH_2OCH_2), 3.77 (s, 6H, OCH_3), 4.42 (s, 2H, Ar-CH_2OCH_2), 6.35 (t, *J* 2.2 Hz, 1H, Ar-4-H), 6.54 (d, *J* 1.9 Hz, 2H, Ar-2-H, Ar-6-H); δ_C(75 MHz, $CDCl_3$) 24.1 ((C-CH_3)$_2$), 28.2 ($C(CH_3)_3$), 45.9 (($C(CH_3)_3$), 50.2 ((C-CH_3)$_2$), 55.4 (OCH_3), 73.2 (Ar-CH_2OCH_2), 78.7 (Ar-CH_2OCH_2), 99.6 (C-4), 105.0(C-2, C-6), 141.2 (C-1), 160.8 (C-3, C-5), 217.4 (C=O); MS (ESI+): *m/z* 309.07 $[M + H]^+$, calcd for $C_{18}H_{28}O_4$: 308.20.

(c) Cyclisation: A solution of 1-(3,5-dimethoxybenzyloxy)-2,2,4,4-tetramethylpentan-3-one (19.15 g, 62 mmol, 1.0 equiv.) in dry DMSO (70 mL) was added dropwise to a suspension of freshly sublimed *t*-BuOK (13.93 g, 124 mmol, 2.0 equiv.) in dry DMSO (140 mL), pre-cooled to 10-15 °C. The resulting reaction mixture was stirred at rt under an Ar atmosphere for 7 h. The reaction mixture was poured into a solution of saturated aq. NH_4Cl (400 mL) and then extracted with EtOAc (3 × 150 mL). The combined organic layers were washed with brine (2 × 150 mL), dried over anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The resulting residue was purified by flash-chromatography on a silica gel column with a mixture of petroleum ether-EtOAc (7 : 1, v/v) as the mobile phase to afforded 3-hydroxy-tetrahydrofuran derivative **4** as yellowish crystals (13.37 g, yield 70%). R_f (Petroleum ether-EtOAc, 5 : 1, v/v) 0.4; mp 63 ± 2 °C; ν_{max}/cm^{-1} 3563, 3551, 2961 (broad), 1595, 1458, 1432, 1369, 1293, 1204, 1152, 1097, 1083, 1064, 1055, 1037, 1015, 1003; δ_H(300 MHz, $CDCl_3$) 0.97 (s, 9H, $C(CH_3)_3$), 1.15 (s, 3H, CH_3), 1.38 (s, 3H, CH_3), 1.66 (s, 1H, OH), 3.48 (d, *J* 7.2 Hz, 1H, OCH_2C), 3.78 (s, 6H, $(OCH_3)_2$), 4.10 (d, *J* 7.2 Hz, 1H, OCH_2C), 5.33 (s, 1H, Ar-CH-O), 6.41 (t, *J* 2.3 Hz, 1H, Ar-4-H), 6.62 (d, *J* 2.3 Hz, 2H, Ar-2,6-H); δ_C(75 MHz, $CDCl_3$) 20.3 ((C-CH_3)$_2$), 26.6 ((C-CH_3)$_2$), 28.0 ($C(CH_3)_3$), 38.1 (($C(CH_3)_2$), 48.2 ((C-$(CH_3)_3$), 55.5 ((OCH_3)$_2$), 82.1 (OCH_2C), 84.2 (Ar-CH-O), 85.0 (C-OH), 100.2 (C-4), 107.2 (C-2,6), 142.7 (C-1), 160.9 (C-3,5); MS (ESI+): *m/z* 309.06 $[M + H]_+$, calcd for $C_{18}H_{28}O_4$: 308.20; elemental analysis (%) calcd: C, 70.10; H, 9.15; found: C, 68.27; H, 8.91.

2-(2-Bromo-3,5-dimethoxyphenyl)-3-*tert*-butyl-4,4-dimethyltetrahydrofuran-3-ol (5)

3-Hydroxy-tetrahydrofuran derivative **4** (2.05 g, 6.66 mmol, 1.0 equiv.) was dissolved in THF (34 mL) and deionised water (1.8 mL) was added. The resulting solution was cooled to 0 °C, and recristallised NBS (1.3 g 7.31 mmol, 1.1 equiv.) was added by portions. After 30 min, the cooled bath was removed and the reaction mixture was stirred at rt under Ar atmosphere overnight. Thereafter, the reaction mixture was poured in brine (30 mL) and then extracted with EtOAc (3 × 150 mL). The combined organic layers were washed with saturated aq. sodium sulfite (30 mL) and brine (2 × 30 mL), dried over anhydrous $MgSO_4$, and finally concentrated under reduced pressure. The resulting residue was purified by flash-chromatography on a silica gel column with a step gradient of EtOAc (12-17%) in petroleum ether as the mobile phase to afford bromobenzene derivative **5** as white crystals (2.35 g, yield 91%). R_f (Petroleum ether-EtOAc, 5 : 1, v/v) 0.5; mp 127 ± 2 °C; ν_{max}/cm^{-1} 3512, 3000 (large), 1586, 1471, 1453, 1416, 1345, 1319, 1294, 1204, 1162, 1075, 1066, 1021, 1000; δ_H(300 MHz, $CDCl_3$) 1.03 (s, 9H, $C(CH_3)_3$), 1.16 (s, 3H, CH_3), 1.40 (s, 3H, CH_3), 1.69 (s, 1H, OH), 3.46 (d, *J* 7.4 Hz, 1H, OCH_2C), 3.82 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 4.14 (d, *J* 7.4 Hz, 1H, OCH_2C), 5.87 (s, 1H, Ar-CH-O), 6.45 (d, *J* 2.8 Hz, 1H, Ar-4-H), 6.86 (d, *J* 2.8 Hz, 2H, Ar-6-H); δ_C(75 MHz, $CDCl_3$) 20.3 ((C-CH_3)$_2$), 27.1 ((C-CH_3)$_2$), 28.0 ($C(CH_3)_3$), 38.5 (($C(CH_3)_2$), 48.4 ((C-$(CH_3)_3$), 55.6 ((C-3-OCH_3), 56.6 (C-5-OCH_3) 82.4 (OCH_2C), 82.4 (Ar-CH-O), 86.7 (C-OH), 99.7 (C-4), 105.9 (C-2), 107.3 (C-6), 142.3 (C-1), 156.5 (C-3), 159.5 (C-5); MS

