Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

FRET-based Cyanine Probes for Monitoring Ligation Reactions and their Applications to Mechanistic Studies and Catalyst Screening

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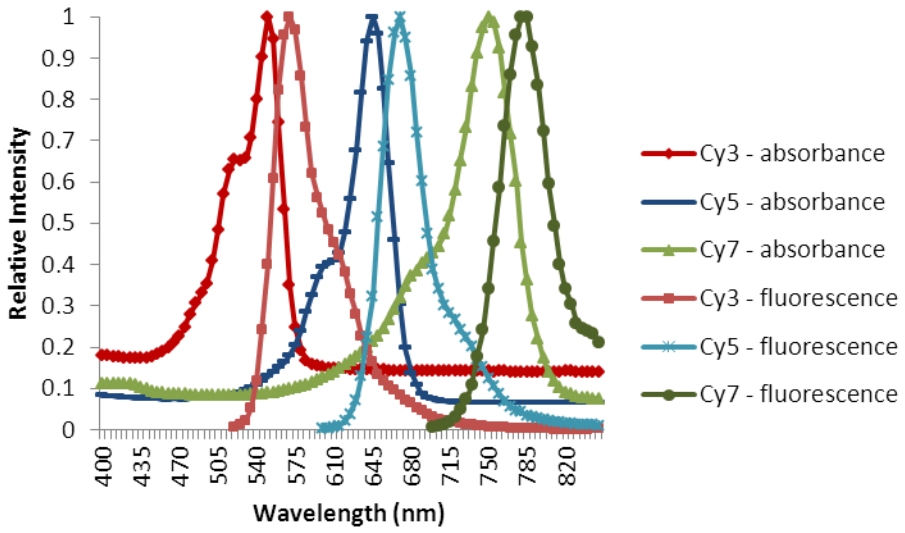
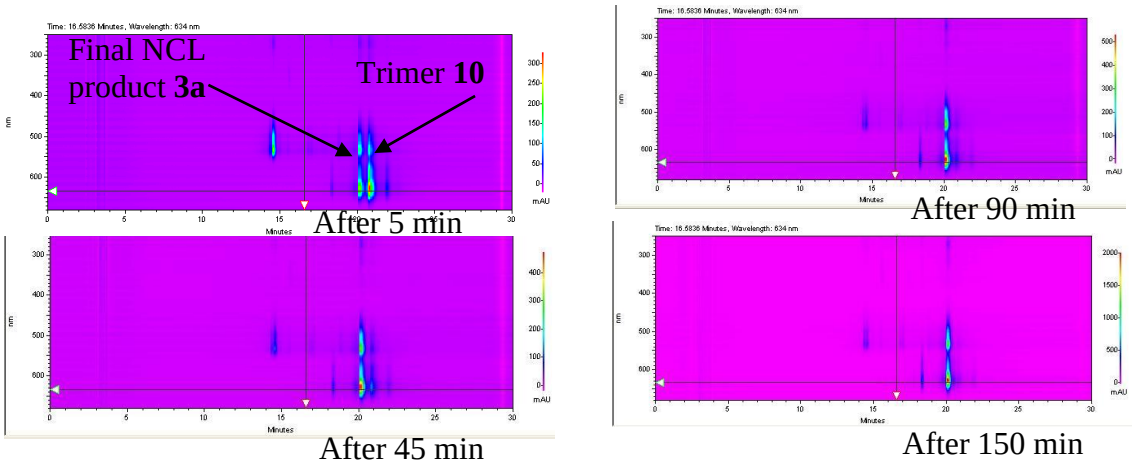
Supporting Information

