

# Novel Water-Soluble Near-Infrared Cyanine Dyes: Synthesis, Spectral Properties, and Use in the Preparation of Internally Quenched Fluorescent Probes

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## SUPPORTING INFORMATION

- S1** Experimental section : Synthesis of thiol-reactive **NIR5.5-1** derivative **24** and fluorogenic **NIR5.5-1/NIR7.0-2** hexapeptide **25**.
- S2** MALDI-TOF mass spectrum of probe **25**.
- S3** Absorption spectra of probe **25** in PBS and EtOH.
- S4** Emission (Ex.  $\lambda = 670$  or  $750$  nm) spectra of probe **25** in PBS and EtOH.
- S5** Emission (Ex.  $\lambda = 780$  nm) spectra of probe **19** in PBS and DMSO.

**-S1- Experimental section : Synthesis of thiol-reactive NIR5.5-1 derivative 24 and fluorogenic NIR5.5-1/NIR7.0-2 hexapeptide 25.**

**HPLC separations.** Several chromatographic systems were used for the analytical experiments and the purification steps. Each one of these systems was optimised in order to improve separation conditions.

**System A:** RP-HPLC (Thermo Hypersil GOLD C<sub>18</sub> column, 5  $\mu$ m, 4.6 x 150 mm) with CH<sub>3</sub>CN and 0.1% aq. trifluoroacetic acid (aq. TFA, 0.1%, v/v, pH 2.0) as the eluents, at a flow rate of 1 mL/min, with the following gradients:

A1 : [100% TFA (5 min), linear gradient from 0 to 20% (8 min) and 20 to 80 (27 min) of CH<sub>3</sub>CN]. Dual UV-Visible detection was achieved at 260 and 680 nm.

A2 : [100% TFA (5 min), linear gradient from 0 to 40% (20 min) and 40 to 80% (40 min) of CH<sub>3</sub>CN]. Triple UV-Visible detection was achieved at 260, 680 and 750 nm.

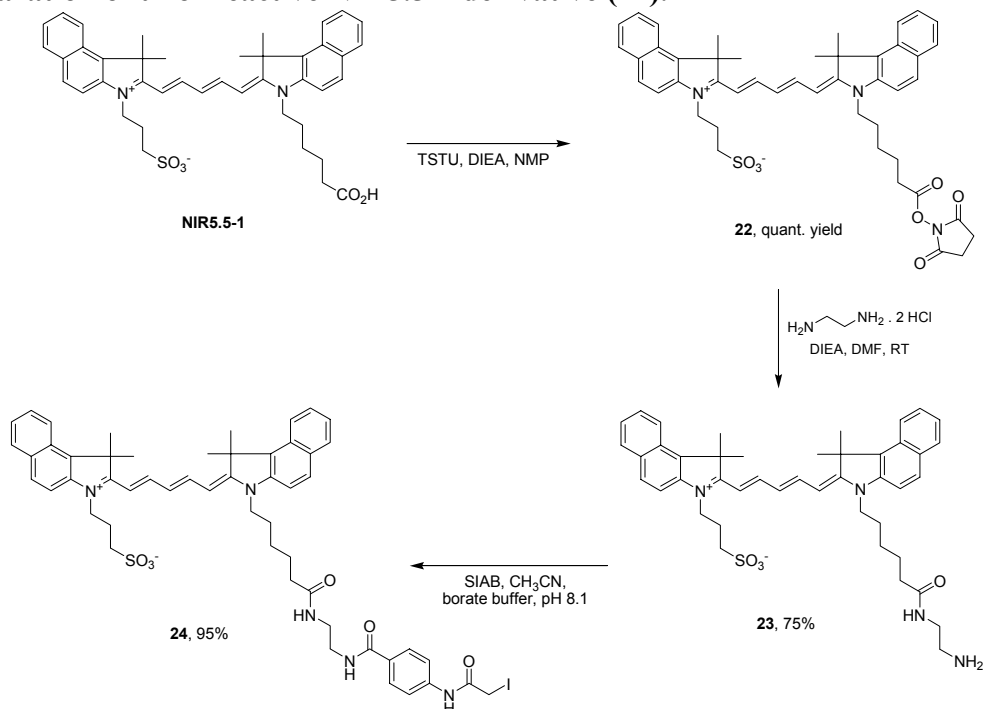
**System B:** RP-HPLC (Waters XTerra MS C<sub>18</sub> column, 5  $\mu$ m, 7.8 x 100 mm) with CH<sub>3</sub>CN and 0.1% aq. TFA as the eluents, at a flow rate of 2.5 mL/min, with the following gradients:

B1 : [85% TFA (5 min), linear gradient from 15 to 60% (45 min) of CH<sub>3</sub>CN]. UV detection was achieved at 260 nm.

B2 : [80% TFA (5 min), linear gradient from 20 to 80% (60 min) of CH<sub>3</sub>CN]. UV detection was achieved at 260 nm.

B3 : [85% TFA (5 min), linear gradient from 15 to 35% (10 min) and 35 to 80% (45 min) of CH<sub>3</sub>CN]. UV detection was achieved at 260 nm.

**Preparation of thiol-reactive NIR5.5-1 derivative (24).**



Synthetic reactions used for the preparation of thiol-reactive NIR5.5-1 derivative 24.

(a) Preparation of NIR5.5-1 carboxylic acid, Succinimidyl Ester 22. Free carboxylic acid dye NIR5.5-1 (6.6 mg, 9.6  $\mu$ mol) was introduced into a Reacti-Vial<sup>TM</sup> and dissolved in 140  $\mu$ L of

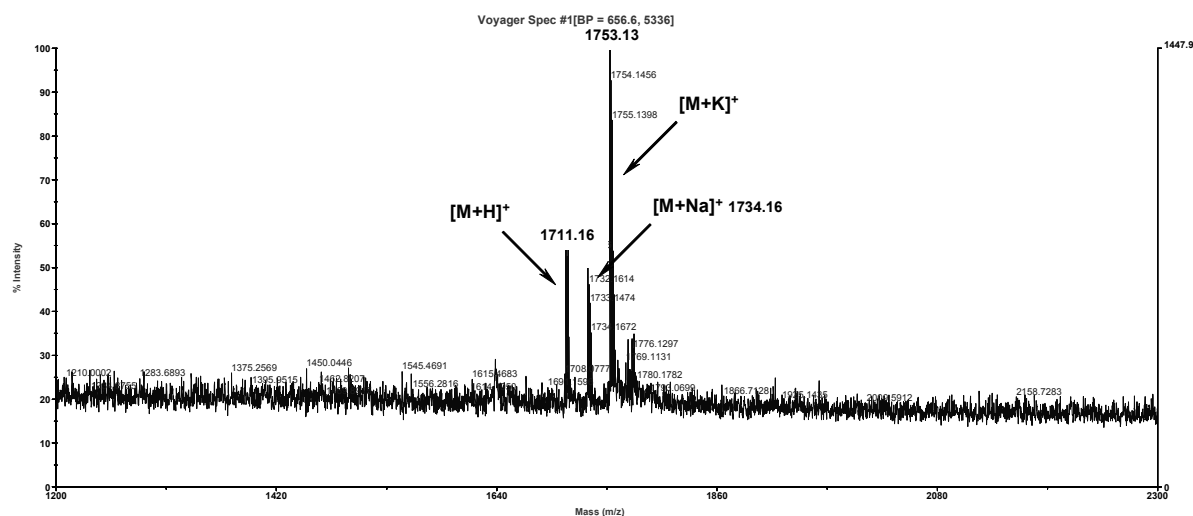
dry NMP. 20  $\mu\text{L}$  of a solution of TSTU reagent in dry NMP (2.88 mg, 9.6  $\mu\text{mol}$ ) and 11.7  $\mu\text{L}$  of DIEA (28.8  $\mu\text{mol}$ ) were added and the resulting reaction mixture was protected from light and stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system A1) and the resulting succinimidyl ester **22** was used without further purification. HPLC (system A1):  $t_{\text{R}} = 33.6$  min (compared to  $t_{\text{R}} = 32.6$  min for **NIR5.5-1** carboxylic acid).

