Electronic Supplementary Information

Two-photon absorbing 8-hydroxy-benzo[g]coumarins with giant Stokes shift: An environment insensitive dye platform for probing biomolecules

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General information
All chemicals were purchased from Sigma-Aldrich or Alfa-Aesar. Commercially available reagents were used without any further purification. All solvents were purified and dried by standard methods prior to use. Thin-layer chromatography (TLC) was performed on precoated silica gel 60F-254 glass plates. $^1$H and $^{13}$C NMR spectra were recorded on Bruker 300 MHz and 500 MHz respectively using tetramethylsilane as the internal reference. Coupling constants (J value) are reported in Hertz. All chemical shifts are reported in the standard notation of parts per million (ppm) using residual solvent protons as the internal standard. Mass spectral analysis was recorded with Jeol JMS 700 and was reported in units of mass to charge (m/z).

Spectroscopic analysis
UV-Vis absorption spectra were obtained using a HP 8453 UV-Vis spectrophotometer. Fluorescence spectra were recorded on a Photon Technical International Fluorescence System with a 3 ml quartz cell with 1–cm standard path length. 1 mM stock solutions were prepared in DMSO. All photo-physical studies were carried out in a concentration of 5 μM probe solution.

Quantum yield was calculated using the following equation (1) considering 9,10-diphenylanthracene (Φ = 0.9 in cyclohexane) as standard at a fixed excitation wavelength of 350 nm.

$$\Phi = \Phi_r \left( \frac{n^2}{n_r^2} \right)^2 \left( \frac{l}{l_r} \right) \left( \frac{A}{A_r} \right) \ldots \ldots \ldots (1)$$

Where, Φ is the quantum yield

\( l \) = measured integrated emission intensity
\( \eta \) = refractive index of the media
\( A \) = absorbance

The Lippert-Mataga plot was obtained by plotting Stoke’s shift with orientation polarizability using equation (2).

$$\nu_A - \nu_F = \frac{2}{h c a} (\mu_E - \mu_G) \Delta f + \text{Constant} \ldots \ldots \ldots \ldots (2)$$

Where, \( \Delta f = \left( \frac{\epsilon - 1}{2\epsilon + 1} \right) - \left( \frac{\eta^2 - 1}{2\eta^2 + 1} \right) \)
\( \nu_A - \nu_F \) = Stokes shift in cm$^{-1}$
\( h \) = Plank’s constant
\( \Delta f \) = Orientation polarizability
\( \epsilon \) = Dielectric constant
\( \eta \) = Refractive index

TPACS value was calculated using equation (3) considering Rhodamine 6G in MeOH as a reference.

$$\sigma_{2\text{new}}(\lambda)\eta_{2\text{new}}(\lambda) = \frac{\Phi_{\text{cal}}\eta_{\text{2cal}}(\lambda)C_{\text{cal}}}{\Phi_{\text{new}}C_{\text{new}}} \frac{<p(t)_{\text{cal}}>_2^2}{<p(t)_{\text{new}}>_2^2} \frac{<F(t)_{\text{cal}}>}{<F(t)_{\text{new}}>} \frac{n_{\text{cal}}}{n_{\text{new}}} \ldots \ldots \ldots \ldots (3)$$

Where, \( \sigma_2 \) = Two Photon Absorption Cross Section
\( \eta \) = Quantum Efficiency
\( C \) = Fluorophore Concentration
\( <p(t)> \) = Time Averaged Laser Power
\( <F(t)> \) = Time Averaged Fluorescence Emission
\( n \) = Reflective Index of Sample
\( \Phi \) = Fluorescence Collection Efficiency
Synthesis

Scheme S1 Synthesis of hydroxybenzocoumarin dyes 5a–5h and esterase probe 5p via key intermediate 4.

2,7-Bis(methoxymethoxy)naphthalene (2). To a solution of compound 1 (3 g, 18.75 mmol) dissolved in anhydrous DMF (50 mL) was added NaH (1.87 g, 46.75 mmol) at –20 °C. The resulting mixture was stirred for 30 min, and then treated with chloromethyl methyl ether (3.56 mL, 46.75 mmol). The reaction mixture was quenched with saturated ammonium chloride and was subjected to an extractive work-up using ethyl acetate (three times). The combined organic phase was washed with brine, dried over anhydrous Na$_2$SO$_4$, and concentrated. The residue was purified by silica gel column chromatography using 2% ethyl acetate in hexane to afford compound 2 as a white solid (3.4 g, 73%).