(ESI+): *m/z* 404.07 and 405.93 calcd $[M + H_2O]^{+\bullet}$ (water cluster fromed during the ionisation process), calcd for $C_{18}H_{27}BrO_4$: 386.11 and 388.11. elemental analysis (%) calcd: C, 55.82; H, 7.03; found: C, 55.66; H, 7.04.

5-(2-Bromo-3,5-dimethoxyphenyl)-4-*tert*-butyl-3,3-dimethyl-2,3-dihydrofuran (6)

Thionyl chloride (4.28 mL, 58.6 mmol, 10.0 equiv.) was added dropwise to a solution of compound **5** (2.27 g, 5.86 mmol, 1.0 equiv.) and dry pyridine (4.74 mL, 58.6 mmol, 10.0 equiv.) in dry CH_2Cl_2 (35 mL) at 0 °C under an Ar atmosphere. After stirring at rt for 2 h, the reaction mixture was poured cautiously into saturated aq. $NaHCO_3$ (200 mL) and then extracted with EtOAc (4 × 50 mL). The combined organic layers were washed with brine (2 × 50 mL), dried over anhydrous $MgSO_4$, and finally concentrated under reduced pressure. The resulting residue was purified by flash-chromatography on a silica gel column with a mixture of petroleum ether-EtOAc (7 : 1, v/v) as the mobile phase to afford dihydrofuran **6** as white crystals (1.55 g, yield 72%). R_f (Petroleum ether-EtOAc, 5 : 1, v/v) 0.7; mp 89 ± 2 °C; ν_{max}/cm^{-1} 2957 (broad), 1579, 1450, 1428, 1341, 1219, 1201, 1162, 1138, 1075, 1054, 1022; δ_H(300 MHz, $CDCl_3$) 1.06 (s, 9H, $C(CH_3)_3$), 1.33 (s, 3H, CH_3), 1.37 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.91 (d, *J* = 12.1 Hz, 2H, OCH_2C), 6.44 (d, *J* = 2.8 Hz, 1H, Ar-4-H), 6.47 (d, *J* = 2.8 Hz, 2H, Ar-6-H); δ_C(75 MHz, $CDCl_3$) 27.1 ($(C-CH_3)_2$), 27.2 ($(C-CH_3)_2$), 31.8 ($C(CH_3)_3$)**,** 32.7 ($(C(CH_3)_2$)**,** 47.2 ($(C-(CH_3)_3$), 55.8 (C-3-OCH_3), 56.4 (C-5-OCH_3) 83.5 (OCH_2C), 99.9 (C-4), 105.3 (C-2), 108.2 (C-6), 126.2 (Ar-C=C), 139.0 (Ar-C=C), 147.8 (C-1), 156.8 (C-3), 159.4 (C-5); MS (ESI+): *m/z* 369.01 and 371.01 $[M + H]^+$, calcd for $C_{18}H_{25}BrO_3$: 368.10 and 370.10; elemental analysis (%) calcd: C, 58.54; H, 6.82; found: C, 58.30; H, 6.81.

Methyl 2-(3-*tert*-butyl-4,4-dimethyl-4,5-dihydrofuran-2-yl)-4,6-dimethoxybenzoate (7)

