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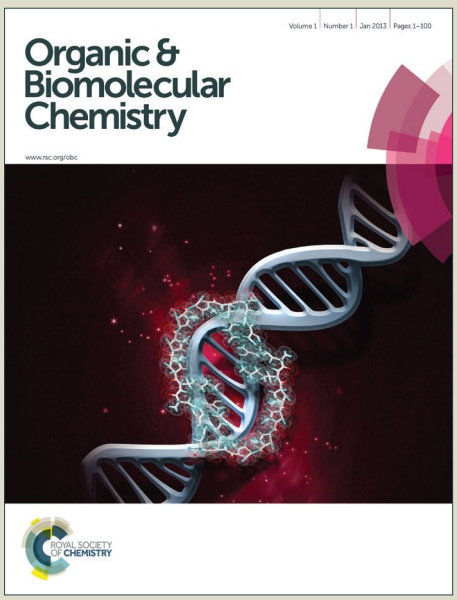

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ARTICLE

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

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There is an ever-increasing need to find better methods to selectively connect two molecules under mild aqueous conditions on small scale. The process of finding such methods significantly relies upon the employment of an appropriate assay. We report here a modular FRET-based assay to monitor such reactions and exemplify how the assay is used to monitor two particular reactions: native chemical ligation (NCL) and the oxime ligation. For both reactions we show that by employing appropriately designed probes FRET measurements could be used to monitor the reaction's progress. We additionally demonstrate the usefulness of the developed probe system to study the mechanism of the ligation reactions. For example in monitoring the formation of a trimeric intermediate in the NCL reaction. Finally, we demonstrate that FRET measurements conducted in our system allow the quantification of the reaction yield and we show the application of our FRET-based assay to catalyst screening for the oxime ligation.

Introduction

Reactions used to selectively connect two molecules in aqueous solutions under mild conditions are sought after for various applications in the continuously expanding field of chemical biology. For example, in order to monitor a biomolecule, such as a protein, a lipid or a nucleic acid in a living cell, a site-specific reaction is needed to connect the reporter molecule and the biomolecule.[1,2] Other common applications include conjugating two biomolecules, such as a protein and an oligonucleotide,[3] or connecting together two peptides,[4] with the promise of obtaining fully functioning synthetic enzymes.[5] However, site-specific and high yielding chemistry in biomolecules is very often challenging. For one reason, biomolecules frequently contain a rich variety of functional groups. Additionally, many biomolecules are unstable at high temperatures and harsh reaction conditions and often have limited solubility in both organic solvents and water. Also, biomolecules are often only available in very small quantities due to associated substantial cost and synthetic challenges. This limits the scope of transformations to connect such molecules together to reactions that could be carried out in low concentrations in aqueous conditions. The required low concentration therefore implies that 2nd-order reactions between such molecules have to be extremely rapid.

Existing reactions that are frequently used to selectively connect molecules in aqueous solutions under mild conditions are the native chemical ligation,[6] the [3 + 2] azide-alkyne (click) cycloaddition,[7,8] the Staudinger ligation,[9] and the oxime ligation.[10] However, these commonly employed synthetic ligation methods are of second order with a rate constant in the range of 10^{-3}-10^{3} $M^{-1}S^{-1}$, whereas biological linking methods, such as the biotin streptavidin interaction have rate constants as fast as 10^{6} $M^{-1}S^{-1}$.[11,12] The importance of ligation reactions in chemical biology research demands improved kinetics of current methods.[4]

The discovery of novel catalysts or alternative conditions for existing methods has the potential of improving the current ligations reactions. In order to access the vast majority of possible combinations of different catalysts and reaction conditions a high-throughput assay is needed. In the optimal case the assay would be modular, so that it could be easily adapted to improve many different ligation reactions. We therefore turned to develop such an assay and decided to exemplify its modularity and usefulness on two ligation reactions, native chemical ligation and the oxime ligation.