$^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 3.52 (s, 6H), 5.28 (s, 4H), 7.08 (dd, $J = 9.0, 2.4$ Hz, 2H), 7.31 (d, $J = 2.4$ Hz, 2H), 7.7 (d, $J = 9.0$ Hz, 2H). $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 55.71, 94.18, 109.13, 116.74, 125.24, 128.94, 135.58, 155.43.

3,6-Bis(methoxymethoxy)-2-naphthaldehyde (3). Compound 2 (2 g, 8 mmol) dissolved in anhydrous diethyl ether (30 mL) was treated with tert-butyl lithium (1.7 M in pentane, 5.93 mL, 10.1 mmol) dropwise over a period of 15 min at –20 °C. The resulting mixture was stirred at –20 °C for 30 min and then treated with anhydrous DMF (726 µL, 9.27 mmol) dropwise to give a pale brown suspension, which was stirred at the same temperature for 1 h. The reaction mixture was treated with ice water and then subjected to an extractive work-up using ethyl acetate (three times). The combined organic phase was dried over Na$_2$SO$_4$ and concentrated under reduced pressure to give a crude product, which was purified by silica gel column chromatography using 5% ethyl acetate in hexane as eluent to afford compound 3 as a white solid (1.11 g, 50%).
1H NMR (300 MHz, CDCl3): δ 3.52 (s, 3H), 3.56 (s, 3H), 5.3 (s, 2H), 5.39 (s, 2H), 7.1 (dd, J = 8.7, 2.1 Hz, 1H), 7.26 (s, 1H), 7.3 (d, J = 2.1 Hz, 1H), 7.38 (s, 1H), 7.79 (d, J = 9.0 Hz, 1H), 8.31 (s, 1H), 10.55 (s, 1H). 13C NMR (75 MHz, CDCl3): δ 56.49, 56.69, 94.49, 94.99, 108.97, 109.51, 118.58, 124.4, 124.45, 130.71, 131.82, 139.36, 156.02, 156.06, 190.11.

3,6-Dihydroxy-2-naphthaldehyde (4). Compound 3 (600 mg, 2.17 mmol) dissolved in anhydrous acetonitrile (15 mL) was treated with NbCl₃ (1.23 g, 4.56 mmol) dissolved in the same solvent (10 mL) at -20 °C. The resulting mixture was stirred for 45 minutes to give a dark brown solution, which was quenched by adding a minimum volume of saturated NaHCO₃ solution. The mixture was extracted with ethyl acetate three times, and the combined organic phase was washed with brine, dried over anhydrous Na₂SO₄, and concentrated under reduced pressure. The residue was purified by column chromatography over silica gel using 5% to 20% ethyl acetate in hexane to afford 4 as a bright yellow solid (220 mg, 54%).

1H NMR (300 MHz, MeOD): δ 6.9–6.94 (m, 3H), 7.75 (d, J = 9.0 Hz, 1H), 8.13 (s, 1H), 10.01 (s, 1H). 13C NMR (75 MHz, MeOD): δ 108.46, 110.19, 118.51, 121.99, 124.18, 132.95, 138.29, 142.15, 157.85, 160.96, 197.28.

tert-Butyl 8-hydroxy-2-oxo-2H-benzo[g]chromene-3-carboxylate (5a). A solution of compound 4 (40 mg, 0.21 mmole), di-tert-butyl malonate (18 μL, 0.234 mmole), 10 mole% piperidine, and one drop of acetic acid in dry ethanol (3 mL) was treated with diethylmalonate (5.7 mg, 0.234 mmole) dissolved in the same solvent (10 mL) at room temperature. The reaction mixture was treated with hexane to afford compound 5a as a yellow solid (50 mg, 76%).

1H NMR (300 MHz, DMSO): δ 1.54 (s, 9H), 7.14 (dd, J = 9, 2.1 Hz, 1H), 7.19 (s, 1H), 7.63 (s, 1H), 7.93 (d, J = 9 Hz, 1H), 8.39 (s, 1H), 8.69 (s, 1H), 10.45 (s, 1H). 13C NMR (75 MHz, DMSO): δ 27.76, 81.66, 108.08, 109.57, 115.12, 117.54, 119.43, 124.72, 131.21, 131.44, 137.76, 148.22, 150.84, 156.43, 158.63, 161.94. HRMS (EI positive): m/z: calculated for C₁₈H₁₆O₃ [M]+ 312.0996; found 312.0996.