To a stirred solution of dihydrofuran **6** (2.0 g, 5.43 mmol, 1.0 equiv.) in dry THF (50 ml), *n*-BuLi (1.6 M in hexane, 4.07 mL, 6.51 mmol, 1.2 equiv.) was added dropwise at -78 °C under an Ar atmosphere. After stirring for 30 min, freshly distilled methyl chloroformate (1.5 mL, 19.0 mmol, 3.5 equiv.) was added dropwise. The resulting reaction mixture was allowed to warm up to -5 °C within 4 h. Thereafter, the reaction was quenched by addition of saturated aq. NH_4Cl (40 mL), extracted with EtOAc (3 × 30 mL), washed with brine (2 × 20 mL), dried over anhydrous $MgSO_4$, and finally concentrated under reduced pressure. The resulting residue was purified by flash-chromatography on a silica gel column with a mixture of petroleum ether-EtOAc (5 : 1, v/v) as the mobile phase to afford methyl ester **7** as a colorless oil (1.57 g, yield 83%). R_f (Petroleum ether-EtOAc, 6 : 1, v/v) 0.2; mp 84 ± 2 °C; ν_{max}/cm^{-1} 2950 (broad), 1721, 1596, 1582, 1419, 1347, 1282, 1273, 1218, 1202, 1159, 1139, 1104, 1052, 1043, 1030, 1017; δ_H(300 MHz, $CDCl_3$) 1.04 (s, 9H, $C(CH_3)_3$), 1.29 (s, 6H, $(CH_3)_2$), 3.81-3.84 (m, 9H, Ar-OCH_3, $COOCH_3$), 3.88 (s, 2H, OCH_2C), 6.39 (d, *J* 2.3 Hz, 1H, Ar-5-H), 6.45 (d, *J* 2.3 Hz, 1H, Ar-3-H); δ_C(75 MHz, $CDCl_3$) 27.1 ($(C-CH_3)_2$), 32.0 ($C(CH_3)_3$), 32.6 ($(C(CH_3)_2$), 47.1 ($(C-(CH_3)_3$), 52.1 (CH_3), 55.6 (OCH_3), 56.1 (OCH_3), 83.5 (OCH_2C), 99.0, 107.2, 116.7 (Ar-C=C), 126.5 (Ar-C=C), 137.0, 147.0, 158.4, 161.3, 167.3; MS (ESI+): *m/z* 349.12 $[M + H]^+$, calcd for $C_{20}H_{28}O_5$: 348.19; elemental analysis (%) calcd: C, 68.94; H, 8.10; found: C, 68.43; H, 8.07.

2-(3-*tert*-Butyl-4,4-dimethyl-4,5-dihydrofuran-2-yl)-6-hydroxy-4-methoxybenzaldehyde (8)

(a) Reduction: To a stirred solution of methyl ester **7** (1.57 g, 4.49 mmol, 1.0 equiv.) in dry THF (50 mL), DIBAL-H (1.0 M in toluene, 11.2 mL, 11.2 mmol, 2.5 equiv.) was added dropwise at -78 °C under an Ar atmosphere. Then, the reaction mixture was warmed up gradually to rt within 6 h, and stirred at this temperature overnight. The reaction was quenched by addition of deionised water (20 mL), followed by addition of a 1.0 M aq.

HCl (30 mL), stirred for 10 min and extracted with EtOAc (3 × 30 mL), washed with brine (2 × 35 mL), dried over anhydrous $MgSO_4$, and finally concentrated under reduced pressure. The resulting residue was purified by flash-chromatography on a silica gel column with a mixture of petroleum ether-EtOAc (5 : 1, v/v) as the mobile phase to afford benzyl alcohol derivative as a colorless oil (1.05 g, yield 73%). R_f (Petroleum ether-EtOAc, 3 : 1, v/v) 0.2; δ_H(300 MHz, $CDCl_3$) 1.06 (s, 9H, $C(CH_3)_3$), 1.35 (s, 6H, $(CH_3)_2$), 2.54 (bs, 1H, CH_2OH), 3.81 (s, 3H, Ar-OCH_3), 3.84 (s, 3H, OCH_3), 3.88 (d, 2H, OCH_2C), 4.61 (bm, 1H, CH_2OH), 6.42 (d, *J* 2.4 Hz, 1H, Ar-5-H), 6.46 (d, *J* 2.5 Hz, 1H, Ar-3-H); δ_C(75 MHz, $CDCl_3$) 27.6, 27.1, 32.2 ((C-$CH_3)_2$), 32.6 ($C(CH_3)_3$), 47.3 (($C(CH_3)_3$), 55.6 (OCH_3), 55.7 (OCH_3), 58.2, 83.1, 99.0, 106.9, 121.3, 126.9, 137.4, 147.8, 159.0, 159.8; MS (ESI+): *m/z* 663.07 [2M + Na^+] calcd for $C_{19}H_{28}O_4$: 320.20.

(b) Oxidation: Benzyl alcohol derivative (1.05 g, 4.49 mmol, 1.0 equiv.) was dissolved in dry toluene (35 mL) and MnO_2 (activated) (2.71 g, 11.2 mmol, 10 equiv.) was added. The resulting reaction mixture was heated to reflux for 2 h, then cooled to rt, filtered through a Celite® 545 pad. The filtrate was concentrated under reduced pressure and the resulting residue was purified by flash-chromatography on a silica gel column with a mixture of petroleum ether-EtOAc (4 : 1, v/v) as the mobile phase to afford benzaldehyde derivative as white crystals (1.05 g, yield 73%). R_f (Petroleum ether-EtOAc, 3 : 1, v/v) 0.2; δ_H(300 MHz, $CDCl_3$) 1.03 (s, 9H, $C(CH_3)_3$), 1.35 (s, 6H, $(CH_3)_2$), 3.87 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 3.92 (s, 2H, CH_2), 6.44 (d, *J* 2.3 Hz, 1H, Ar-5-H), 6.47 (d, *J* 2.3 Hz, 1H, Ar-3-H), 10.18 (1H, s, CHO); δ_C(75 MHz, $CDCl_3$) 27.2 ((C-$CH_3)_2$), 32.1 ($C(CH_3)_3$), 32.6 (($C(CH_3)_2$), 47.4 ((C-$(CH_3)_3$), 55.8 (OCH_3), 56.1 (OCH_3), 83.7 (OCH_2C), 98.9 (C-5), 108.9 (C-3), 117.7 (Ar-C=C), 127.1 (Ar-C=C), 142.8 (C-1), 146.3 (C-2), 162.8 (C-6), 164.5 (C-4), 189.6 (CHO); MS (ESI+): *m/z* 319.10 $[M + H]^+$, calcd for $C_{19}H_{26}O_4$: 318.18.