(b) Synthesis of **NIR5.5-1** amine **23**. Ethylenediamine dihydrochloride (150 mg, 1.152 mmol) was dissolved in a mixture of deionised water (0.9 mL) and DMF (10 mL). The crude reaction mixture containing the succinimidyl ester **22** and a 10% solution of DIEA in DMF (0.9 mL, 537  $\mu\text{mol}$ ) were sequentially added and the resulting reaction mixture was protected from light and stirred at room temperature for 1 h. The reaction was checked for completion by RP-HPLC (system A1) and the mixture was evaporated to dryness. The resulting residue was purified by RP-HPLC (system B1, 2 injections). The product-containing fractions were lyophilised to give the **NIR5.5-1** amine **23** as a blue amorphous powder (5.2 mg, yield 75%). HPLC (system A1):  $t_{\text{R}} = 29.8$  min, purity > 95%. MS (MALDI-TOF, positive mode, CHCA matrix):  $m/z$  733.82  $[\text{M}+\text{H}]^+$ , calcd exact mass for  $\text{C}_{44}\text{H}_{52}\text{N}_4\text{O}_4\text{S}$  732.99.

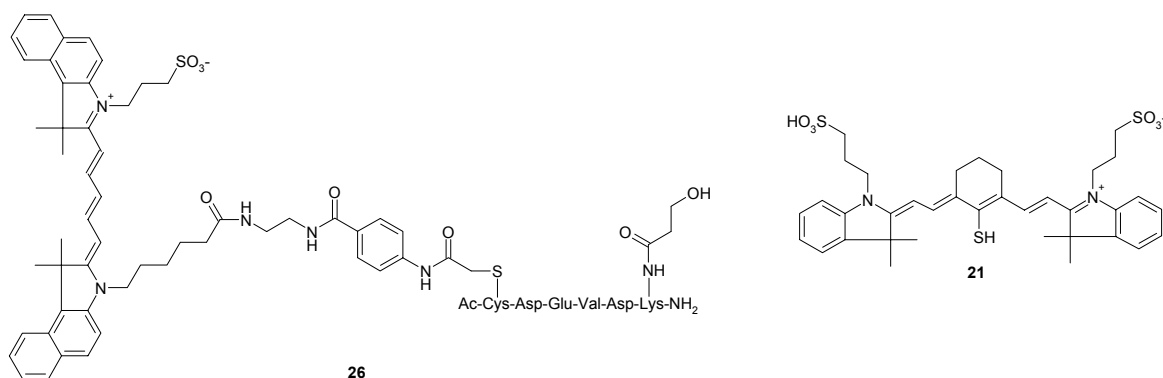
(c) Preparation of **NIR5.5-1** SIAB derivative **24**. **NIR5.5-1** amine **23** (9.6  $\mu\text{mol}$ ) was dissolved in a mixture of  $\text{CH}_3\text{CN}$  (250  $\mu\text{L}$ ) and borate buffer (140  $\mu\text{L}$ , 50 mM, pH 8.1). A solution of SIAB reagent (4.14 mg, 10.3  $\mu\text{mol}$ ) in a mixture of  $\text{CH}_3\text{CN}$  (400  $\mu\text{L}$ ) and borate buffer (350  $\mu\text{L}$ ) was added. The reaction mixture was protected from light and stirred at room temperature for 90 min. The reaction was checked for completion by RP-HPLC (system A1). Finally, the reaction mixture was quenched by dilution with aq. TFA 0.1% and purified by RP-HPLC (system B2, 2 injections). The product-containing fractions were lyophilised to give the thiol-reactive **NIR5.5-1** derivative **24** as a blue amorphous powder (9.8 mg, yield 95%). HPLC (system A1):  $t_{\text{R}} = 32.4$  min, purity > 95%. MS (MALDI-TOF, positive mode, CHCA matrix):  $m/z$  1020.88  $[\text{M}+\text{H}]^+$ , 1042.86  $[\text{M}+\text{Na}]^+$ , calcd exact mass for  $\text{C}_{53}\text{H}_{58}\text{IN}_5\text{O}_6\text{S}$  1020.05.

