Supporting Information

A sensitive colorimetric and fluorescent sensor based on imidazolium-functionalized squaraines for the detection of GTP and alkaline phosphatase in aqueous solution

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I. General Remarks

NMR spectra were obtained on a Bruker AV II-400 MHz or a Varian Inova 400 spectrometer. The $^1$H NMR (400 MHz) chemical shifts were measured relative to CDCl$_3$, D$_2$O or DMSO-$d_6$ as the internal reference (CDCl$_3$: $\delta = 7.26$ ppm; D$_2$O: $\delta = 4.79$ ppm; DMSO-$d_6$: $\delta = 2.50$ ppm). The $^{13}$C NMR (100 MHz) chemical shifts were given using CDCl$_3$ or DMSO-$d_6$ as the internal standard (CDCl$_3$: $\delta = 77.16$ ppm; DMSO-$d_6$: $\delta = 39.52$ ppm). High-resolution mass spectra (HRMS) were obtained with a Waters-Q-TOF-Premier (ESI). Melting points were determined with XRC-1 and are uncorrected. Absorption spectra were detected on a HITACHI U-2910 absorption spectrophotometer. Fluorescence spectra were collected on a Horiba Jobin Yvon-Edison Fluoromax-4 fluorescence spectrometer. TEM studies were carried out on a HITACHI H-600IV, operating at 75 kV. Dynamic light scattering (DLS) experiments were recorded on a Brookhaven BI-200SM Laser Light Scattering Goniometer. Fluorescence images was examined under a fluorescence microscopy (OLYMPUS IX71) irradiated by green light source (540-580 nm).

Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Tetrahydrofuran was dried by heating at reflux over sodium/benzophenone and freshly distilled prior to use. $n$-Butanol was dried by heating at reflux over sodium and distilled prior to use. Benzene was treated firstly by shaking with concentrated H$_2$SO$_4$ until free from thiophene, and then with water, dilute NaOH and water again. This mixture was then dried with sodium and distilled prior to use. All syntheses and manipulations were carried out under dry N$_2$ atmosphere. The semisquaraine 7 was synthesized according to the reference.$^{[1]}$

Water was distilled for twice in the optical spectroscopic studies. The sodium salts of ATP and GTP were purchased from J&K Scientific Ltd. (Beijing, China). The sodium salts of other nucleotides were purchased from Biotogether Co., Ltd. (Nanjing, China). Stock solutions (6 mM) of nucleotides (the sodium salts of ATP, GTP, ADP, GDP, AMP, GMP, UMP, CMP) and anions (CH$_3$CO$_2$Na, Na$_2$SO$_4$, NaF, NaCl, NaBr, NaI, NaNO$_3$, NaHCO$_3$, NaH$_2$PO$_4$·2H$_2$O, Na$_4$P$_2$O$_7$) in HEPES buffer (10 mM, pH = 7.2) were stored at
-20 °C and used within a few days. Stock solutions of host (10 µM) were prepared in HEPES buffer (10 mM, pH = 7.2). Each time a 3 mL of host solution was filled in a quartz cell of 1 cm of optical path length and the stock solution of anion was dropped into a quartz cell using a microsyringe. The volume of anions stock solution added was less than 100 µL to remain the concentration of host constant. For all measurements, the emission slits of fluorescence spectra were set at 5.0 nm if not specified.

II. Synthesis and characterization of ImSQ4, ImSQ8 and ImSQ12

1

1 was synthesized according to the procedure described in the previous literature. A mixture of phloroglucinol dihydrate (8.0 g, 49 mmol) and diethanolamine (4.6 g, 44 mmol) were dissolved in a mixture of toluene (50 mL) and n-butanol (50 mL). The mixture was refluxed with azeotropic distillation of water for 3 h, during this time period reaction solution turned to red. After being cooled, the solvent was removed under reduced pressure. The crude residue was purified by silica column chromatography (EtOAc/petroleum = 1/30~1/5, v/v) to afford the product 1 as a pink solid (5.8 g, 62% yield).

2

To a suspension of K$_2$CO$_3$ (14.5 g, 105 mmol) in DMF (100 mL) was added 1 (5.6 g, 26.3 mmol) and benzyl chloride (12.1 mL, 105 mmol). The resulting mixture was then reflux for 4 h. Water (60 mL) was added and the mixture was extracted with EtOAc (3 × 60 mL). The combined organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated under reduced pressure. The crude material was purified by column chromatography on silica gel (CH$_2$Cl$_2$/EtOAc = 2/1, v/v) to afford the desired product 2 as an off-white solid (5.6 g, 54% yield). M.p.: 90-91 °C. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$
= 3.42 (s, 2H), 3.50 (t, \( J = 4.8 \) Hz, 4H), 3.77 (t, \( J = 4.8 \) Hz, 4H), 5.01 (s, 4H), 5.95 (s, 2H), 6.10 (s, 1H), 7.30-7.42 (m, 10H) ppm. \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta = 55.7, 61.0, 70.2, 90.8, 93.6, 127.7, 128.1, 128.7, 137.2, 149.8, 161.0 \) ppm. HRMS (ESI\(^+\)) : calcd for C\(_{24}\)H\(_{28}\)NO\(_4\) [M+H]\(^+\) 394.2018, found 394.2010.