(c) Removal of methoxy group: BCl_3 (1.0 M in hexane, 3.1 mL, 3.12 mmol, 1.6 equiv.) was added dropwise during 15 min (*Boron trichloride should be used fresh and temperature should not exceeded -5°C)* to a stirred solution of benzaldehyde derivative (0.62 g, 1.95 mmol, 1.0 equiv.) in dry CH_2Cl_2 (48 mL) and cooled to -20 °C, under an Ar atmosphere. The resulting reaction mixture was warmed up to -5 °C over 2 h, and then poured with stirring into brine (50 mL). The CH_2Cl_2 layer was separated and aq. layer extracted with further amount of CH_2Cl_2 (3 × 10 mL). The collected dichloromethane phases were combined, washed with brine (2 × 50 mL), dried over anhydrous $MgSO_4$, and finally concentrated under reduced pressure. The resulting resiude was purified by flash-chromatography on a silica gel column with a mixture of petroleum ether-EtOAc (5 : 1, v/v) as the mobile phase to afford phenol **8** as white crystals (0.46 g, yield 78%). R_f (Petroleum ether-EtOAc, 3 : 1, v/v) 0.8; mp 80 ± 2 °C; ν_{max}/cm^{-1} 2950 (broad), 1617, 1373, 1301, 1261, 1218, 1202, 1160, 1128, 1103, 1054, 1038, 1018, 1007; δ_H(300 MHz, $CDCl_3$) 1.06 (s, 9H, $C(CH_3)_3$), 1.28 (s, 6H, $(CH_3)_2$), 3.85 (s, 3H, OCH_3), 3.90 (s, 2H, CH_2), 6.40 (d, *J* 2.3 Hz, 1H, Ar-4-H), 6.42 (d, *J* 2.5 Hz, 1H, Ar-6-H), 9.83 (1H, s, CHO), 12.06 (s, 1H, OH); δ_C(75 MHz, $CDCl_3$) 27.2 ((C-$CH_3)_2$), 27.6 ((C-$CH_3)_2$), 32.2 ($C(CH_3)_3$), 32.7 (($C(CH_3)_2$), 47.6 ((C-$(CH_3)_3$), 55.9 (OCH_3), 83.6 (OCH_2C), 101.0 (C-4), 111.4 (C-6), 113.2 (Ar-C=C), 129.6 (Ar-C=C), 142.0 (C-1), 144.6 (C-2), 165. 5 (C-6), 166.1 (C-4), 195.0 (CHO); MS (ESI+): *m/z* 305.12 $[M + H]^+$, (ESI-): *m/z* 303.32 $[M - H]^-$, calcd for $C_{18}H_{24}O_4$: 304.17; elemental analysis (%) calcd: C, 71.03; H, 7.95; found: C, 71.00; H, 7.93.

5-(3-*tert*-Butyl-4,4-dimethyl-4,5-dihydrofuran-2-yl)-7-methoxy-2H-chromen-2-one (9)

To a stirred solution of *ortho*-hydroxybenzaldehyde **8** (0.46 g, 1.52 mmol, 1.0 equiv.) in dry N,N-diethylaniline (10 mL), carbethoxymethylenetriphenylphosphorane (0.64 g, 1.83 mmol, 1.2 equiv.) was added and the reaction mixture was heated to 175 °C for 50 min and then to 185 °C for 30 min under an Ar atmosphere. Thereafter, the reaction mixture was cooled, poured into 1.0 M aq. HCl (20 mL) and extracted with EtOAc (3 × 20 mL). Combined organic phases were washed with 1.0 M aq. HCl (2 × 20 mL), brine (2 × 20 mL), dried over anhydrous $MgSO_4$, and finally concentrated under reduced pressure. The resulting residue was purified by flash-chromatography on a silica gel column with a mixture of petroleum ether-EtOAc (5 : 1, v/v) as the mobile phase to afford coumarin **9** as yellowish crystals (0.42 g, yield 83%). R_f (Petroleum ether-EtOAc, 3 : 1, v/v) 0.5; mp 128 ± 2 °C; ν_{max}/cm^{-1} 2950 (broad), 1742, 1596, 1464, 1384, 1360, 1355, 1314, 1234, 1212, 1161, 1154, 1120, 1095, 1034, 1012; δ_H(300 MHz, $CDCl_3$) 1.02 (s, 9H, $C(CH_3)_3$), 1.38 (s, 6H, $(CH_3)_2$), 3.87 (s, 3H, OCH_3), 3.93 (s, 2H, CH_2), 6.27 (d, *J* 9.6 Hz, 1H, Ar-3-H), 6.79 (s, 2H, Ar-6,8-H), 7.67 (d, *J* 9.6 Hz, 1H, Ar-4-H); δ_C(75 MHz, $CDCl_3$) 27.2 ((C-CH_3)$_2$), 27.4 ((C-CH_3)$_2$), 32.2 ($C(CH_3)_3$)**,** 32.7 (($C(CH_3)_2$)**,** 47.6 ((C-$(CH_3)_3$), 56.0 (OCH_3), 83.6 (OCH_2C), 101.2 (C-6), 112.1 ((Ar-C=C), 113.5 (C-3), 114.9 (C-8), 129.7 ((Ar-C=C)), 135.9 (C-5), 141.8 (C-4), 145.0 (C-10), 156.1 (C-9), 161.2 (C-7), 162.1 (C-2); MS (ESI+): *m/z* 329.46 $[M + H]^+$, calcd for $C_{20}H_{24}O_4$: 328.17; elemental analysis (%) calcd: C, 73.15; H, 7.37; found: C, 71.27; H, 7.28.