Native chemical ligation (NCL), the most commonly used peptide ligation method, is an efficient method to join together two unprotected polypeptides by an amide bond.[13] This reaction takes place between a peptide that contains a thioester fragment at its *C*-terminus and another peptide that bears a cysteine residue at its *N*-terminus. The oxime ligation presents another powerful way to link two molecules under physiological conditions in the µM range.[14,15] The oxime bond is formed by reacting a carbonyl, such as a ketone or an aldehyde, with an aminooxy nucleophile. Contrary to its imine counterpart, the oxime bond formation is irreversible under physiological conditions.[11,16] In order to achieve effective reaction rates, the oxime ligation requires the use of a catalyst when performed in physiological pH.[17] Since the original report of catalysis of the ligation of two peptide fragments by aniline and 4-methoxyaniline,[16] various aniline derivatives have been

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introduced as alternative oxime ligation catalysts.[17] Examples include 4-aminophenylalanine as a biocompatible aniline derivative,[18] and *m*-phenylenediamine, a highly water-soluble catalyst that could be introduced in high concentrations.[19]

The assay used to discover *m*-phenylenediamine as a catalyst for the oxime reaction was based on a fluorescence enhancement of the aminooxy-dansyl conjugate upon oxime bond formation with a hydrophobic aldehyde. In a different assay, nitrobenzoxadiazole (NBD) hydrazine, which is known to undergo a red shift when it forms a hydrazone, was used to discover catalysts for the hydrazone ligation.[20,21] However, catalysts that were found in this screen to outperform aniline in the hydrazone ligation did not necessarily outperform aniline in the oxime ligation. The substrate scope of both assays described above is inherently limited. The dansyl-based assay is limited to the use of hydrophobic electrophiles, while the NBD-based assay is limited to the use of the NBD hydrazine nucleophile. Thus, both assays do not allow the use of peptides and other common biomolecules employed in the biological applications of the oxime ligation.[17] A recent screening assay for oxime-ligation catalysts has a much wider potential substrate scope. However, the use of HPLC monitoring in this assay would challenge its use in high-throughput screening.[22]

The excitement about discovering new oxime ligation catalysts has prompted us to develop a FRET-based assay for monitoring ligation reactions. We envisioned this assay to be both modular and high-throughput. Every probe systems we designed contains two molecules, each of which includes a fluorophore moiety and an active functional group appropriate to the reaction we wished to study (Fig. 1). The separation between the fluorophore reporting moiety and the reacting moiety is the source of modularity in our system. Upon bond formation, a quantitative FRET signal can be conveniently measured by a spectrophotometer, thus providing an easy way of obtaining kinetic data for ligation reactions in various conditions. The fluorescence of many dyes, including cyanines, is influenced by various factors, such as dye concentration, photobleaching, and hydrophobic interactions.[23] We have therefore employed in this research ratiometric molecular probes. Such probes provide a built-in correction for these factors by detecting the fluorescence signal of both reacted and unreacted probes.[24] The use of near-IR dyes in the assay we present here would promote the assay's usefulness in studying ligation reactions *in vivo* in the future.[25,26]

Results and discussion

Organic synthesis

The probe system to monitor the native chemical ligation reaction includes Cy3 covalently bound to the amino acid cysteine (compound **1**), and Cy5 covalently bound to a thioester activated glycine (compound **2a**, Fig. 2). The synthesis of compounds **1** and **2a** is presented in Scheme 1. Activated ester **4** was reacted with glycine in the presence of Et_3N to form compound **5a**. The latter was further reacted with DCC and thiophenol to afford the desired product **2a**. Carboxylic acid

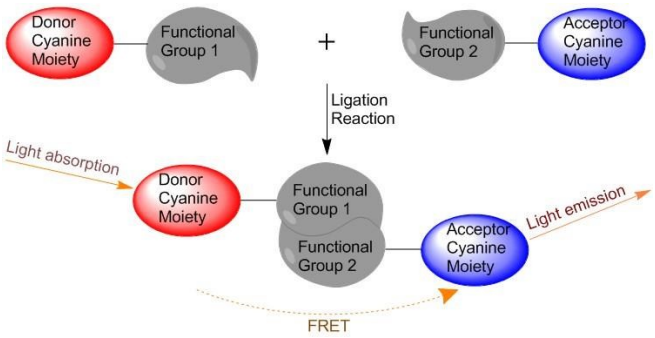


Fig. 1 A schematic illustration of a ligation reaction between two molecular probes and the resulting acceptor emission as a result of illumination at the donor absorption wavelength upon bond formation