Tosyl chloride (4.6 g, 24.1 mmol) in dichloromethane (20 mL) was added dropwise to a solution of 2 (3.8 g, 9.7 mmol) and triethylamine (4.1 mL, 29.5 mmol) in dichloromethane (20 mL). The solution was then stirred at room temperature overnight. After the triethylamine hydrochloride salt had been removed by filtration, the filtrate was evaporated to dryness. The crude material was purified by column chromatography on silica gel (petroleum ether/EtOAc = 2/1, v/v) to afford 3 as colorless oil (5.4 g, 79% yield). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \( \delta = 2.38 \) (s, 6H), 3.49 (t, \( J = 6.0 \) Hz, 4H), 4.05 (t, \( J = 6.0 \) Hz, 4H), 4.97 (s, 4H), 5.71 (s, 2H), 6.05 (s, 1H), 7.24 (d, \( J = 8.4 \) Hz, 4H), 7.31-7.42 (m, 10H), 7.69 (d, \( J = 8.0 \) Hz, 4H) ppm. \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \( \delta = 21.8, 50.6, 66.8, 70.2, 91.5, 92.8, 127.7, 128.0, 128.2, 128.8, 130.0, 132.8, 137.1, 145.1, 147.8, 161.1 \) ppm. HRMS (ESI\(^+\)) : calcd for C\(_{38}\)H\(_{40}\)NO\(_8\)S\(_2\) [M+H]\(^+\) 702.2195, found 702.2171.

Sodium hydride (60% in mineral oil, 0.88 g, 21.9 mmol) was added to a solution of imidazole (1.35 g, 19.9 mmol) in THF (20 mL). After the solution had been stirred for 30 min at 40 °C, the THF (15 mL) solution of 3 (5.95 g, 8.5 mmol) was added dropwise over 30 min. The resulting mixture was then stirred vigorously for 12 h at 75 °C. Subsequently, the reaction was quenched by water and extracted with CH\(_2\)Cl\(_2\) (3×15 mL). The combined organic layers was concentrated in vacuo and purified by flash column
chromatography on silica gel (CH₂Cl₂/CH₃OH = 16/1, v/v) to give 4 as a light yellow powder (3.5 g, 83% yield). M.p.: 126-127 °C. ¹H NMR (400 MHz, CDCl₃): δ = 3.13 (t, J = 5.2 Hz, 4H), 3.83 (t, J = 5.2 Hz, 4H), 5.04 (s, 4H), 5.86 (s, 2H), 6.18 (s, 1H), 6.74 (s, 2H), 7.04 (s, 2H), 7.26 (m, 2H), 7.31-7.41 (m, 10H) ppm. ¹³C NMR (100 MHz, DMSO-d₆): δ = 43.5, 51.4, 69.1, 91.1, 92.1, 119.6, 127.7, 127.8, 128.4, 128.5, 137.4, 137.5, 148.4, 160.5 ppm. HRMS (ESI⁺): calcd for C₃0H₃₂N₅O₂ [M+H]⁺ 494.2556, found 494.2558.

A mixture of 5% Pd/C (0.24 g) and 4 (2.5 g, 5.0 mmol) in MeOH (15 mL) was hydrogenated at 1.5 MPa in an autoclave and stirred at room temperature overnight. Subsequently, the reaction mixture was filtered and washed with MeOH. The solid residues containing product and catalyst were heated with DMF at 120 °C, filtered immediately. The filtrate was removed in vacuo to obtain 5 as an off-white solid (1.5 g, 96% yield), which was carried forward to further reactions without further purifications.

**General procedure for the synthesis of 6a–c**

n-Bromoalkane (1 mL) was added to a solution of 5 (0.31 g, 1.0 mmol) in DMF (6 mL). The solution was then stirred vigorously for 24 h at 75 °C. Subsequently, the reaction mixture was concentrated under reduced pressure and purified by column chromatography on silica gel (CH₂Cl₂/CH₃OH = 10/1, v/v) to afford the desired product as an off-white solid. Further purification was taken by recrystallization from isopropanol and hexane.
**6a:** 0.46 g, 78%. M.p.: 200-201 °C. $^1$H NMR (400 MHz, D$_2$O): $\delta =$ 0.85 (t, $J =$ 7.2 Hz, 6H), 1.07-1.18 (m, 4H), 1.50-1.57 (m, 4H), 3.80 (t, $J =$ 4.8 Hz, 4H), 3.98 (t, $J =$ 7.2 Hz, 4H), 4.40 (t, $J =$ 4.8 Hz, 4H), 5.48 (s, 2H), 5.66 (s, 1H), 7.37 (t, $J =$ 1.6 Hz, 2H), 7.52 (t, $J =$ 1.6 Hz, 2H), 8.54 (s, 2H) ppm. $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta =$ 13.3, 18.7, 31.3, 46.1, 48.5, 48.9, 91.1, 92.9, 122.2, 122.9, 136.5, 148.3, 159.2 ppm. HRMS (ESI$^+$): calcd for C$_{24}$H$_{37}$N$_5$O$_2$Br $[M-Br]^+$ 506.2131, found 506.2131.