8-Hydroxy-2-imino-2H-benzo[g]chromene-3-carbonitrile (5b). Compound 4 (40 mg, 0.21 mmole) dispersed in dry ethanol (3 mL) was treated with malononitrile (7.7 mg, 0.234 mmole) followed by 10 mole% piperidine. The reaction mixture was stirred at room temperature for 1 h to give a turbid, orange solid. The solid was filtered under vacuum and washed with cold ethanol followed by hexane to afford compound 5b as an orange solid (42 mg, 85%).

1H NMR (300 MHz, DMSO): δ 7.07 (dd, J = 9.0, 2.4 Hz, 1H), 7.13 (d, J = 1.8 Hz, 1H), 7.87 (d, J = 8.7 Hz, 1H), 8.05 (s, 1H), 8.43 (s, 1H), 8.89 (d, J = 1.2, 1H), 10.27 (s, 1H). 13C NMR (75 MHz, DMSO): δ 102.59, 108.37, 109.1, 114.85, 115.62, 118.94, 124.25, 130.58, 131.12, 147.03, 150.08, 151.71, 158.6. HRMS (EI positive): m/z: calculated for C₁₈H₁₆N₂O₂ [M]+ 236.0586; found 236.0583.

3-Acetyl-8-hydroxy-2H-benzo[g]chromen-2-one (5c). A solution of compound 4 (30 mg, 0.16 mmol) in dry ethanol (3 mL) was treated with diethylmalonate (30 ml, 0.24 mmol) and 10 mole% piperidine, and the resulting mixture was stirred for 4 h at room temperature. The reaction mixture was treated with hexane to provide precipitates, which were filtered under reduced pressure, and the filtrate was washed with 10% ethyl acetate in hexane to afford compound 5c as an orange-yellow solid (37 mg, 90%).

1H NMR (300 MHz, DMSO): δ 2.59 (s, 3H), 7.14 (dd, J = 9.0, 2.4 Hz, 1H), 7.2 (d, J = 2.1 Hz, 1H), 7.66 (s, 1H), 7.94 (d, J = 9.0 Hz, 1H), 8.45 (s, 1H), 8.7 (s, 1H). 13C NMR (75 MHz, DMSO): δ 30.18, 108.2, 109.59, 115.43, 119.57, 123.05, 124.89, 131.34, 132.42, 138, 147.63, 151.01, 158.85, 158.89, 194.95. HRMS (EI positive): m/z: calculated for C₁₃H₁₀O₄ [M]+ 254.0579; found 254.0577.
(Z)-8-Hydroxy-3-(1-hydroxy-3-oxobut-1-enyl)-2H-benzo[g]chromen-2-one (5d). A mixture of compound 4 (30 mg, 0.16 mmol), 4-hydroxy-6-methyl-2-pyrene (24 mg, 0.19 mmol), and benzyltrimethylammonium chloride (9 mg, 0.04 mmol) in dry ethanol (3 mL) was refluxed for 15 h. Precipitates were produced, which were filtered and washed with water and then with dichloromethane to afford compound 5d as a yellow solid (39 mg, 85%).

1H NMR (300 MHz, DMSO): δ 2.23 (d, J = 3.9 Hz, 3H), 6.91 (s, 1H), 7.15 (dd, J = 9.0, 2.4 Hz, 1H), 7.21 (d, J = 2.1 Hz, 1H), 7.68 (d, J = 3.3 Hz, 1H), 7.94 (d, J = 9.0 Hz, 1H), 8.47 (s, 1H), 8.87 (s, 1H), 10.51 (s, 1H). 13C NMR (75 MHz, DMSO): δ 2686, 10079, 109.5, 115.59, 118.18, 119.95, 124.87, 131.98, 146.59, 150.44, 157.72, 158.83, 173.79, 198.41. HRMS (EI positive): m/z: calculated for C_{27}H_{12}O_{5} [M]^+ 296.0685; found 296.0686.

3-(Benzo[d]thiazol-2-yl)-2-imino-2H-benzo[g]chromen-8-ol (5e). A solution of compound 4 (40 mg, 0.21 mmol), benzothiazole-2-acetonitrile (48 mg, 0.27 mmol), and 10 mole% piperidine in dry ethanol (3 mL) was stirred for 5 h at room temperature. Precipitates resulted, which were filtered under vacuum and washed with hexane and then with chloroform to afford compound 5e as a yellowish-brown solid (64 mg, 87%).