5-(3-*tert*-Butyl-4,4-dimethyl-4,5-dihydrofuran-2-yl)-2-oxo-2H-chromen-7-yl acetate (10)

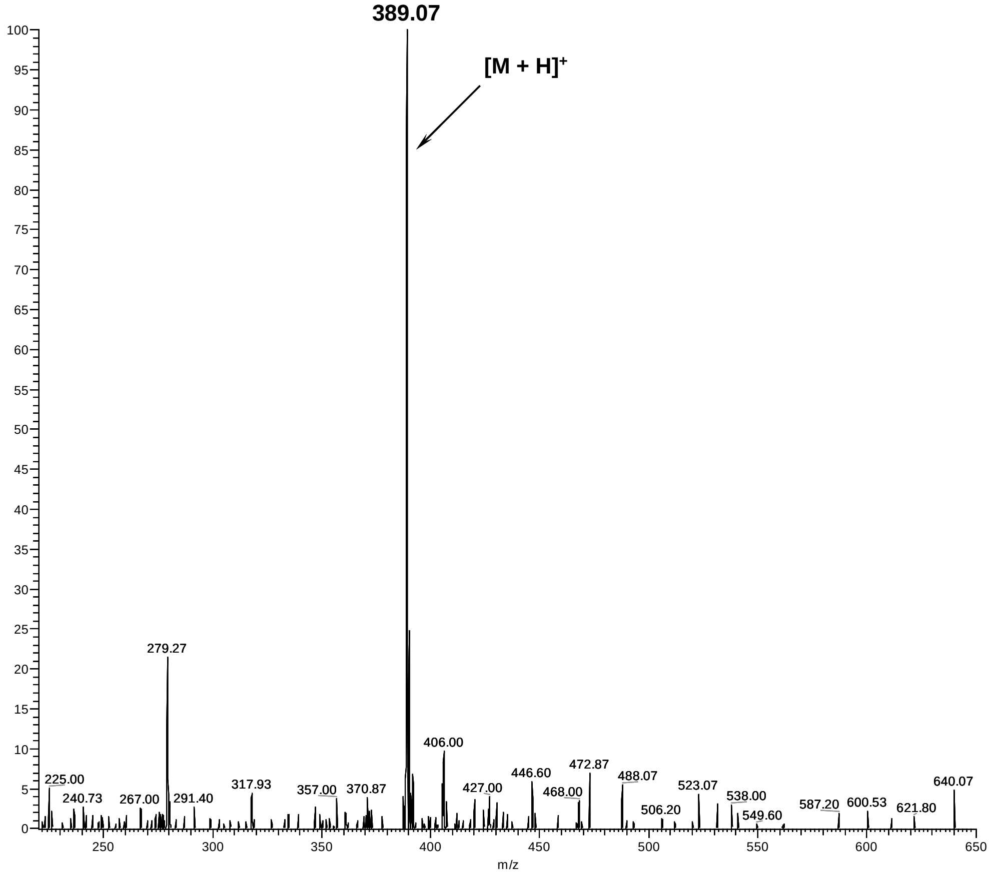
(a) Removal of methoxy group: To a stirred solution of coumarin **9** (0.376 g, 1.14 mmol, 1.0 equiv.) in dry CH_2Cl_2 (48 mL), $AlCl_3$ (1.22 g, 9.16 mmol, 8.0 equiv.) was added at 0 °C and stirred at the same temperature for 10 min. Then, the reaction mixture was heated to reflux for 30 h under an Ar atmosphere. During this time, further amounts $AlCl_3$ (4 equiv.) were added. Thereafter, the reaction mixture was cooled and quenched by adding drowise of a 1.0 M aq. HCl (40 mL). The CH_2Cl_2 layer was separated and aq. layer extracted with EtOAc (4 × 20 mL). The combined organic phases were washed with brine (2 × 50 mL), dried over anhydrous $MgSO_4$, and finally concentrated under reduced pressure. The resulting residue was purified by flash-chromatography on a silica gel column with a step gradient of EtOAc (25-33%) in petroleum ether as the mobile phase to afford phenol derivative as yellowish crystals (0.357 g, quantitative yield). R_f (Petroleum ether-EtOAc, 3 : 1, v/v) 0.2; δ_H(300 MHz, $CDCl_3$) 1.02 (s, 9H, $C(CH_3)_3$), 1.38 (s, 6H, $(CH_3)_2$), 3.93 (s, 2H, CH_2), 6.27 (d, *J* 9.6 Hz, 1H, Ar-3-H), 6.82 (d, *J* 2.5 Hz, 1H, Ar-8-H), 6.96 (d, *J* 2.4 Hz, 1H, Ar-6-H), 7.73 (d, *J* 9.6 Hz, 1H, Ar-4-H); δ_C(75 MHz, $CDCl_3$) 27.2 ((C-CH_3)$_2$), 27.3 ((C-CH_3)$_2$), 32.3 ($C(CH_3)_3$)**,** 32.7 (($C(CH_3)_2$)**,** 47.6 ((C-$(CH_3)_3$), 83.5 (OCH_2C), 103.7 (C-6), 111.9 ((Ar-C=C), 112.6 (C-3), 116.2, 129.9 ((Ar-C=C)), 136.3 (C-5), 142.9 (C-4), 144.7, 155.7 (C-9), 159.7 (C- 7), 162.6 (C-2); MS (ESI+): *m/z* 315.33 $[M + H]^+$, calcd for $C_{19}H_{22}O_4$: 314.15.

