Electronic supporting information (ESI)

Novel Fluorescent Probe for Highly-Sensitive Bioassay using Sequential Enzyme-Linked Immunosorbent Assay - Capillary Isoelectric Focusing (ELISA-cIEF)

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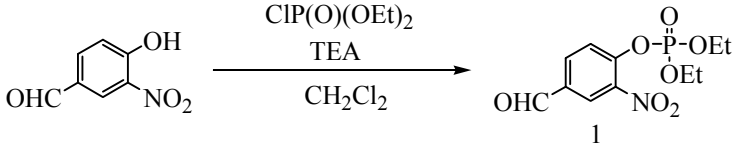
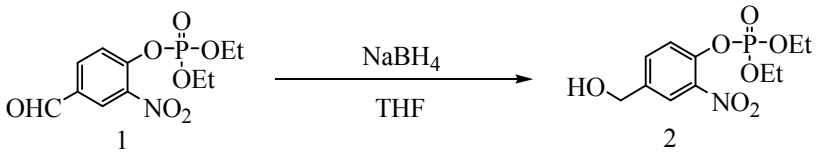
1. Synthesis of Rho 110-DP



1

To a solution of 3-nitro-4-hydroxybenzaldehyde (1.000 g, 5.99 mmol) in TEA (1.25 mL) and dichloromethane (20 mL), $ClP(O)(OEt)_2$ (1.134 g) was slowly added at 0 ºC and the mixture was stirred at room temperature. After stirring for 20.5 h, water was added to the reaction mixture at 0 ºC, and extraction was performed with dichloromethane. The organic phase was washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (SiO_2, hexane:ethyl acetate, 30:70 → 50:50) to yield compound **1** (1.284 g, 83.0 %).

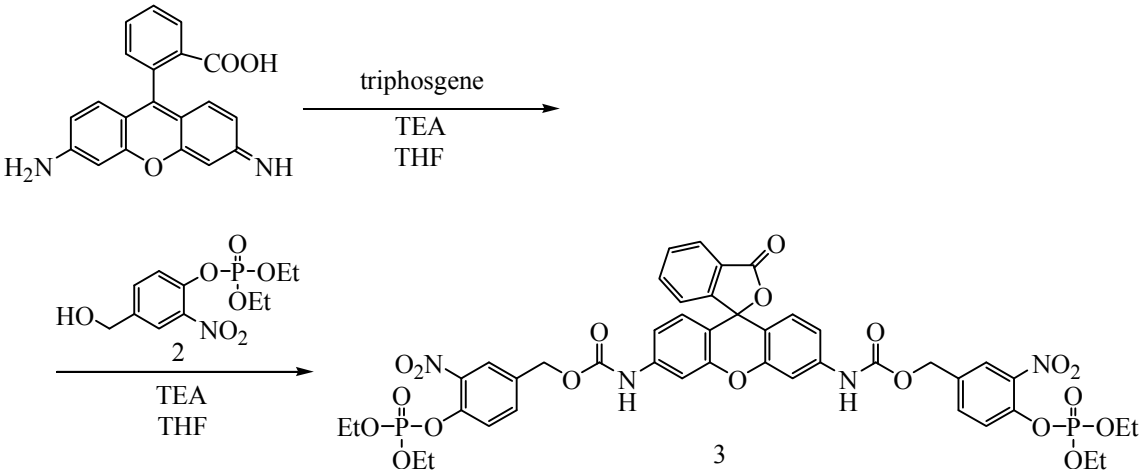
δ_H (500 MHz; $CDCl_3$; Me_4Si) 1.39 (6H, dt, J 0.1, 7.1), 4.30 (4H, dq, J 0.5, 8.0), 7.82 (1H, d, 9.0), 8.11 (1H, dd, J 8.5, 2.2), 8.43 (1H, d, J 1.6), 10.02 (1H, s)