derivative **6** was activated with DCC and NHS and the resulting activated ester was immediately reacted with compound **7**[27] to produce the protected amine **8**. The latter was deprotected with TFA and reacted with N-Boc-S-Trityl-L-cysteine, EDC, HOBt and Et_3N to afford the protected cysteine derivative **9**, which was deprotected to afford compound **1**. TCEP was added to all reactions of **1** in order to maintain its cysteine side chain in its reduced form. DMSO was added as a cosolvent to all ligation reactions to prevent dye aggregation.

Our probe system for monitoring the oxime ligation includes Cy3 covalently bound to the carbonyl moiety and Cy5 covalently bound to the aminooxy moiety (compounds **12** and **11a**, respectively, Fig. 6). We decided to additionally synthesize probe **11b** to evaluate if the use of a Cy3-Cy7 FRET pair would allow better signal to noise ratio than the use of the Cy3-Cy5 FRET pair.[28] We found that for our application the Cy3-Cy7 FRET pair performs as well as the Cy3-Cy5 FRET pair (Fig. S12†). The synthesis of compounds **11a-b** is presented in Scheme 2. Compounds **20** and **21** were synthesized in a similar manner to previously published procedures.[29] Compound **22** was prepared from compound **21** in a similar manner to the synthesis of compound **8** from compound **6**. Compound **22** was deprotected with TFA and reacted with the carboxylic acid derivative **16**,[30] EDC, HOBt and Et_3N to form compound **23**. The latter was deprotected with TFA to form the trifluoroacetate hydroxylammonium salt of **11a** that was used without further purification. The aminooxy moiety of compound **11a** is extremely nucleophilic and demands careful handling to prevent unwanted oxime bond formation reactions with trace carbonyl compounds, such as acetone and formaldehyde that could be also found in trace amounts of common laboratory solvents, or even in common laboratary plastic equipment.[31] Immediately after deprotection, a stock solution of 10 mM in DMSO was made for immediate use or for storage of up to 24 hours in -20 °C. Deprotection using lower concentrations of **23** led to a higher prevalence of side reactions (data not shown). We employed a more convergent route to the synthesis of compound **11b** by first introducing the protected amine functionality to the indolenine moiety in compound **24** and only then reacting it with compound **25.** The synthetic route to compounds **12** and **18a** is presented in Scheme 2. The synthesis of **28** was based on previously published procedures.[29] Compound **29** was deprotected with TFA and reacted with **17** and Et_3N to afford the desired compound **12**.