Supporting figures

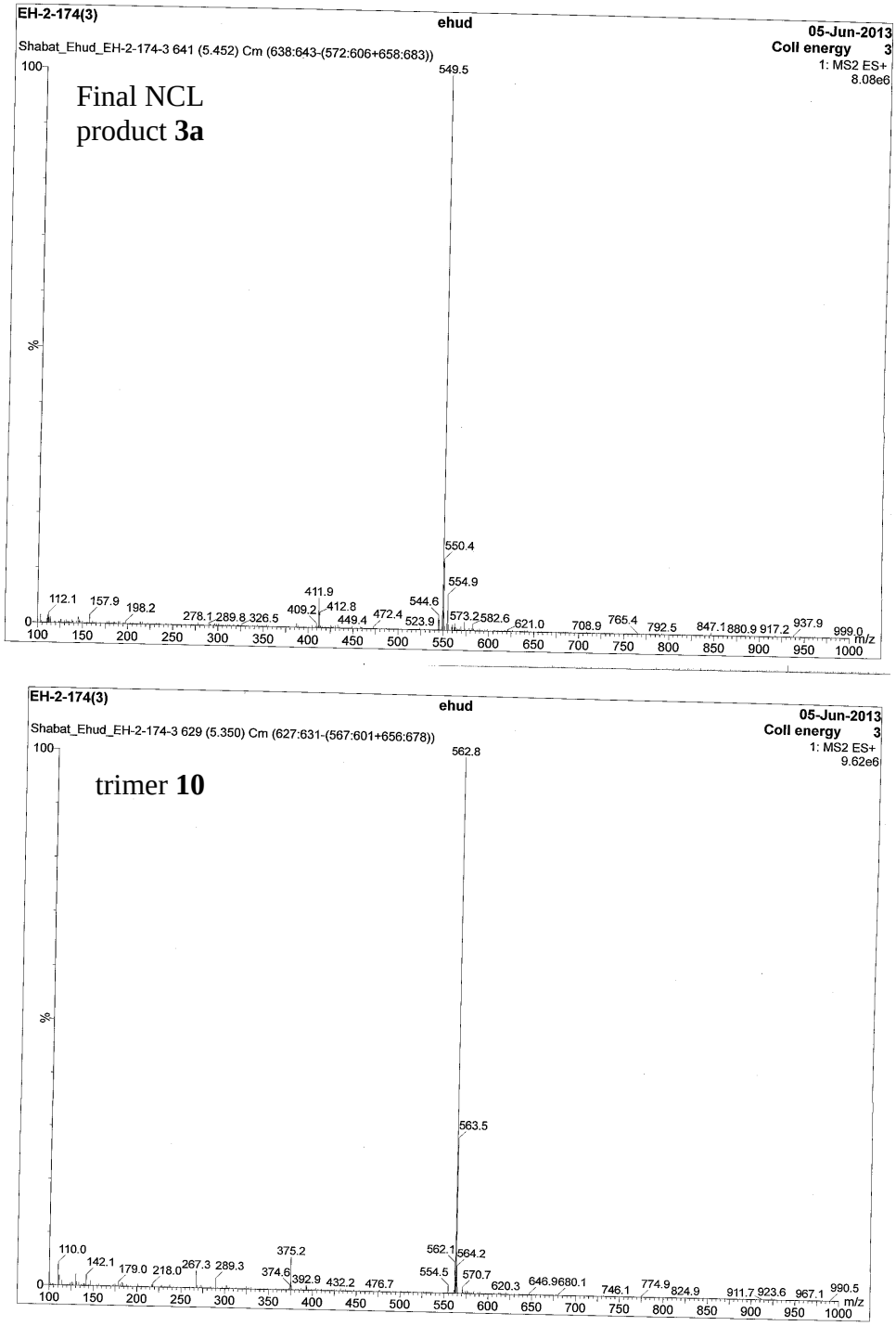


Supporting Figure 1. The absorption and emission spectra of Cy3, Cy5 and Cy7. All the maximum absorption and emission values were defined as 1 and the other values were normalized accordingly. The measurements were carried out in phosphate buffered saline (PBS) pH 7.4 with a 10 μM dye concentration. Compounds 12, 11a, 11b served as models of Cy3, Cy5, and Cy7 dyes, respectively.

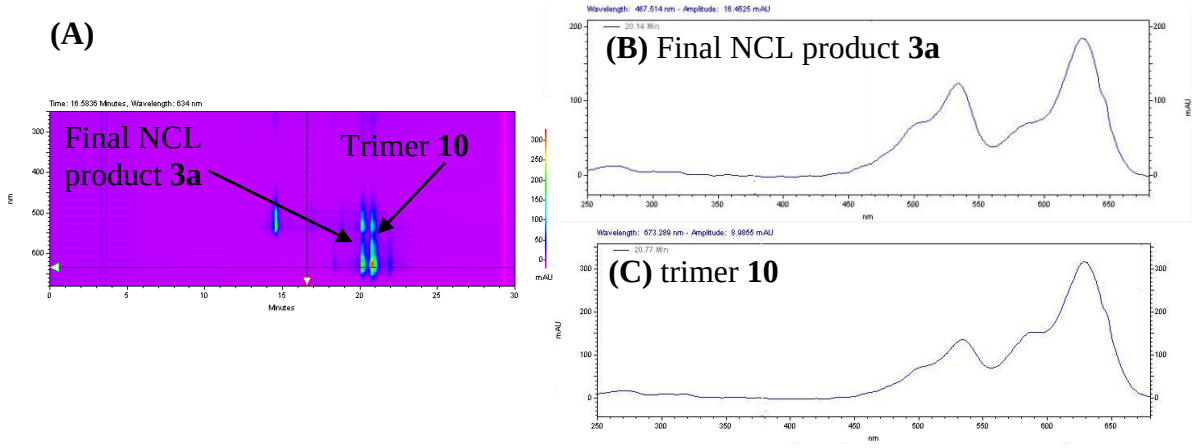


Supporting Figure 2. The HPLC three-dimensional chromatograms of the NCL reaction mixture between compound **1** and compound **2a** The absorbance scale of the compounds is indicated on the right of each chromatogram and the absorption wavelength is

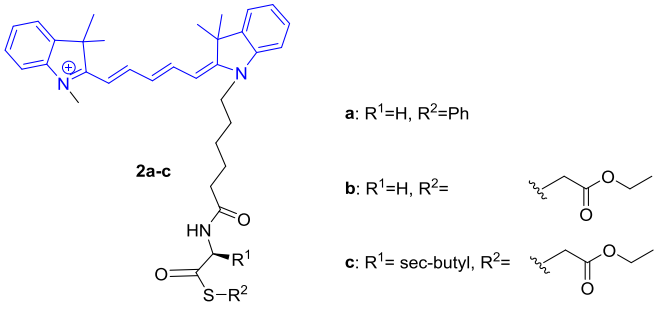
indicated on the left of each chromatogram The separation was performed on a C-18 RP-column (gradient: 10% to 90% ACN in 0.1% (v/v) TFA aqueous solution over 20 min).