(b) Acetylation: Phenol derivative (150 mg, 0.47 mmol, 1.0 equiv.) was dissolved in dry CH_2Cl_2 (6 mL). Then, dry pyridine (76 µL, 0.94 mmol, 2.0 equiv.) and acetic anhydride (58 µL, 0.62 mmol, 1.3 equiv.) were sequentially added. The resulting reaction mixture was stirred at rt overnight. Thereafter, 10% aq. citric acid (10 mL) was added. After decantation, the CH_2Cl_2 layer was dried over anhydrous Na_2SO_4, and finally concentrated under reduced pressure. The resulting residue was purified by flash-chromatography on a silica gel column with a step gradient of EtOAc (0-30%) in cyclohexane as the mobile phase to afford 7-O-protected coumarin **10** as white crystals (151 mg, yield 90%). R_f (Cyclohexane-EtOAc, 7 : 3, v/v) 0.5; mp 128 ± 2°C; ν_{max}/cm^{-1} 2950 (broad), 1770, 1731, 1603, 1367, 1193, 1124, 1106, 1058, 1041, 1020, 1007; δ_H(300 MHz, $CDCl_3$) 1.03 (s, 9H, $C(CH_3)_3$), 1.38 (s, 6H, $(CH_3)_2$), 2.33 (s, 3H, $COCH_3$), 3.93 (s, 2H, CH_2), 6.81 (d, *J* 9.6 Hz, 1H, Ar-3-H), 7.02 (d, *J* 2.5 Hz, 1H, Ar-8-H), 7.11 (d, *J* 2.4 Hz, 1H, Ar-6-H), 7.72 (d, *J* 9.6 Hz, 1H, Ar-4-H); δ_C(75 MHz, $CDCl_3$) 21.2 (C=OCH_3), 27.2 ((C-CH_3)$_2$), 27.3 ((C-CH_3)$_2$), 32.3 ($C(CH_3)_3$)**,** 32.7 (($C(CH_3)_2$)**,** 47.6 ((C-$(CH_3)_3$), 83.5 (OCH_2C), 110.5, 116.0, 116.3, 120.7, 130.4, 135.7, 141.3, 144.3, 152.4, 154.7, 160.3, 168.5; MS (ESI+): *m/z* 357.33 $[M + H]^+$, calcd for $C_{21}H_{24}O_5$: 356.16; elemental analysis (%) calcd: C, 70.77; H, 6.79; found: C, 70.92; H, 6.85.

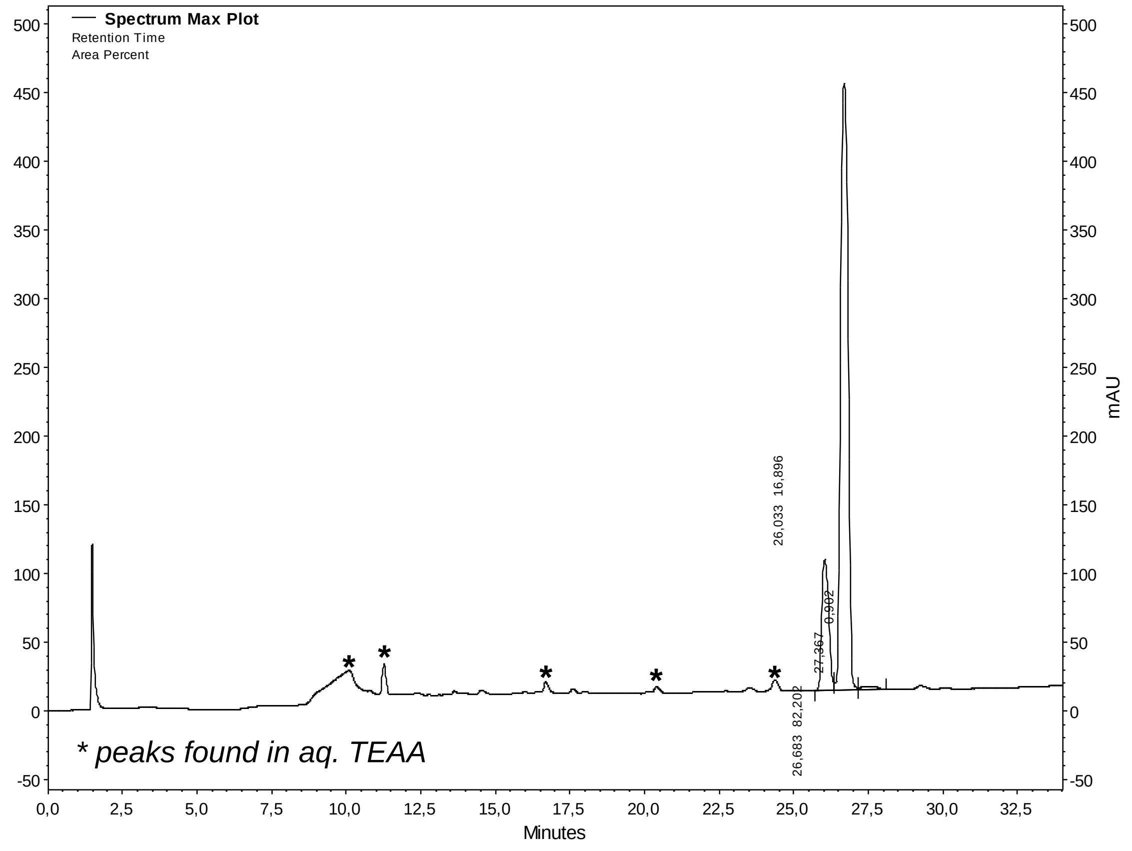
5-(5-*tert*-Butyl-4,4-dimethyl-2,6,7-trioxabicyclo[3.2.0]heptan-1-yl)-2-oxo-2H-chromen-7-yl acetate (1)