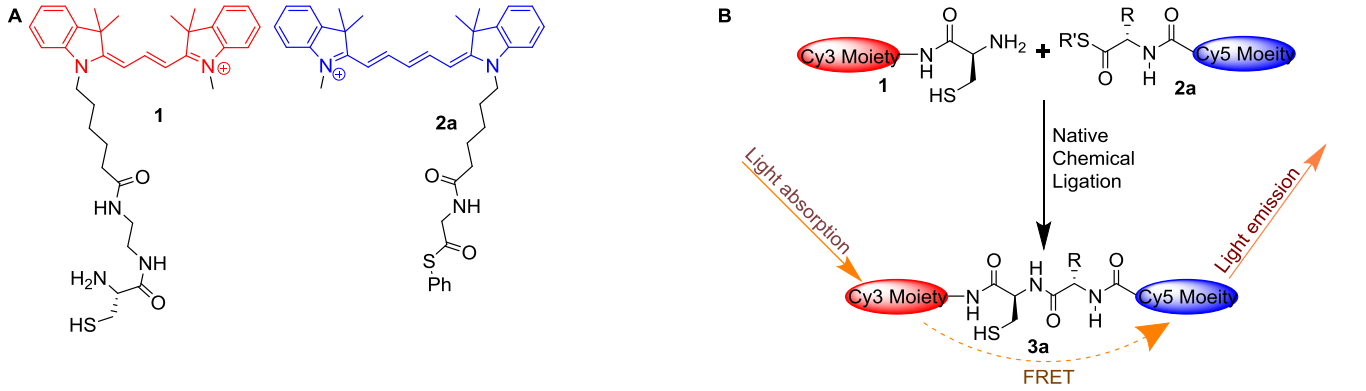
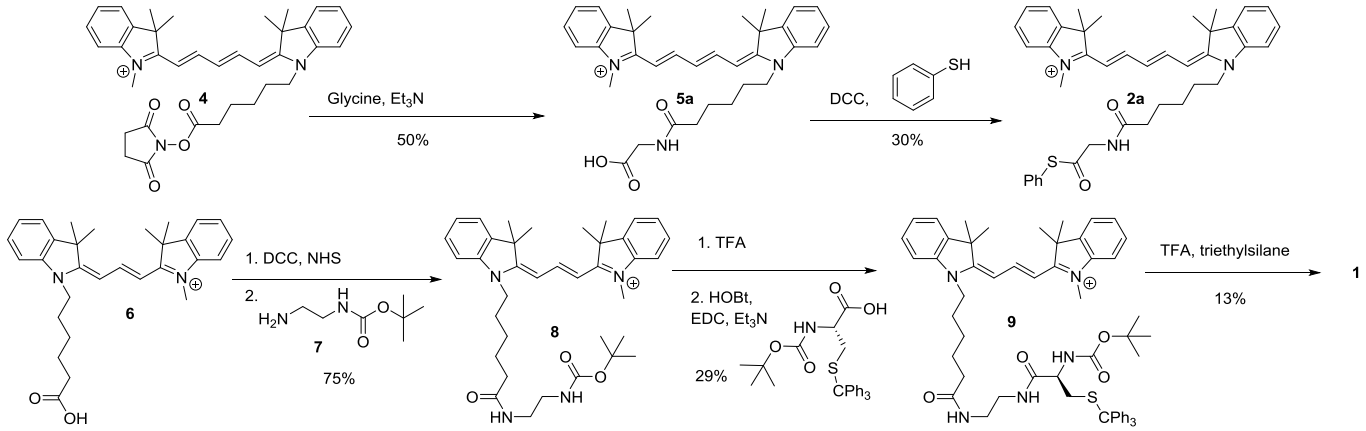


Fig. 2 (a) The chemical structure of the FRET-based probe system for monitoring the native chemical ligation. (b) A schematic illustration of a native chemical ligation reaction between two probes, such as **1** and **2a**



Scheme 1 The chemical synthesis of compounds **1** and **2a**.

Evaluation of the FRET-based probe system for monitoring the NCL reaction

The reaction of **1** and **2a** in PBS 7.4 leads to a fluorescent signal in the Cy5 emission spectrum upon excitation in the Cy3 absorbance spectrum when our NCL probes are used but not when unfunctionalized Cy3-COOH and Cy5-COOH probes are used in the same conditions (Fig. 3). This confirms our hypothesis that our probes could be used to monitor the NCL reaction by FRET.

Isolation and identification of trimer formation in the NCL reaction

Monitoring the progress of the reaction between **1** and **2a** by HPLC (Fig. 4 and Fig. S2†) shows that an intermediate is formed in considerable quantity before the formation of the final product in the reaction. We conducted LCMS experiments and identified two compounds that have both Cy3 and Cy5 absorption (550 nm and 650 nm, respectively). The experimental m/z values for the two compounds were 562.8 and 549.5 respectively (Fig. S3†). The 562.8 value corresponds to the calculated m/z value of 562.33 for compound **3a** ($[M]^{2+}$) – the ligation product of compounds **1** and **2a**. The 549.5 value corresponds to the m/z calculated value of 549.0 for trimer **10** ($[M]^{3+}$). The formation of this trimer could be reasoned by a transthioesterification reaction between compound **3a** and compound **2a** following the reaction of compound **1** and compound **2a** to form compound **3a** (Fig. 5).