**Synthesis of Ac-Cys(NIR5.5-1)-Asp-Glu-Val-Asp-Lys(NIR7.0-2)-NH<sub>2</sub> (25).** Peptide Ac-Cys-Asp-Glu-Val-Asp-Lys(NIR7.0-2)-NH<sub>2</sub> **18** (3.4 mg, 2.3  $\mu\text{mol}$ ) was introduced into a Reacti-Vial<sup>TM</sup> and dissolved in 600  $\mu\text{L}$  of sodium bicarbonate buffer (0.1 M, pH 8.5). 400  $\mu\text{L}$  of a solution of iodoacetyl derivative **24** (0.8 mg, 0.77  $\mu\text{mol}$ ) in DMF was added. The reaction mixture was protected from light and stirred at room temperature for 2 h. The reaction was checked for completion by RP-HPLC (system A2) and purified by RP-HPLC (system B3, 3 injections,  $t_{\text{R}} = 30.0$  min). The product-containing fractions were lyophilised to give the peptide Ac-Cys(NIR5.5-2)-Asp-Glu-Val-Asp-Lys(NIR7.0-2)-NH<sub>2</sub> **25** as a blue-green amorphous powder. This fluorogenic caspase-3 substrate was found to be not soluble in aqueous buffers even after conversion into the triethylammonium salt. Consequently, stock solution of **25** was prepared in HPLC grade water (containing 10% DMSO) and UV-Visible quantification was achieved in EtOH at  $\lambda_{\text{max}}$  of the **NIR7.0-2** by using the  $\epsilon$  value 171 000  $\text{M}^{-1}\text{cm}^{-1}$  (yield after RP-HPLC purification: 24%). HPLC (system A2):  $t_{\text{R}} = 41.5$  min, purity > 95%. UV/Visible (water, 25°C):  $\lambda_{\text{max}} = 646$  (broad) and 801 nm. UV-Visible (EtOH, 25°C):  $\lambda_{\text{max}} = 682$  and 793 nm. MS (MALDI-TOF, positive mode, CHCA matrix):  $m/z$  1711.16  $[\text{M}+\text{H}]^+$ , 1734.16  $[\text{M}+\text{Na}]^+$ , 1753.13  $[\text{M}+\text{K}]^+$ , calcd exact mass for  $\text{C}_{85}\text{H}_{109}\text{N}_{13}\text{O}_{21}\text{S}_2$  1713.02.