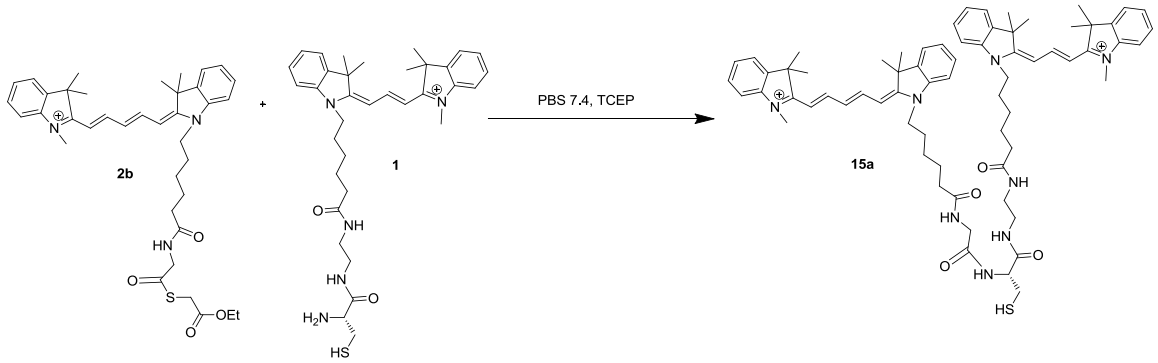
Supporting Figure 3. The MS spectra (ESI+) of the final NCL product **3a** and of trimer **10**. The separation was performed on a C-18 RP-column (gradient: 0% to 100% ACN in 0.1% (v/v) TFA aqueous solution over 12 min).



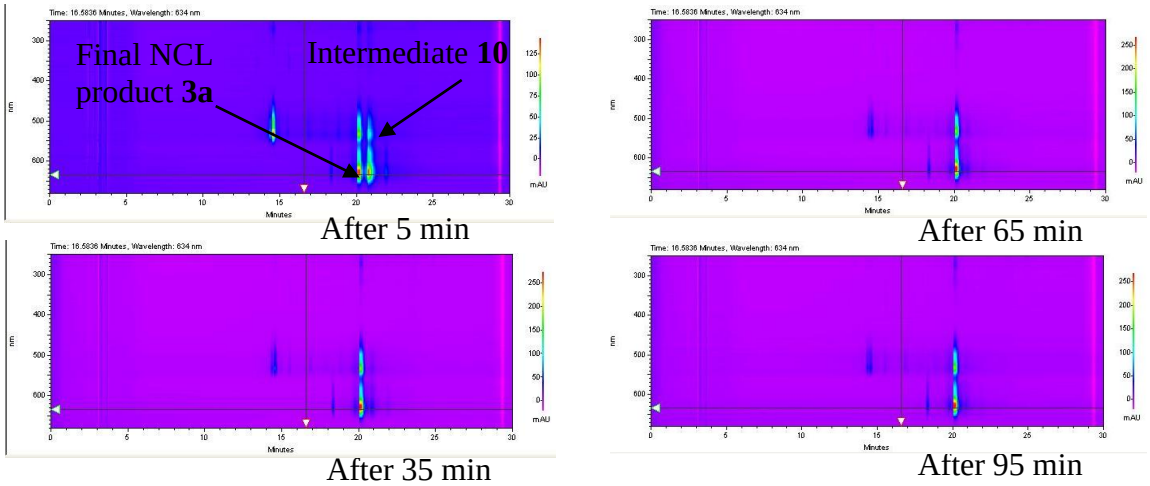
Supporting Figure 4. (A) The HPLC three-dimensional chromatogram after 5 minutes of the NCL reaction between compound **1** and compound **2a**. The NCL final product **3a** has a retention time of 20.1 minutes and trimer **10** has a retention time of 20.7 minutes**.** (B) The absorbance spectrum of the final NCL product **3a**. (C) The absorbance spectrum of trimer **10**. Trimer **10** presents a much higher 650 nm absorbance to 550 nm absorbance ratio than the final NCL product **3a**. The separation was performed on a C-18 RP-column (gradient: 10% to 90% ACN in 0.1% (v/v) TFA aqueous solution over 20 min).



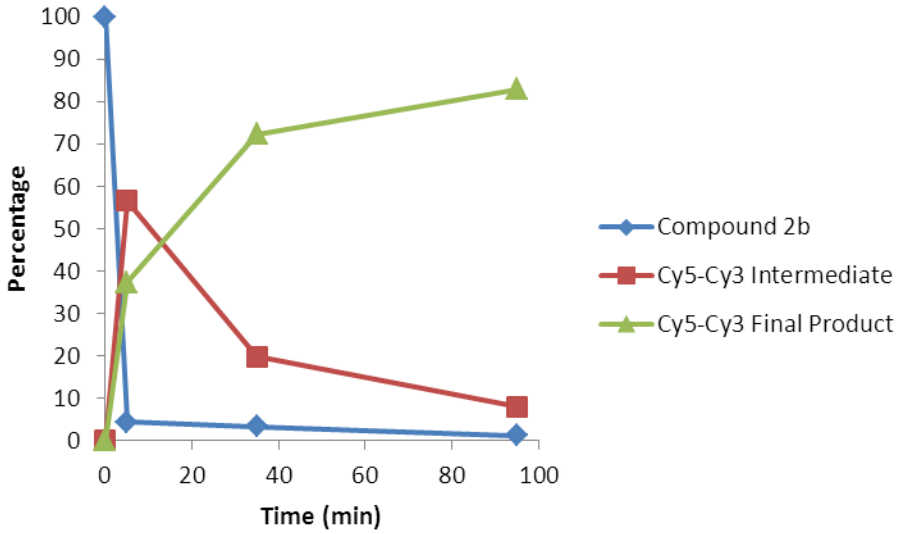
Supporting Figure 5. The chemical structure of compounds **2a-c**. The synthesis of **2b** and **2c** was performed in a similar manner to that of **2a** (see section 4.2. chemical synthesis).