Using an independent measurement, we wished to confirm the hypothesis that trimer **10** is the intermediate formed during the course of the NCL reaction between **1** and **2a**. Trimer **10**

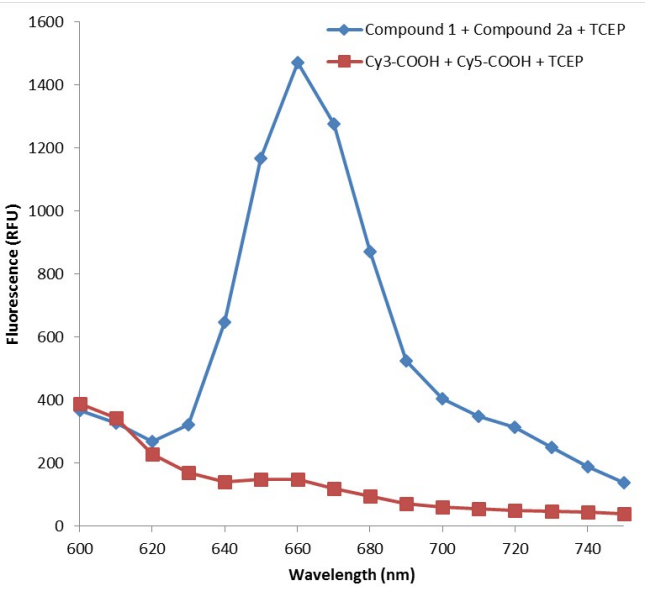
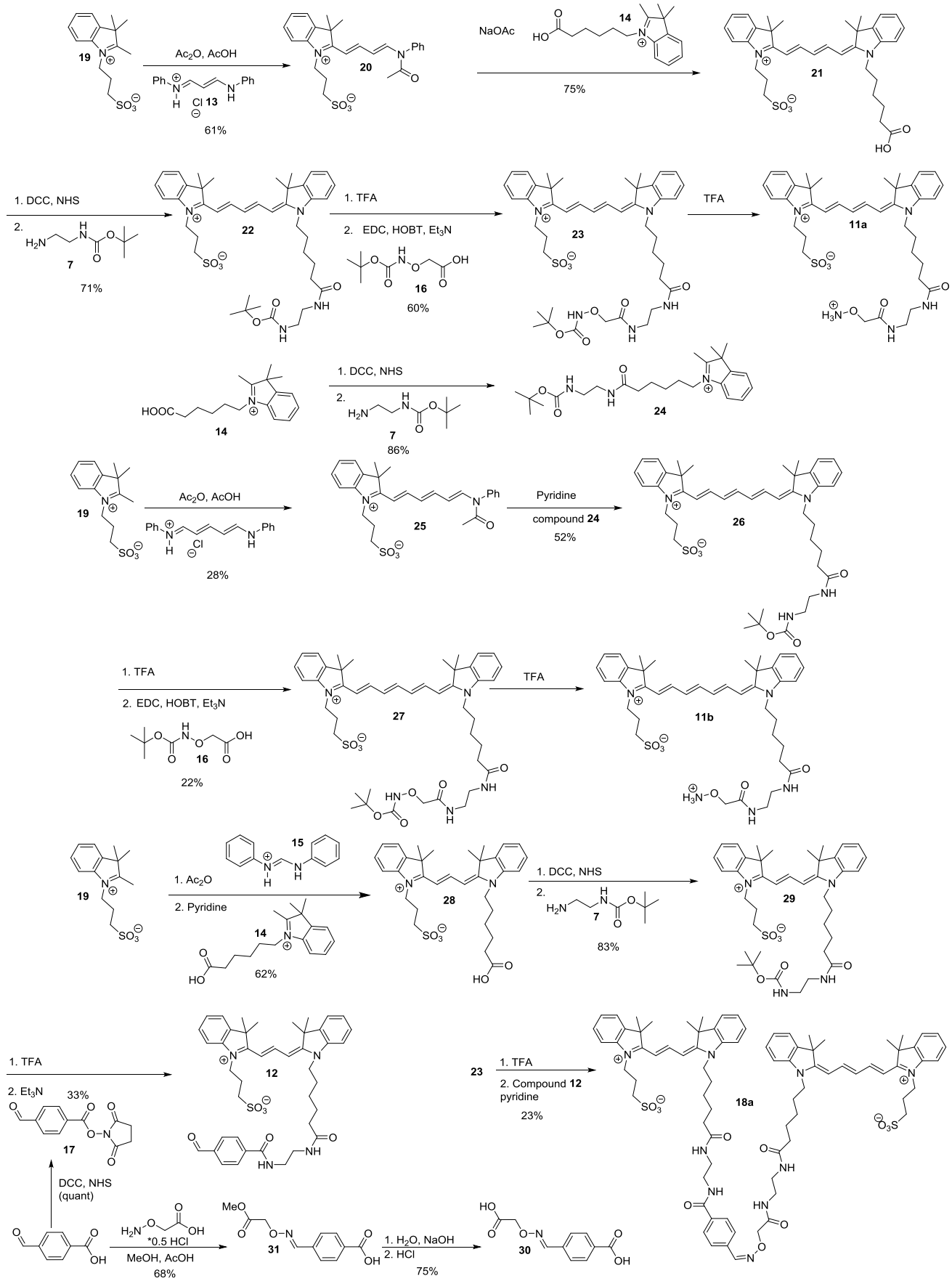