1 2

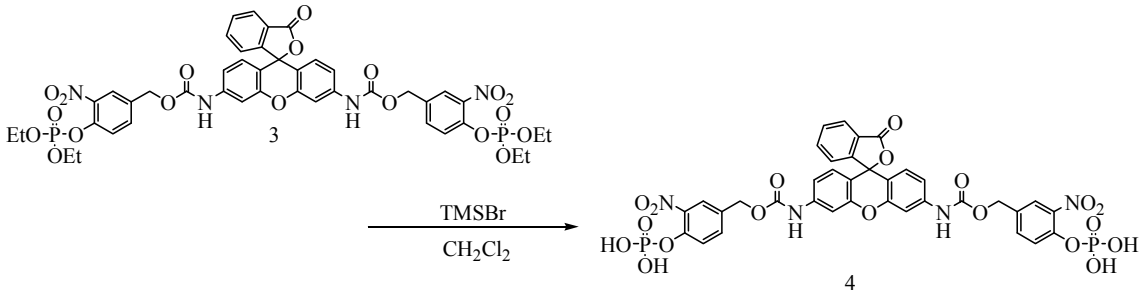
To a solution of **1** (0.972 g, 3.21 mmol) in THF (25 mL), $NaBH_4$ (90 wt%) (0.140 g) was slowly added at 0 ºC and the mixture was stirred at room temperature. After stirring for 1 h, acetone was added to the reaction mixture, before concentrating in vacuo. The residue was purified by column chromatography (SiO_2, chloroform:ethyl acetate, 70:30 → 50:50) to yield compound **2** (0.890 g, 90.8 %).

δ_H (500 MHz; $CDCl_3$; Me_4Si) 1.37 (6H, t, J 7.0), 3.59 (1H, s), 4.27 (4H, m), 4.66 (2H, d, J 3.0), 7.44 (1H, d, J 8.6), 7.51 (1H, d, J 8.3), 7.94 (1H, s)



To a solution of triphosgene (94.4 mg, 0.159 mmol) in THF (3 mL), rhodamine 110 chloride (58.4 mg, 0.197 mmol) in TEA (2 mL) and THF (30 mL) was added over a period of 30 min. Then, compound **2** (154.6 mg, 0.507 mmol) in THF (10 mL) was added over a period of 1 h, and the mixture was stirred at room temperature. After stirring for 19 h, additional triphosgene (47.2 mg, 0.080 mmol) in THF (3 mL) and compound **2** (147.8 mg, 0.484 mmol) in THF (10 mL) was added to the reaction mixture. Stirring was continued for a further 24 h, before the reaction mixture was concentrated *in vacuo*. The residue was extracted with dichloromethane and the organic phase was washed with water and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by column chromatography (SiO_2, chloroform:methanol = 97:3 → 90:10) and gel permeation chromatography to generate compound **3** (34.8 mg, 22.0 %).

δ_H (500 MHz; $CDCl_3$; Me_4Si) 1.37 (12H, t, J 7.0), 4.27 (8H, m), 5.16 (4H, s), 6.58 (2H, d, J 8.6), 6.79 (2H, dd, J 2.3, 6.3), 7.14 (1H, d, J 7.8), 7.50 (6H, dd, J 2.1, 8.5), 7.65 (2H, m), 7.80 (2H, s), 8.02 (1H, d, J 7.5)