In a solution of coumarin **10** (42 mg, 0.118 mmol, 1.0 equiv) in $CDCl_3$ (1.5 ml), a point of spatula of methylene blue was added (< 1 mg) at 0 °C. The mixture was irradiated with a 300W-halogen-lamp under a flux of O_2 for 390 min and the reaction was checked to complete by TLC. Thereafter, volatiles were removed without heating thanks of a flux of N_2. The resulting crude was purified on a silica gel column (10 g) by means of an automated flash purification system (Biotage Isolera One), and by using a linear gradient of EtOAc (0-30%) in cyclohexane to give the targeted dioxetane **1** as a white solid (29 mg, yield 63%). R_f (Cyclohexane-EtOAc, 7 : 3, v/v) 0.4; mp 142 ± 5°C ; ν_{max}/cm^{-1} 2950 (broad), 1769, 1734, 1714, 1683, 1607, 1365, 1305, 1227, 1193, 1158, 1127, 1050, 1033, 1010 ; δ_H(300 MHz, $CDCl_3$) 0.95 (s, 9H, $C(CH_3)_3$), 1.24 (s, 3H, $(CH_3)_2$), 1.41 (s, 3H, $(CH_3)_2$), 2.34 (s, 3H, $COCH_3$), 3.98 (d, *J* 8.5 Hz , 1H, CH_2), 4.61 (d, *J* 8.5 Hz , 1H, CH_2), 6.40 (d, *J* 9.6 Hz, 1H, Ar-3-H), 7.22 (d, *J* 9.6 Hz, 1H, Ar-4-H), 7.64 (bs, 1H, Ar-6-H), 8.51 (bs, 1H, Ar-8-H); δ_C(75 MHz, $CDCl_3$) 21.2 ($C{=}OCH_3$), 26.1 ($(C\text{-}CH_3)_2$), 26.7 ($C(CH_3)_3$), 28.2 ($(C\text{-}CH_3)_2$), 36.9 ($(C(CH_3)_2)$), 45.7 ($(C\text{-}(CH_3)_3)$), 80.6 (OCH_2C), 106.1, 112.5, 115.5, 115.9, 121.7, 134.6, 141.2, 152.0, 155.8, 159.6, 168.6; MS (ESI+): *m/z* 389. 07 $[M + H]^+$, calcd for $C_{21}H_{24}O_7$: 388.15; HPLC: t_R = 26.7 min, purity 82% (max-plot 220-450 nm); elemental analysis (%) calcd: C, 64.94; H, 6.23; found: C, 64.68; H, 6.20.

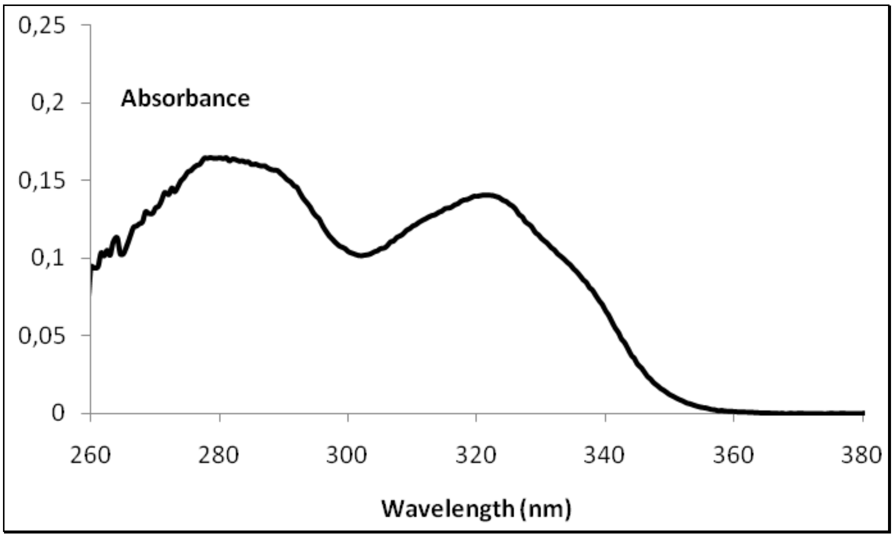
ESI mass spectrum of chemiluminophore 1 recorded in the positive mode