Fig. 3 Fluorescence (λex= 490 nm). Reaction conditions between compound **1** and compound **2a**: 50 μM of **1**, 50 μM of **2a**, and 500 μM of TCEP in PBS (pH 7.4) with 7.5% DMSO (v/v) at r.t. for 120 minutes. Negative control reaction conditions between Cy3-COOH and Cy5-COOH: 50 μM of Cy3-COOH (**6**), 50 μM of Cy5-COOH,[38] and 500 μM of TCEP in PBS (pH 7.4) with 7.5% DMSO (v/v), at r.t. for 120 minutes.



Scheme 2 The chemical synthesis of compounds **11a-b**, **12**, **18a** and **30**

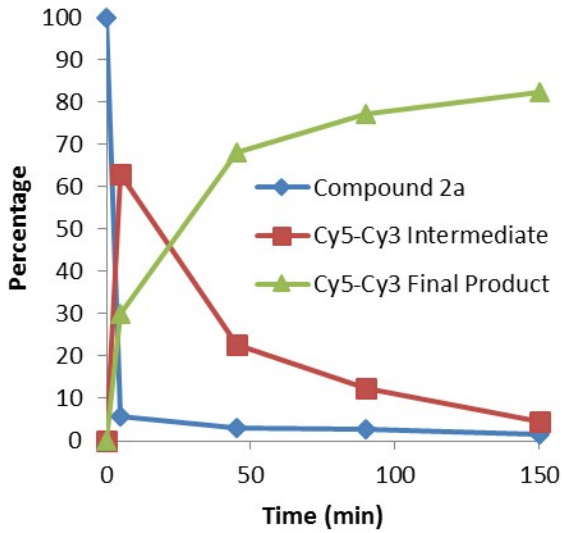


Fig. 4 Quantification of relative Cy5 absorption across the HPLC chromatograms of the NCL reaction between compound **1** and compound **2a**. Conditions: 50 µM of compound **1**, 50 µM of compound **2a** and 500 µM of tris(2-carboxyethyl)phosphine (TCEP) in PBS (pH 7.4) with 7.5% DMSO (v/v), at room 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).

contains two Cy5 moieties and one Cy3 moiety, whereas the ligation product **3a** has one Cy3 moiety and one Cy5 moiety. Thus, the ratio of 650 nm absorption to 550 nm absorption is expected to be significantly higher in trimer **10** than in the ligation product **3a**. Indeed, we observed the expected difference at the 2D HPLC chromatogram (Fig. S4†). The ratio between the absorption peak area of Cy5 (650 nm) to the absorption peak area of Cy3 (550 nm) was 35% higher in trimer **10** than in the final NCL compound **3a**.