**6b:** 0.50 g, 71%. M.p.: 188-189 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta =$ 0.86 (t, $J =$ 6.4 Hz, 6H), 1.24 (s, 20H), 1.70 (t, $J =$ 6.8 Hz, 4H), 3.61 (s, 4H), 4.10 (t, $J =$ 6.8 Hz, 4H), 4.30 (s, 4H), 5.55 (s, 2H), 5.65 (s, 1H), 7.78 (s, 2H), 7.81 (s, 2H), 8.92 (s, 2H), 9.21 (s, 2H) ppm. $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta =$ 14.2, 22.3, 25.7, 28.5, 28.7, 29.6, 31.4, 46.2, 49.0, 49.3, 91.3, 93.0, 122.4, 123.1, 136.7, 148.4, 159.4 ppm. HRMS (ESI$^+$): calcd for C$_{32}$H$_{52}$N$_5$O$_2$ $[M-2Br-H]^+$ 538.4121, found 538.4119.

**6c:** 0.65 g, 80%. M.p.: 180-182 °C. $^1$H NMR (400 MHz, CDCl$_3$): $\delta =$ 0.87 (t, $J =$ 7.2 Hz, 6H), 1.25-1.33 (m, 36H), 1.91 (s, 4H), 3.78 (s, 4H), 4.19 (t, $J =$ 7.2 Hz, 4H), 4.44 (s, 4H), 5.65 (s, 1H), 6.23 (s, 2H), 7.19 (s, 2H), 8.21 (s, 2H), 8.28 (s, 2H), 10.02 (s, 2H) ppm. $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta =$ 13.9, 22.1, 25.5, 28.4, 28.7, 28.8, 28.98, 29.02, 29.4, 31.3, 46.1, 49.8, 49.2, 91.2, 93.0, 122.2, 122.9, 136.4, 148.2, 159.2 ppm. HRMS (ESI$^+$): calcd for C$_{40}$H$_{69}$N$_5$O$_2$Br $[M-Br]^+$ 730.4635, found 730.4634.

**General procedure for the synthesis of ImSQ4, ImSQ8 and ImSQ12**
3-(4-(dibutylamino)phenyl)-4-hydroxycyclobut-3-ene-1,2-dione 7 (150 mg, 0.50 mmol) was reacted with the imidazolium salt 6 (0.60 mmol) in a mixture of distilled n-butanol (3 mL) and benzene (3 mL) at reflux for 10 h. The solvent was removed in vacuo after the reaction mixture was cooled down to room temperature. The residue was washed with ether and filtrated. Further purification was taken by recrystallization from isopropanol and hexane, the desired product was gained as a green solid.

**ImSQ4**: 147 mg, 34%. M.p.: 195-198 °C. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 0.91 (t, $J$ = 7.2 Hz, 6H), 0.97 (t, $J$ = 7.2 Hz, 6H), 1.31-1.38 (m, 8H), 1.59-1.62 (m, 4H), 1.84-1.86 (m, 4H), 3.36 (s, 4H), 4.24 (s, 4H), 4.73 (s, 4H), 5.81 (s, 2H), 6.66 (d, $J$ = 8.8 Hz, 2H), 7.35 (s, 2H), 7.97 (d, $J$ = 8.8 Hz, 2H), 8.06 (s, 2H), 10.11 (s, 2H), 12.44 (s, 2H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 13.6, 14.0, 19.6, 20.3, 29.6, 31.9, 46.8, 49.8, 50.1, 51.3, 93.3, 106.2, 112.6, 117.5, 122.1, 123.9, 132.6, 137.1, 152.9, 159.4, 164.8, 166.8, 178.0, 182.2 ppm. HRMS (ESI$^+$): calcd for C$_{42}$H$_{58}$N$_6$O$_4$Br [M-Br]$^+$ 789.3703, found 789.3696.