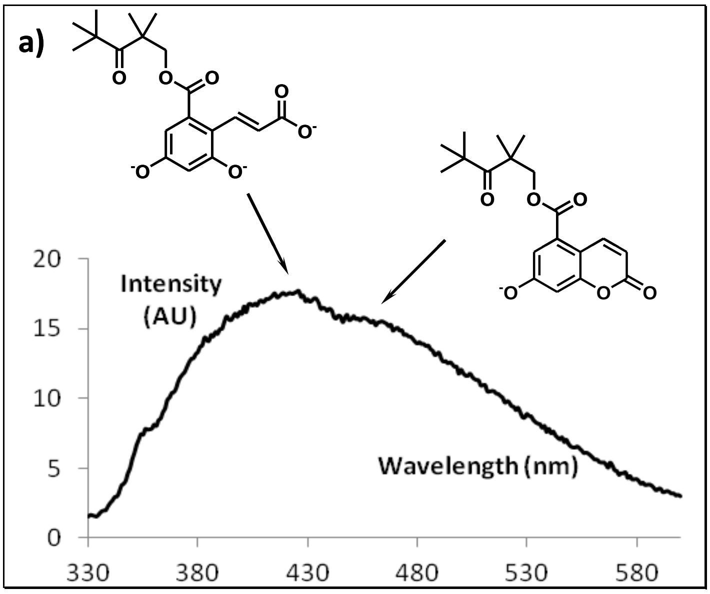
RP-HPLC elution profile of chemiluminophore 1



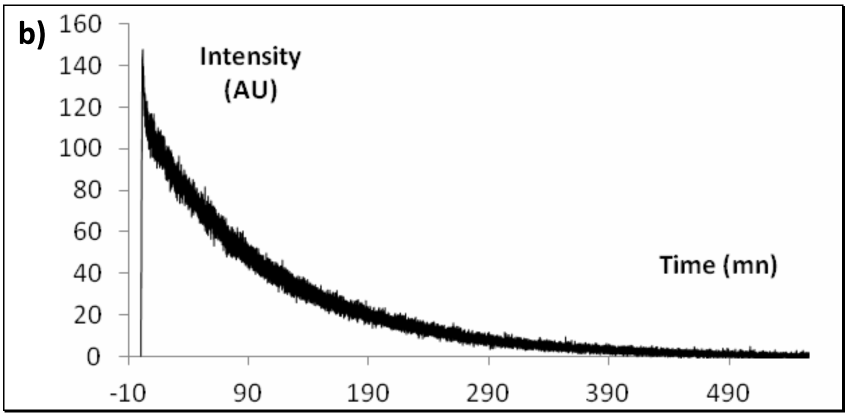
Absorption spectrum of chemiluminophore 1 in DMSO (concentration: 17 μM) at 25 °C



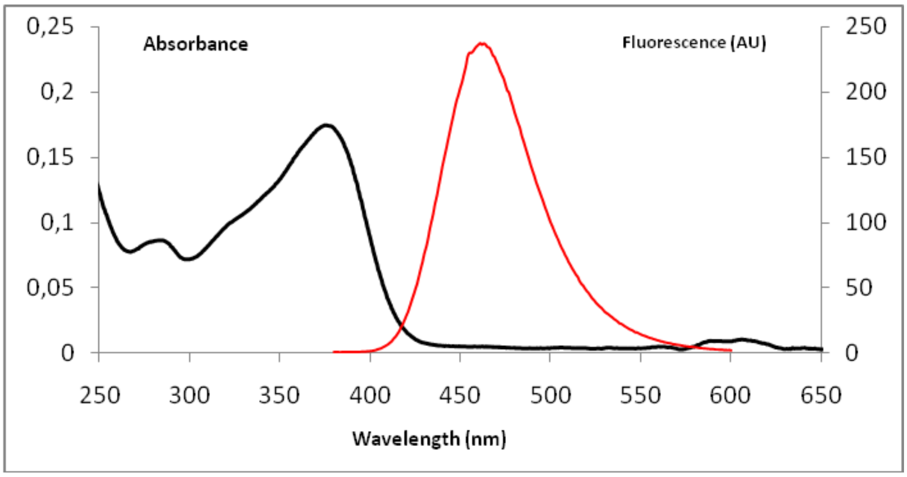
Chemiluminescence spectra of 1 in DMSO (after adding aq. 1.0 M NaOH, 3% v/v)[a] at 25 °C (concentration: 170 μM): (a) scan mode, (b) kinetic mode at λ = 470 nm.



[a] In such aq. alkaline conditions, both acetyl removal and lactone ring-opening may have occurred to produce two different emitting species: cinnamate derivative and compound **11** whose Em. maxima are 430 and 470 nm respectively.



Normalised absorption (—) and emission (—) spectra of keto ester coumarin 11 in DMSO at 25 °C



A quantum yield of 57% was determined for this coumarinic keto ester (78% in PBS).