According to previously published studies,[32,33] changing the phenolate leaving group to an aliphatic thiolate leaving group is expected to slow down the transthioesterification stage of NCL. A rate reduction of greater magnitude is expected for changing the amino acid side chain of the C-terminal amino acid to a bulkier one. We hypothesized that slowing down the transthioesterification stage would reduce the prevalence of a trimer such as **10**. We therefore synthesized compounds **2b** with an aliphatic thiolate leaving group, and compound **2c** with an aliphatic thiolate leaving group as well as an isoleucine as the C-terminus amino acid (Fig. S5†). We measured the reaction progress of compounds **2b** and **2c** with compound **1** by HPLC (Fig. S6-S9†). Surprisingly, compound **2b** reacted with compound **1** in a comparable rate to the case of compound **2a**. However, as expected, the reaction between compound **2c** and **1** was significantly slower than in the cases of **2b** and **2a**. Trimer **10** formed in the reaction of **2b** and **1** (Fig. S7-S9†). In accordance with our hypothesis, no trimer formation was identified in the reaction of **2c** and **1** in various reaction conditions (Fig. S10, S11†). Unproductive internal thioester formation was previously reported to form between an internal cysteine residue and a *C*-terminal thioester and to slow down reaction rates.[34,35] However, to the best of our knowledge, we show here the first characterization of such an internal thioester formation in the NCL reaction.

Evaluation of the FRET-based probe system for monitoring the oxime ligation

The oxime ligation was performed between compound **11a** and compound **12** (Fig. 6, Table 1) and the change over time in the fluorescence spectrum of the reaction was measured (Fig. 7). We observed a gradual quenching of the fluorescence of Cy3 molecules, and a gradual increase in the Cy5 fluorescence, as could be expected with gradual formation of product **18a.** This pattern of change in fluorescence allowed the probes to be used as ratiometric probes, and thus provided the desired built-in correction for factors that affect the fluorescence. The structure of the oxime conjugate **18a** was ascertained by a larger scale synthesis (Scheme 2), followed by NMR and mass spectroscopy. This shows that the oxime ligation could be monitored on small-scale by conducting FRET measurements.

Insights from the correlation between the FRET- and the HPLC-monitoring of the oxime ligation

In order to estimate percent conversion to product based upon the easily conducted FRET-measurements, efforts to correlate the FRET measurements of the reaction progress with HPLC measurements of the reaction progress followed. In initial HPLC measurements with 0.1 % TFA as the aqueous mobile phase, the conversion of **11b** to product was shown to exceed 90% after 5 minutes only (Fig. 8). We suspected that the seemingly rapid reaction progress might result from acid catalysis of oxime bond formation by the 0.1% TFA aqueous phase (pH 2) as the oxime ligation reaction rate is known to be enhanced in acidic conditions.[16] Indeed, replacing the 0.1% TFA aqueous mobile phase in the HPLC measurements of Reaction 3 to PBS 7.4 has led to a much slower measured rate of conversion to product

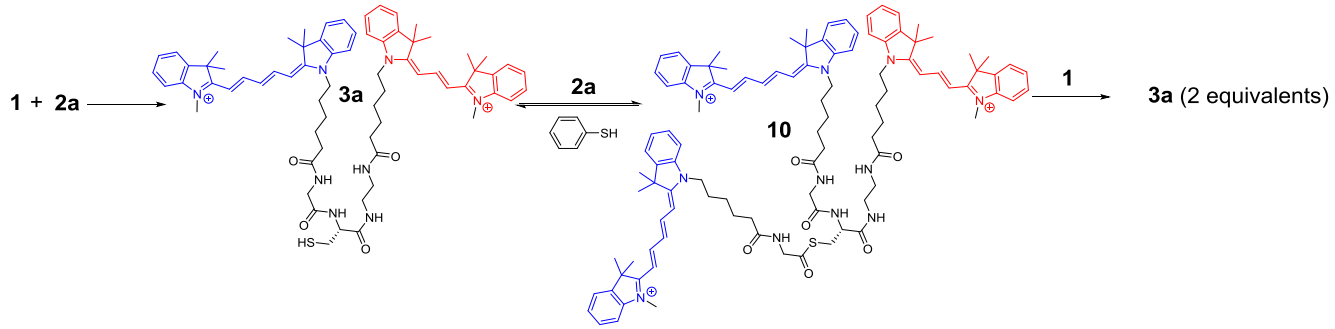


Fig. 5 The suggested reaction sequence leading to the NCL product **3a** through trimer **10**