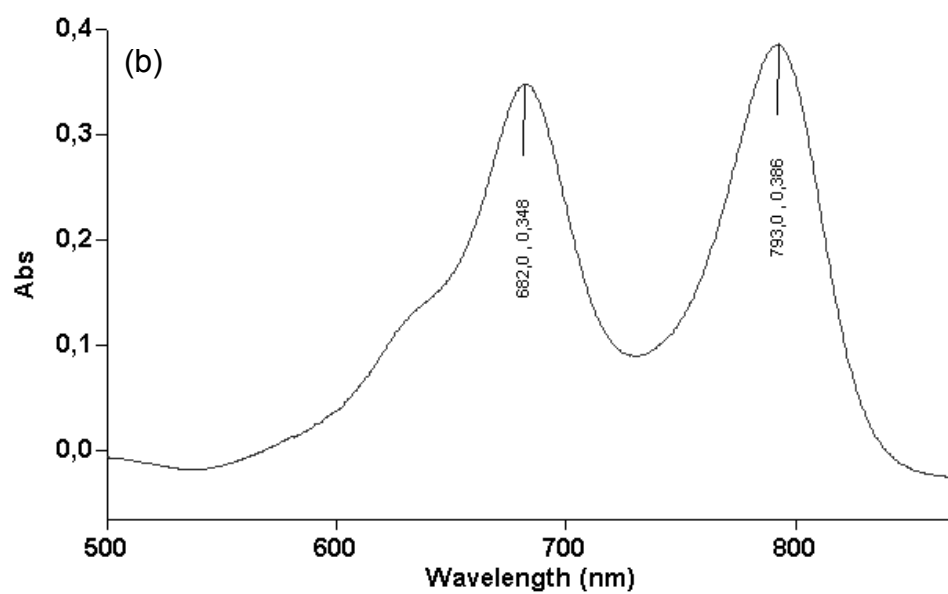
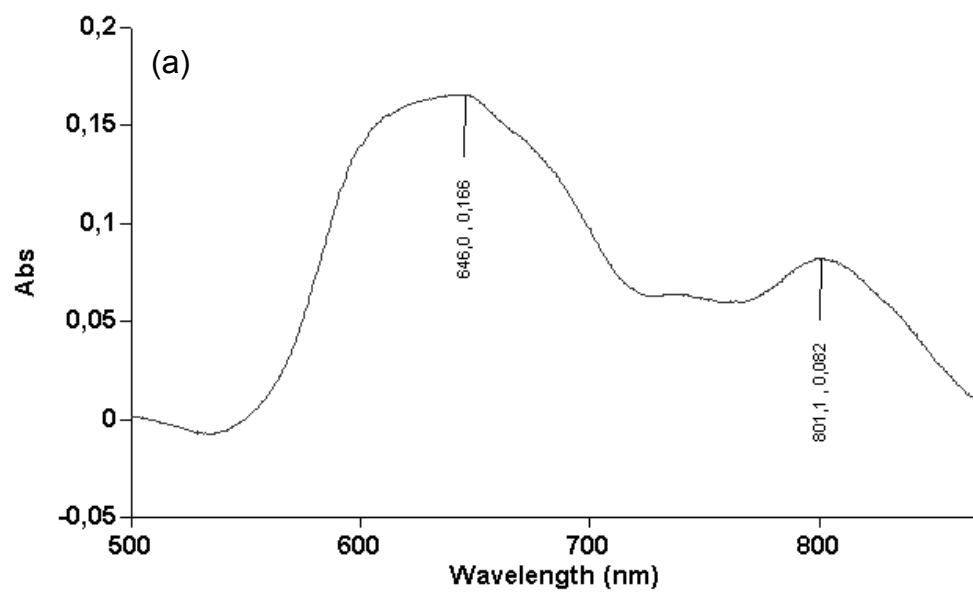
**-S2-** MALDI-TOF mass spectrum of the fluorogenic substrate of caspase-3 protease **25<sup>a</sup>**, in the positive mode,  $[M+H]^+$ :  $m/z$ : calcd  $C_{85}H_{109}N_{13}O_{21}S_2$  1713.02, found 1711.16. Structures of peptide **26** and thiol NIR dye **21**.