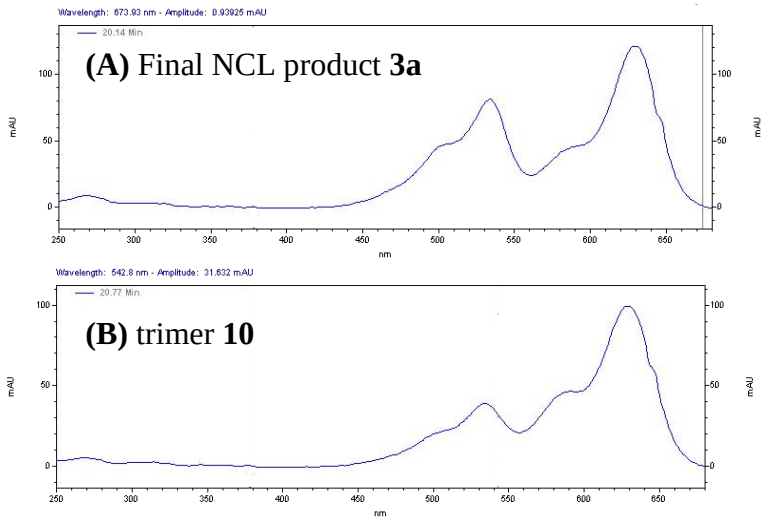
Supporting Figure 6. The NCL reaction between compounds **1** and **2b** to afford compound **3a**. Conditions: 50 μM of compound **1**, 50 μM of compound **2b** and 500 μM of TCEP in PBS (pH 7.4) with 7.5% DMSO (v/v), at room temperature. As with the ligation between compounds **1** and **2a,** an intermediate is formed in considerable quantities in the case of the reaction of compounds **1** and **2b** as well (Supporting Figures 7-9). As expected, the intermediate formed in the reaction between **1** and **2b** has the same retention time as trimer **10** and the final ligation product in this case has the same retention time as **3a** (Supporting Figure 8)**.** In a similar manner to trimer **10,** the intermediate formed in the reaction between compound **1** and compound **2a**, the intermediate formed in the reaction between **1** and **2b** also has a higher 650 nm to 550 nm absorption ratio than ligation product **15a** (Supporting Figure 9).



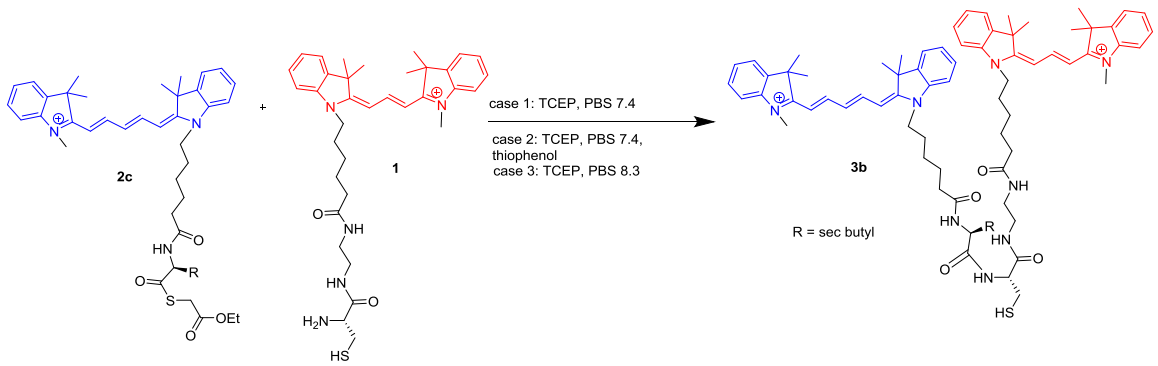
Supporting Figure 7. The HPLC three-dimensional chromatograms of the NCL reaction mixture between compound **1** and compound **2b** The absorbance scale of the compounds is indicated on the right of each chromatogram and the absorption wavelength is indicated on the left of each chromatogram The separation was performed on a C-18 RP-column (gradient: 10% to 90% ACN in 0.1% (v/v) TFA aqueous solution over 20 min).