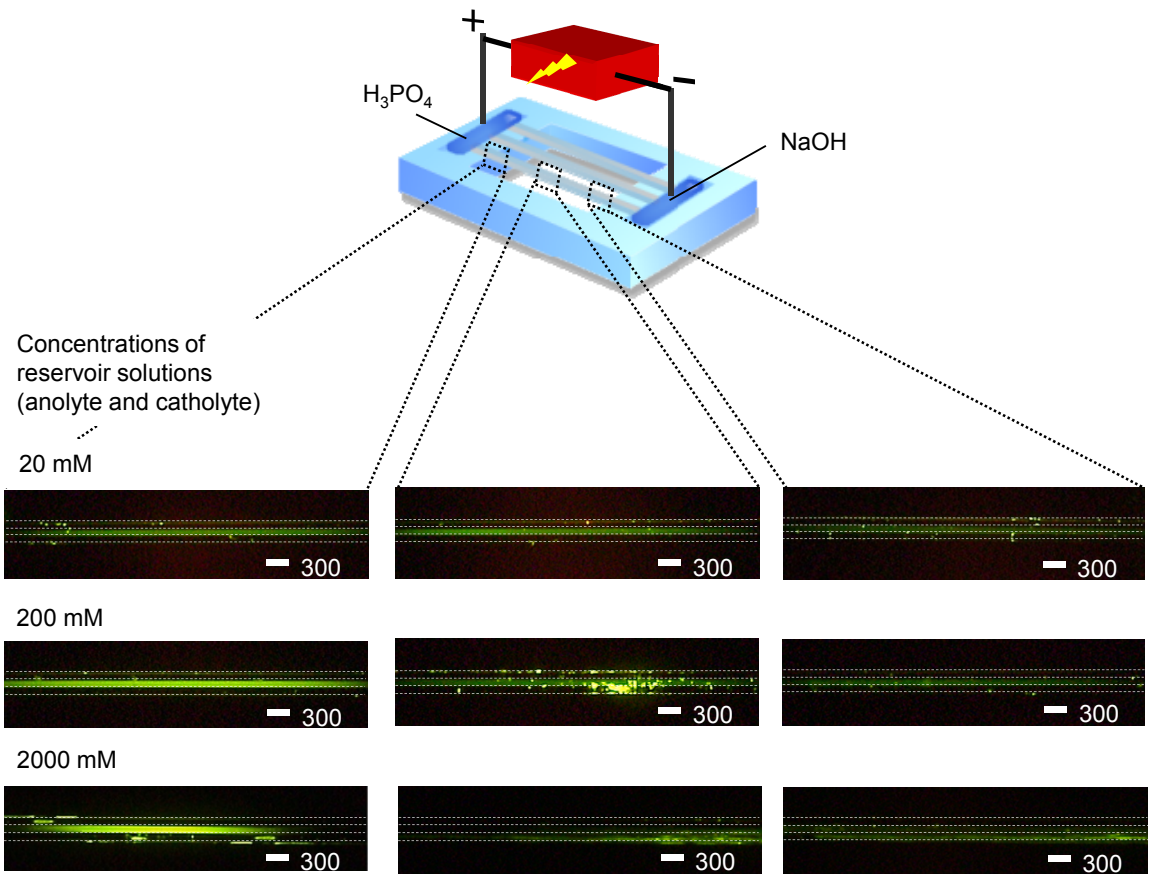


To a solution of **3** (6.6 mg, 0.007 mmol) in dichloromethane (2.0 mL), bromotrimethylsilane (1.0 mL) was added and the mixture was stirred at room temperature for 22.5 h, before being

concentrated *in vacuo*. The residue was purified by reverse-phase preparative thin-layer chromatography (methanol:water:acetone, 1.8:1:1) to give compound **4** (0.17 mg, 3.4 %).
δ_H (500 MHz; DMSO-d6; Me_4Si) 5.13 (4H, s), 6.69 (2H, d, J 8.6), 7.15 (2H, d, J 8.6), 7.29 (1H, d, J 7.5), 7.57 (4H, d, J 7.5), 7.71 (2H, m), 7.79 (4H, d, J 8.0), 8.00 (1H, d, J 7.5), 10.13 (2H, s)

2. Effect of the concentrations of reservoir solutions on IEF behavior

Fluorescence images showed concentration of Rho 110 at different positions of the capillary. When the concentrations of reservoir solutions were relatively low (20 mM), the concentrated band was very broad. However, increasing the concentrations of reservoir solutions resulted in an increase in fluorescence due to the pre-concentration by cIEF.



3. Fluorescence intensity profiles of the capillaries used for evaluating the peak area shown in Fig.4

Along the 4cm-long capillary, the signal from the fluorescent products that appeared at 0-1 cm from the anode side was integrated to calculate the peak area. In each case, the background signal (data obtained for 0 ng/mL Human IgG) was subtracted.