1H NMR (300 MHz, DMSO): δ 7.08 (dd, J = 9.0, 2.4 Hz, 1H), 7.15 (d, J = 1.8 Hz, 1H), 7.41–7.57 (m, 3H), 7.84 (d, J = 9.0 Hz, 1H), 8.07 (d, J = 7.8 Hz, 1H), 8.13 (d, J = 7.8 Hz, 1H), 8.29 (s, 1H), 8.81 (s, 1H), 9.17 (d, J = 1.8 Hz, 1H), 10.24 (s, 1H). 13C NMR (75 MHz, DMSO): δ 108.31, 108.42, 116.47, 118.6, 120.5, 121.91, 122.33, 124.95, 126.29, 130.59, 135.71, 136.87, 136.9, 150.08, 151.76, 153.37, 157.88, 161.04. HRMS (FAB positive): m/z: calculated for C_{20}H_{13}NO_{2}S [M+H]^+ 345.0692; found 345.0695.

3-(Benzo[d]thiazol-2-yl)-8-hydroxy-2H-benzo[g]chromen-2-one (5f). Compound 5f (40 mg, 0.116 mmol) in glacial acetic acid (3 mL) was refluxed for 3 h. The reaction mixture was concentrated, and the residue was treated with ethyl acetate and hexane (1:1) to give precipitates, which were filtered and washed with hexane to afford compound 5f as a saffron solid (32 mg, 80%).

1H NMR (300 MHz, DMSO): δ 7.17 (dd, J = 9.0, 2.4 Hz, 1H), 7.24 (d, J = 2.1 Hz, 1H), 7.45–7.51 (m, 2H), 7.56 (d, J = 1.2 Hz, 1H), 7.6 (dd, J = 7.2, 1.2 Hz, 1H), 7.77–8.56 (m, 2H), 9.28 (s, 1H), 10.48 (s, 1H). 13C NMR (75 MHz, DMSO): δ 108.23, 109.88, 115.82, 117.82, 122.18, 119.59, 122.18, 122.43, 125.06, 125.62, 125.62, 131.1, 131.38, 135.88, 137.43, 142.42, 150.06, 151.96, 158.55, 159.74, 160.05. HRMS (EI positive): m/z: calculated for C_{20}H_{11}NO_{2}S [M+H]^+ 345.0640; found 345.0456.

8-Hydroxy-3-(pyridin-4-yl)-2H-benzo[g]chromen-2-one (5g). To a mixture of compound 4 (100 mg, 0.53 mmol) and 4-pyridineacetic acid hydrochloride (138 mg, 0.8 mmol) suspended in dichloromethane (30 ml), was added triethylamine (370 μL, 2.65 mmol) followed by 4-dimethylaminopyridine (6.5 mg, 0.053 mmol) and EDC (153 mg, 0.8 mmol), and the mixture was stirred at room temperature overnight. Yellow precipitates formed were filtered under reduced pressure, and the filtrate was washed thoroughly with water and then with ethyl acetate to afford compound 5g as a yellow solid (130 mg, 87%).

1H NMR (300 MHz, DMSO): δ 7.14 (dd, J = 9.0, 2.1 Hz, 1H), 7.21 (s, 1H), 7.69 (s, 1H), 7.78 (d, J = 6.0 Hz, 2H), 7.95 (d, J = 9 Hz, 1H), 8.27 (s, 1H), 8.53 (s, 1H), 8.67 (d, J = 6 Hz, 2H), 10.33 (s, 1H). 13C NMR (75 MHz, DMSO): δ 108.05, 109.54, 116.35, 119.3, 122.75, 124.79, 129.59, 130.72, 136.76, 142.21, 142.25, 142.76, 149.67, 150.26, 157.93, 159.28. HRMS (EI positive): m/z: calculated for C_{18}H_{11}NO_{3} [M]^+ 289.0739; found 289.074.

8-Hydroxy-3-(N-methyl-pyridinum-4-yl)-2H-benzo[g]chromen-2-one trifluorosulfonate (5h). To a solution of compound 5g (100 mg, 0.346 mmol) in dichloromethane, was added methyl trifluoromethanesulfonate (80 ml, 0.7 mmol) dropwise. The reaction mixture was allowed to stir for 7 h at room temperature.
Precipitates formed were filtered under reduced pressure, and the filtrate was washed with ethyl acetate and then with hexane to afford compound 5h as an orange solid (130 mg, 87%).

\(^{1}\)H NMR (300 MHz, MeOD): \(\delta 4.4\ (s, 3H), 7.13–7.18\ (m, 2H), 7.59\ (s, 1H), 7.93\ (d, \(J = 9\) Hz, 1H), 8.3\ (s, 1H), 8.57\ (s, 1H), 8.6\ (s, 1H), 8.82\ (s, 1H), 8.87\ (s, 1H), 8.89\ (s, 1H). 13C NMR (75 MHz, DMSO): \(\delta 47.38, 108.24, 109.76, 109.87, 116.01, 119.46, 119.66, 124.93, 125.61, 131.25, 131.37, 137.78, 145.12, 146.53, 150.07, 150.41, 158.83\). HRMS (FAB positive): m/z: calculated for C_{19}H_{14}NO_{3} (cationic part only) [M]\(^{+}\) 304.0974; found 304.336.