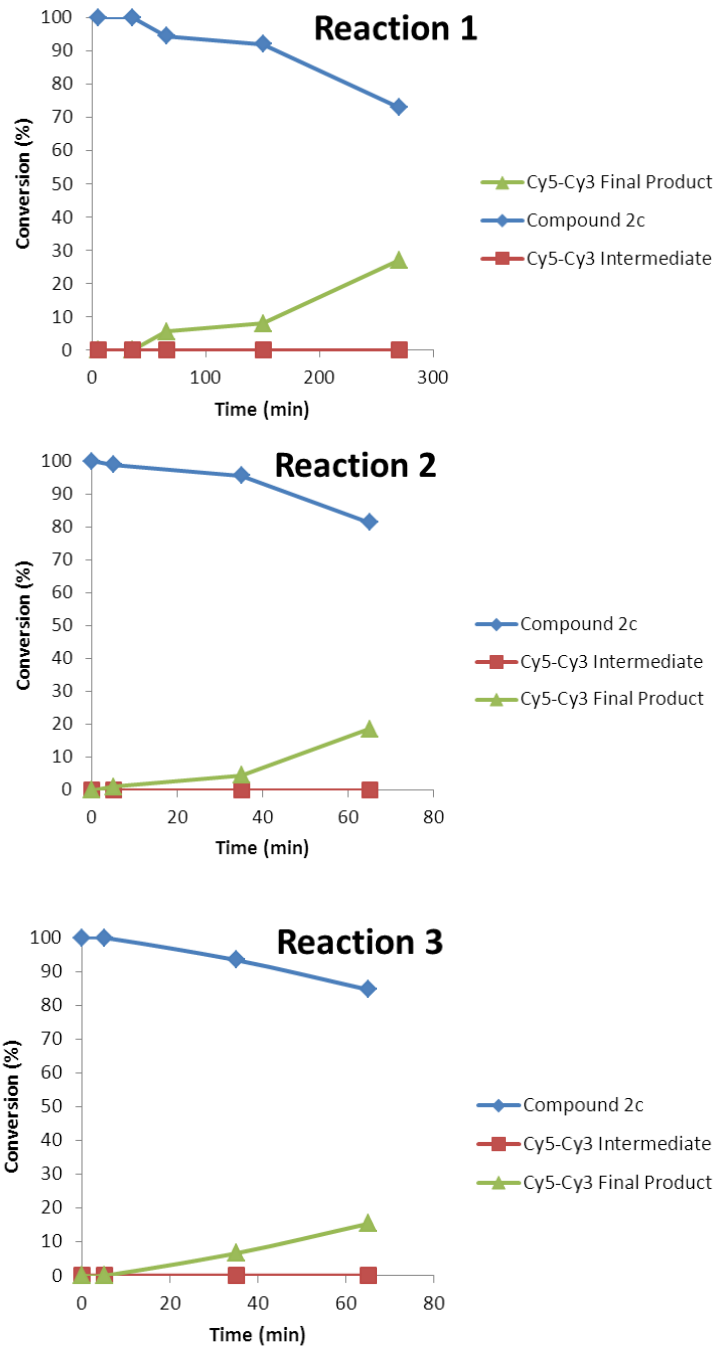
Supporting Figure 8. HPLC analysis of the NCL reaction between compound **1** and compound **2b** at the conditions mentioned in Supporting Figure 6. The percent conversion was measured by integrating the 650 nm absorption over the HPLC chromatogram. The separation was performed on a C-18 RP-column (gradient: 10% to 90% ACN in 0.1% (v/v) TFA aqueous solution over 20 min).



Supporting Figure 9. (A) The absorbance spectrum of the final NCL product **3a** (retention time 20.1). (B) The absorbance spectrum of trimer **10** (retention time 20.7). The separation was performed on a C-18 RP-column (gradient: 10% to 90% ACN in 0.1% (v/v) TFA aqueous solution over 20 min).



Supporting Figure 10. The NCL reaction between compounds **1** and **2c** to afford compound **3b**. Conditions: **Reaction 1** –50 μM of compound **1**, 50 μM of compound **2c** and 500 μM of TCEP in PBS (pH 7.4), with 7.5% DMSO (v/v), at room temperature; **Reaction 2** –50 μM of compound **1**, 50 μM of compound **2c**, 500 μM of thiophenol and 500 μM of TCEP in PBS (pH 7.4), with 10% DMSO (v/v), at room temperature; **Reaction 3** –50 μM of compound **1**, 50 μM of compound **2c** and 500 μM of TCEP in PBS (pH 8.3), with 7.5% DMSO (v/v), at room temperature.



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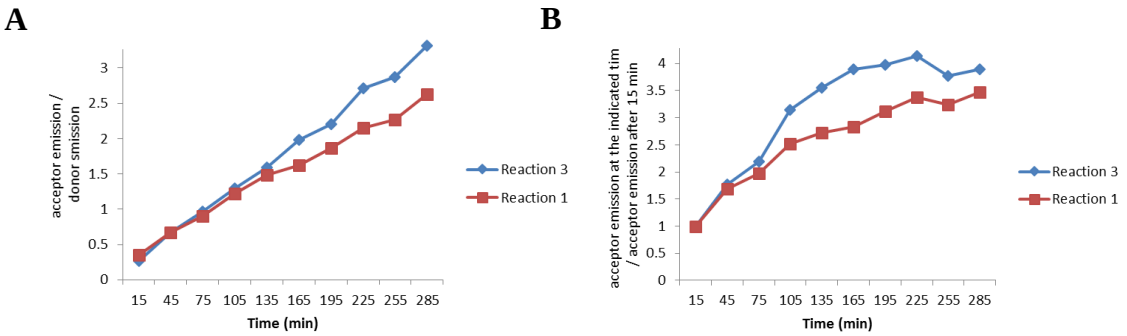
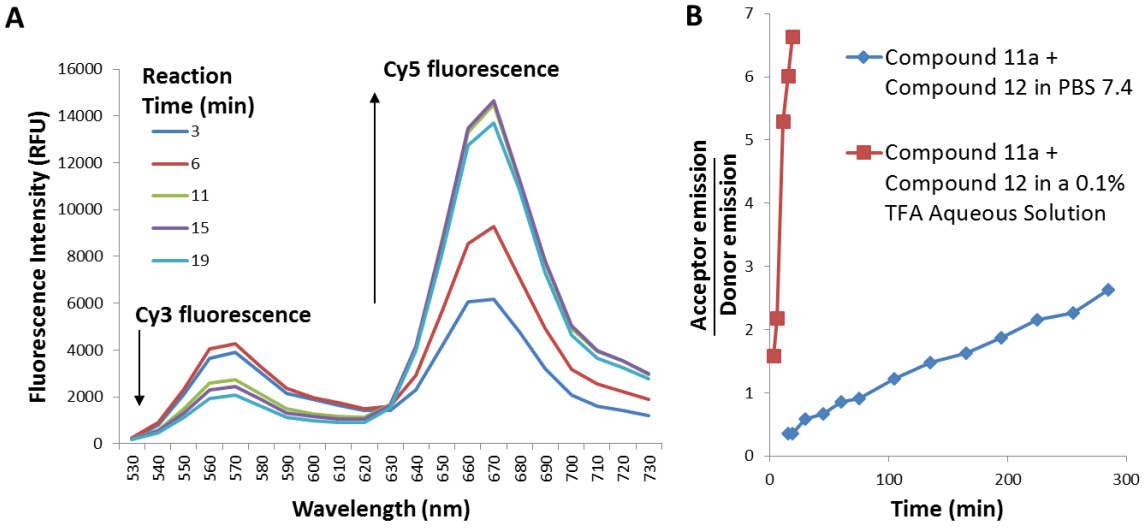
Supporting Figure 11. HPLC analysis of the NCL reaction between compound **1** and compound **2c**. Conditions: **Reaction 1** –50 μM of compound **1**, 50 μM of compound **2c** and 500 μM of TCEP in PBS (pH 7.4), with 7.5% DMSO (v/v), at room temperature; **Reaction 2** –50 μM of compound **1**, 50 μM of compound **2c**, 500 μM of thiophenol and 500 μM of TCEP in PBS (pH 7.4), with 10% DMSO (v/v), at room temperature; **Reaction 3** –50 μM of compound **1**, 50 μM of compound **2c** and 500 μM of TCEP in PBS (pH 8.3), with 7.5% DMSO (v/v), at room temperature. The percent conversion was measured by integrating the 650 nm absorption over the HPLC chromatogram. The