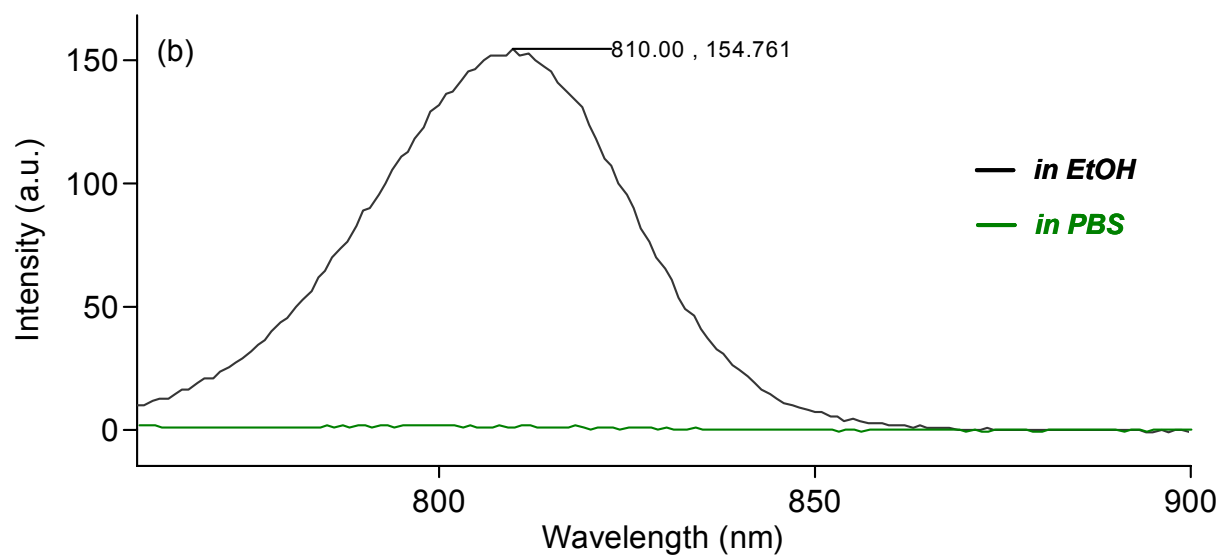
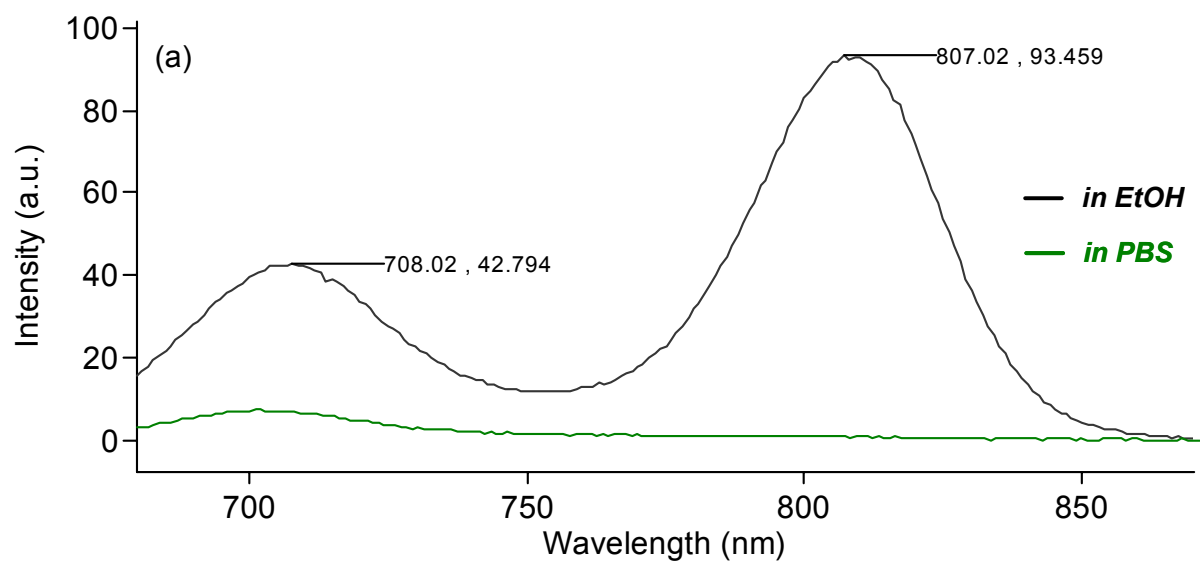
<sup>a</sup>Loss of thiol NIR dye **21** occurred during the ionisation process.



**-S3-** Absorption spectra of probe **25** at 25°C (concentration 2.2  $\mu\text{M}$ ). (a) in PBS. (b) in EtOH.



**-S4-** Emission spectra of probe **25** at 25°C (concentration 2.2  $\mu\text{M}$ ) (a) Ex.  $\lambda = 670$  nm (in PBS and EtOH). (b) Ex.  $\lambda = 750$  nm (in PBS and EtOH).



**-S5-** Emission spectra of probe **19** at 25°C (concentration 2.6  $\mu\text{M}$ ). Ex.  $\lambda = 780$  nm (in PBS and DMSO).