4-(8-Acetoxy-2-oxo-2H-benzo[g]chromen-3-yl)-1-methylpyridinium trifluoromethanesulfonate (5P). A suspension of compound 5g (40 mg, 0.14 mmol) and triethylamine (58 \(\mu\)L, 0.42 mmol) in dichloromethane was treated with acetic anhydride (16 \(\mu\)L, 0.17 mmol), and the resulting mixture was allowed to stir for 5 h at room temperature. Finally, the solvent was evaporated to give white precipitates, which were dissolved in dichloromethane and then treated with methyl trifluoromethanesulfonate (80 \(\mu\)L, 0.7 mmol). The mixture was stirred at room temperature for 6 h to give precipitates, which were filtered under reduced pressure and washed with ethyl acetate and then with dichloromethane to afford compound 5P as a bright greenish-yellow solid (50 mg, 72%).

\(^{1}\)H NMR (300 MHz, DMSO): \(\delta 2.36\ (s, 3H), 4.36\ (s, 3H), 7.43\ (dd, \(J = 9, 2.4\) Hz, 1H), 7.84\ (d, \(J = 2.1\) Hz, 1H), 8.02\ (s, 1H), 8.24\ (d, \(J = 9.0\) Hz, 1H), 8.52\ (s, 1H), 8.54\ (s, 2H), 8.95\ (s, 1H), 9.05\ (s, 1H), 9.07\ (s, 1H). 13C NMR (75 MHz, DMSO): \(\delta 20.92, 47.5, 111.82, 118.32, 118.61, 121.92, 122.28, 126.02, 127.86, 130.82, 130.98, 135.81, 145.71, 149.79, 150.27, 150.78, 158.47, 169.22\). HRMS (FAB positive): m/z: calculated for C_{21}H_{16}NO_{4} (cationic part only) [M]\(^{+}\) 346.1079; found 346.301.
UV-Vis absorption spectra of 5a–5h

**Fig. S1** UV-Vis absorption spectra of compounds 5a–5h in a) acetonitrile and in b) ethanol. All the measurements were conducted at 5 μM concentration of each compound in a cuvette with 1-cm path length.

Fluorescence emission spectra of 5a–5h

**Fig. S2** Fluorescence emission spectra of compounds 5a–5h in a) acetonitrile and in b) ethanol upon excitation at their respective maximum absorption wavelength. All the measurements were conducted at 5 μM concentration of each compound in a cuvette with 1-cm path length and the slit was fixed at 2 nm.

Lippert-Mataga plot of 5a–5h

**Fig. S3** Lippert-Mataga plot for 5a–5h constructed from the Stokes shift data obtained in 1,4-dioxane and acetonitrile.
Aqueous solubility data of 5h

![Graph showing solubility of 5h in PBS 7.4 buffer.](image)

Fig. S4 Solubility of 5h in PBS 7.4 buffer.

Photostability of 5a–5h

![Graph showing photostability of 5a–5h compared to Umbelliferone.](image)

Fig. S5 Photostability of 5a–5h in PBS 7.4 in comparison to Umbelliferone. Dyes were irradiated with 365 nm UV light in a WUV-L10 chamber up to 30 minutes.

Table S1 Molar absorptivity of 5a–5h in different solvents.

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<th>Molar absorptivity (ε) (L·Mol⁻¹·cm⁻¹)</th>
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<th>5b</th>
<th>5c</th>
<th>5d</th>
<th>5e</th>
<th>5f</th>
<th>5g</th>
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Table S2 Two-photon absorption cross section (TPACS) values in acetonitrile

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<th>800 nm</th>
<th>820 nm</th>
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<tr>
<td>5h</td>
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<td>92</td>
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</tr>
</tbody>
</table>

*aCalculated considering Rhodamine 6G in methanol as reference.*
$^1$H & $^{13}$C NMR data

dihydroxy CHO_LH_MeOD

Aromatic-H

CHO

-Aromatic-H

11 10 9 8 7 6 5 4 3 2 1 0 ppm

200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm
Aromatic-H & imine-H

-OH
ss-19-40-mesu-pyridinium salt

Aromatic-H

CH₃ on Pyridine

5h
HRMS data