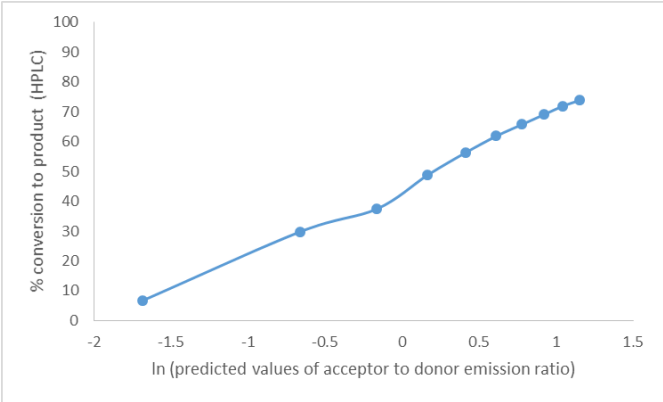
separation was performed on a C-18 RP-column (gradient: 10% to 90% ACN in 0.1% (v/v) TFA aqueous solution over 20 min).



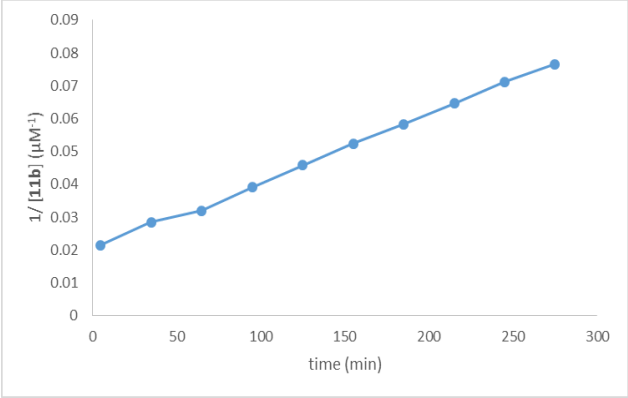
Supporting Figure 12. (A) Changes over time in the ratio of the acceptor emission (670 nm and 780 nm for Reactions 1 and 3, respectively) to the donor emission at 570 nm in the fluorescence spectrum of Reactions 1 and 3 (see Table 1 for the reaction conditions). (B) Changes over time in the ratio of acceptor emission (670 nm and 780 nm in Reactions 1 and 3, respectively) to the initial acceptor emission after 15 minutes.



Supporting Figure 13. (A) Changes over time in the fluorescence spectrum (λex = 460 nm) of Reaction 2. (B) Changes over time in the ratio of the emission at 670 nm to the emission at 570 nm in the fluorescence spectrum of Reaction 2 versus Reaction 1 (see Table 1 for the reaction conditions).



Supporting Figure 14. Correlation between the natural logarithm of the predicted values of acceptor to donor emission ratios, as predicted according to the linear regression formula y = 0.011*x + 0.130 (section 2.5) and the emission ratio data for Reaction 3 (Supporting Figure 12) and percent conversion to product as measured by HPLC with PBS 7.4 aqueous phase (Figure 9).



Supporting Figure 15. Change of 1 / [**11b**] (μM^{-1}) over time in Reaction 3 as measured by HPLC with a PBS 7.4 aqueous mobile phase (Figure 9). Since $[\mathbf{11b}]_0 = [\mathbf{12}]_0$ the second order equation expected for Reaction 3 is $\frac{1}{[\mathbf{11b}]} = kt + \frac{1}{[\mathbf{11b}]_0}$. A pearson linear correlation was calculated between $\frac{1}{[\mathbf{11b}]}$ and time to be r = 0.999 ($\rho < 0.001$).