**ImSQ8**: 149 mg, 30%. M.p.: 218-220 °C. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ = 0.69 (t, $J$ = 6.4 Hz, 6H), 0.91 (t, $J$ = 6.4 Hz, 6H), 1.04-1.10 (m, 20H), 1.31-1.34 (m, 4H), 1.50-1.52 (m, 8H), 3.49 (s, 4H), 4.00-4.04 (m, 8H), 4.48 (s, 4H), 5.47 (s, 2H), 6.96 (d, $J$ = 8.4 Hz, 2H), 7.78 (s, 2H), 7.87-7.92 (m, 4H), 9.19 (s, 2H), 12.32 (s, 2H) ppm. $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ = 13.77, 13.84, 19.5, 22.1, 25.7, 28.7, 29.2, 30.2, 31.4, 46.5, 47.8, 48.9, 50.3, 92.1, 105.0, 113.3, 116.5, 122.5, 123.3, 131.6, 136.5, 153.0, 159.8, 163.5, 164.9, 175.2, 181.8 ppm. HRMS (ESI$^+$): calcd for C$_{56}$H$_{74}$N$_6$O$_4$Br [M-Br]$^+$ 901.4955,
found 901.4952.

**ImSQ12**: 160 mg, 29%. M.p.: 211-214 °C. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ = 0.86 (t, $J$ = 7.2 Hz, 6H), 0.97 (t, $J$ = 7.2 Hz, 6H), 1.20-1.28 (m, 36H), 1.38 (q, $J$ = 7.2 Hz, 4H), 1.61 (s, 4H), 1.85 (s, 4H), 3.35 (s, 4H), 4.23 (s, 4H), 4.29 (s, 4H), 4.78 (s, 4H), 5.82 (s, 2H), 6.65 (d, $J$ = 8.4 Hz, 2H), 7.29 (s, 2H), 7.97 (d, $J$ = 8.4 Hz, 2H), 8.12 (s, 2H), 10.20 (s, 2H), 12.48 (s, 2H) ppm. $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ = 14.0, 14.2, 20.3, 22.8, 26.5, 29.2, 29.4, 29.59, 29.64, 29.7, 30.4, 32.0, 46.9, 49.9, 50.4, 51.3, 93.3, 106.4, 112.6, 117.6, 121.9, 124.0, 132.6, 137.2, 152.9, 159.4, 164.8, 166.9, 178.2, 182.2 ppm. HRMS (ESI$^+$): calcd for C$_{58}$H$_{90}$N$_6$O$_4$Br [M-Br]$^+$ 1013.6207, found 1013.6207.

### III. References


### IV. The failed synthetic route to imidazolium-functionalized squaraines

![Scheme S1](image)

**Scheme S1** The failed synthetic route to imidazolium-functionalized squaraines.

The bisimidazolium-containing aniline derivatives with one or no *meta*-hydroxyl group on the benzene ring could not deliver the desired squaraines.
V. Photophysical properties of ImSQ4, ImSQ8 and ImSQ12

**Fig. S1** Absorption spectra of ImSQ4, ImSQ8 and ImSQ12 (10 µM) in HEPES buffer (10 mM, pH = 7.2).

**Fig. S2** Fluorescent emission spectra of ImSQ4, ImSQ8 and ImSQ12 (10 µM) in HEPES buffer (10 mM, pH = 7.2).
VI. Related experiments of the host-guest recognition

Fig. S3-1 Absorption spectra of ImSQ8 (10 µM) upon addition of different anions in HEPES buffer (10 mM, pH = 7.2).
Fig. S3-2 Absorption spectra of ImSQ8 (10 µM) upon addition of different anions in HEPES buffer (10 mM, pH = 7.2).
**Fig. S3-3** Absorption spectra of ImSQ8 (10 µM) upon addition of different anions in HEPES buffer (10 mM, pH = 7.2).

**Fig. S4** Fluorescent emission spectra of ImSQ8 (10 µM) upon addition of GTP in HEPES buffer (10 mM, pH = 7.2). λex = 613 nm.

**VII. Calculation of the detection limit**

The typical limit of detection (LOD) is obtained by using the equation followed:

\[
\text{LOD} = \frac{3 \times \text{SD}}{S}
\]

Where SD is the standard deviation of the background (ImSQ8 in HEPES buffer (10 mM, pH = 7.2)) and S is the sensitivity. S can be obtained from the slope of the linear fit.
VIII. Fluorescence image of live cells

**Fig. S5** Fluorescence microscopy images of Bel-7402 cells treated with **ImSQ8** (20 µM) in physiological saline containing 1% DMSO: (a) before and (b) after adding GTP (30 equiv). (c)/(d) were the brightfield images corresponding to (a)/(b), respectively.

IX. DLS measurements of aggregates in HEPES buffer

**Fig. S6** CONTIN plot from DLS measurements at 90° on **ImSQ8** (40 µM) in HEPES buffer (10 mM, pH = 7.2) in the presence of GTP (2.0 equiv.).
X. Copies of $^1$H and $^{13}$C NMR spectra