The linear regression formula for predicting $\frac{1}{[\mathbf{11b}]}$ based on time values was calculated to be

$$\frac{1}{[11b]} = 3.442\ s^{-1} \times t + \frac{1}{[11b]_0}.$$

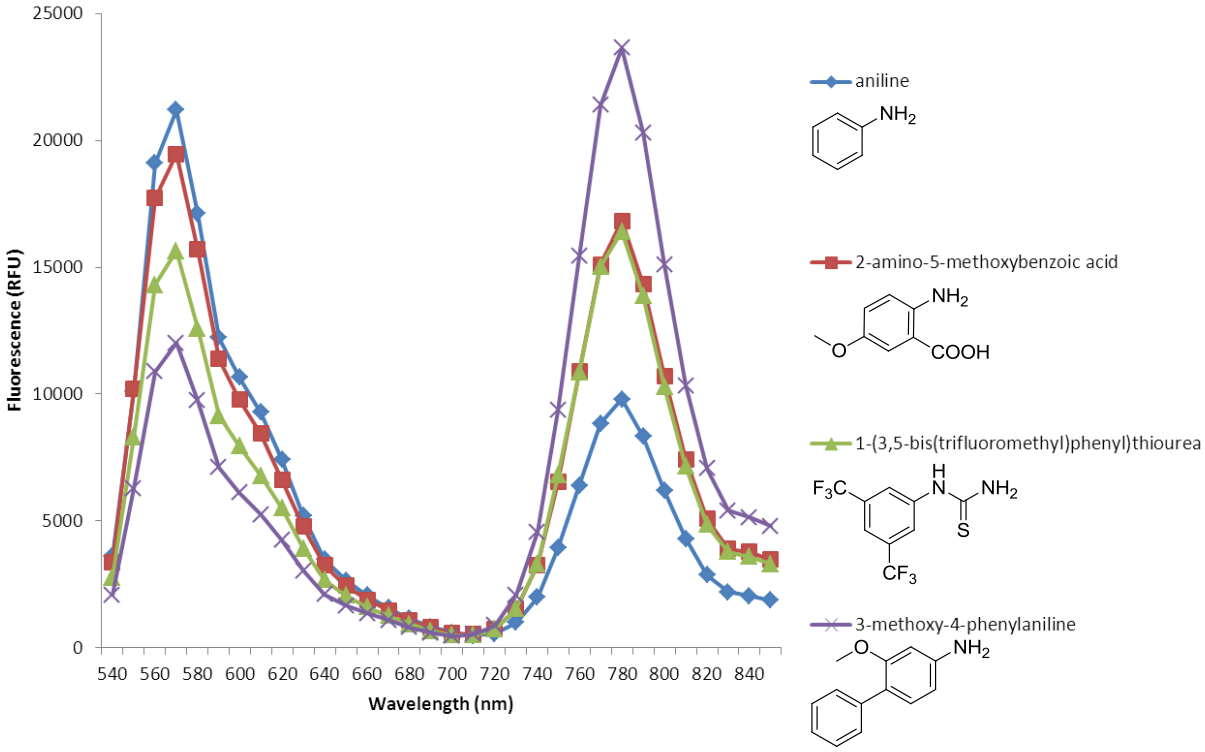
For Reaction 3:

$$\frac{1}{[11b]_0} = 20{,}000\ M^{-1}$$

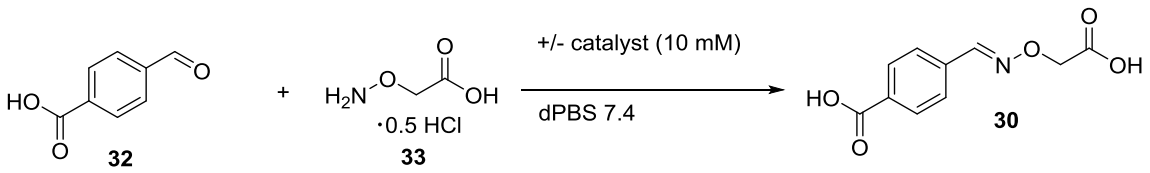
and therefore

$$\frac{1}{[11b]} = 3.442\ s^{-1} \times t + 20{,}000\ M^{-1}$$

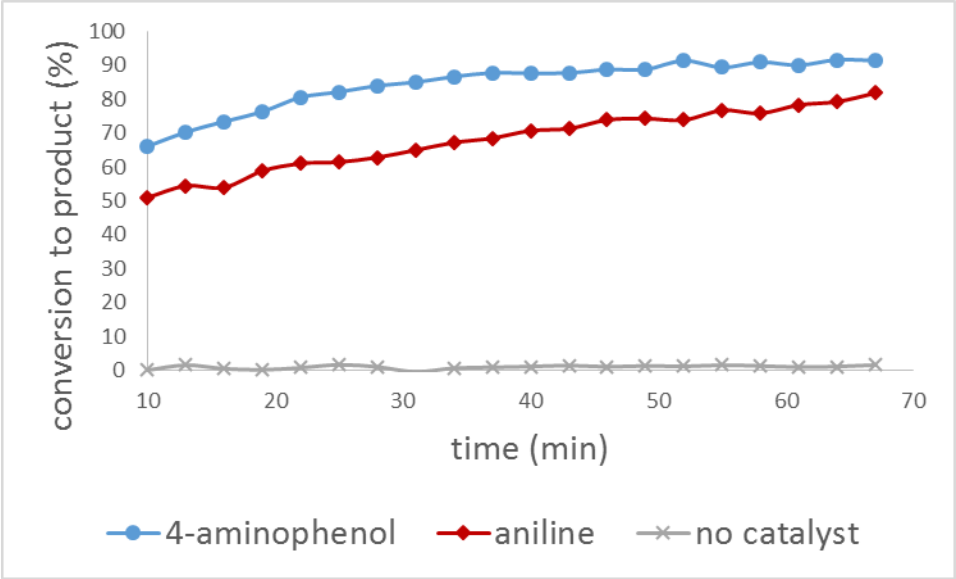
The reaction rate is therefore $k_{obs} = 3.442\ M^{-1}S^{-1}$.



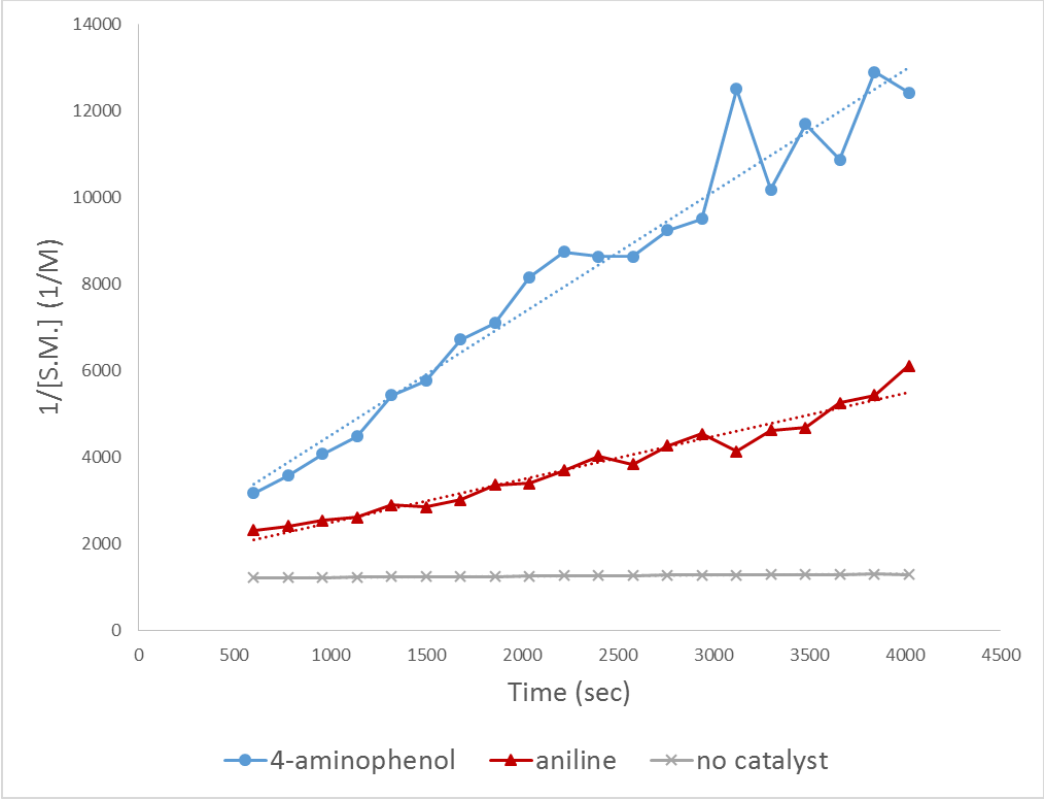
Supporting Figure 16. The fluorescence spectra (λex = 500 nm) of the reaction with selected catalysts after 120 minutes (see entries 1, 3, 5 and 7 in Table 2 for the reaction conditions).



Supporting Figure 17. Oxime bond formation between 4-formylbenzoic acid (compound **32,** 1 mM) and (aminooxy)acetic acid hemihydrochloride (compound **33,** 1 mM) in the presence of a) 10 mM 4-aminophenol b) 10 mM aniline c) no catalyst. The reactions were performed in deuterated PBS 7.4 (dPBS 7.4) with 7.5 % DMSO (V/V). Directly after mixing the reaction components the mixture was transferred to an NMR tube and placed on dry ice. The reaction was then thawed and the reaction progress was measured by ^{1}H-NMR with probe temperature set to 300.0 K.



Supporting Figure 18. Conversion to product over time in the oxime bond formation between 4-formylbenzoic acid and (aminooxy)acetic acid hemihydrochloride in the presence or absence of 10 mM catalyst (Supporting Figure 17). Conversion to product (%) was measured as the sum of integration value of the aromatic protons of oxime product **30** (δ = 7.88 and 7.69) divided by the sum of integration values for the aromatic protons of 4-formylbenzaldehyde and oxime product **30** (δ = 8.00, 7.88 and 7.69).



Supporting Figure 19. Change of 1 / S.M. (1 / [4-formylbenzoic acid] (M^{-1})) over time in the oxime bond formation reaction between 4-formylbenzoic acid (Compound **32**) and (aminooxy)acetic acid hemihydrochloride (Compound **33**) in the presence or absence of 10 mM catalyst (Supporting Figure 17). [4-formylbenzoic acid] was calculated relative to the concentration of DMSO at the same time point, following the formula ($Int_{aromatic}$-**32**/4)/(Int_{methyl}-DMSO/6)*1.056 M, where $Int_{aromatic}$-**32** is the integration of the aromatic protons of compound **32** (δ = 8.00) and Int_{methyl}-DMSO is the integration of the DMSO methyl protons (δ = 2.71). Since $[\mathbf{32}]_0 = [\mathbf{33}]_0$ the second order equation expected for the reactions is $\frac{1}{[32]} = kt + \frac{1}{[32]_0}$.

Pearson linear correlations were calculated between $\frac{1}{[32]}$ and time to be r = 0.976, 0.978, 0.985 for +4-aminophenol, +aniline and –catalyst, respectively ($\rho < 0.001$). K_{obs} was calculated based on the linear regression formula for predicting $\frac{1}{[32]}$ based on time values to be 2.6938 $M^{-1}S^{-1}$, 0.9968 $M^{-1}S^{-1}$ and 0.02449 $M^{-1}S^{-1}$ for +4-aminophenol, +aniline and -catalyst, respectively.