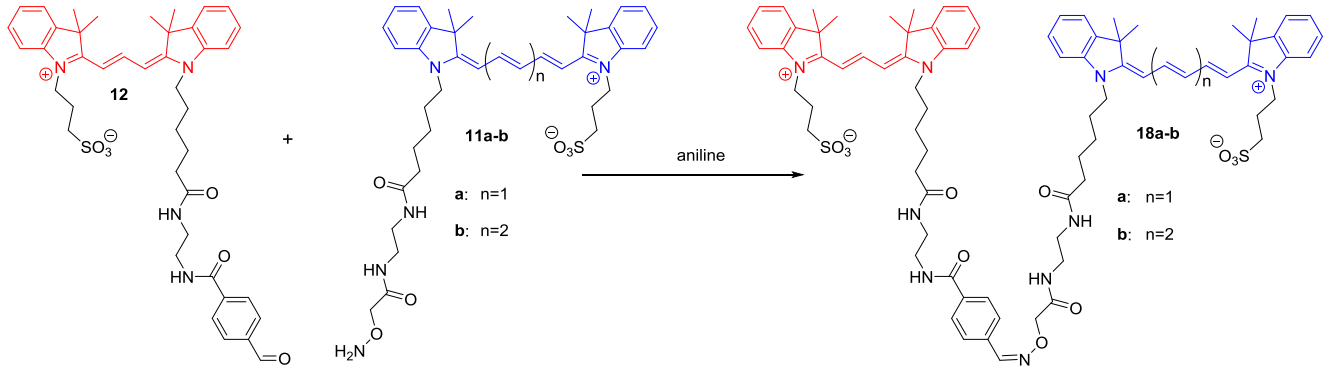


Fig. 6 The oxime ligation reaction between compounds **12** and **11a** or **11b** to afford compound **18a** or **18b**, respectively. Conditions: see Table 1.

Table 1 Reaction conditions for oxime ligation reactions between compound **11a** or **11b** and compound **12**. All the reactions were performed at room temperature with 5 mM aniline and 7.5% DMSO (v/v).

Reaction Number	Solvent	Conc. of **11a**	Conc. of **11b**	Conc. of **12**
1	PBS (pH 7.4)	50 μM	-	50 μM
2	0.1% (v/v) TFA in H_2O	50 μM	-	50 μM
3	PBS (pH 7.4)	-	50 μM	50 μM

(Fig. 8). The rate of Reaction 3, k_{obs} = 3.442 $M^{-1}S^{-1}$, was calculated according to the HPLC monitoring of the reaction (Fig. 8, Fig. S15†). We confirmed the rapid oxime bond formation in a 0.1% TFA aqueous solution by comparing the change in the Cy5 / Cy3 fluorescence ratio over time as measured when the reaction is performed in 0.1% TFA with the change in the same fluorescence ratio over time when the reaction is performed in a PBS 7.4 aqueous solution (Fig. S13†). Our measurements show that at least for some reactants the HPLC measurement of progress of the oxime ligation reaction is not informative when a 0.1% (v/v) TFA aqueous mobile phase is employed. This acidic aqueous solution is commonly employed for studies of the oxime ligation reaction.[16,11,36,37] Therefore, further research should be conducted to examine if the oxime ligation reaction of other reactants is also enhanced in HPLC runs employing 0.1% (v/v) TFA aqueous mobile phase. It is possible that the effect of acid catalysis by the HPLC mobile aqueous phase is more pronounced in faster reactions, such as the one between our probes, which has a higher rate constant compared to other oxime ligation reactions (e.g., k_{obs} = 3.442 M^{-1} S^{-1} vs K_{obs} = 0.061 M^{-1} S^{-1}).[16]

After establishing an appropriate HPLC assay to monitor the reaction we conducted a correlation analysis between the HPLC- and the fluorescence-monitoring of the reaction

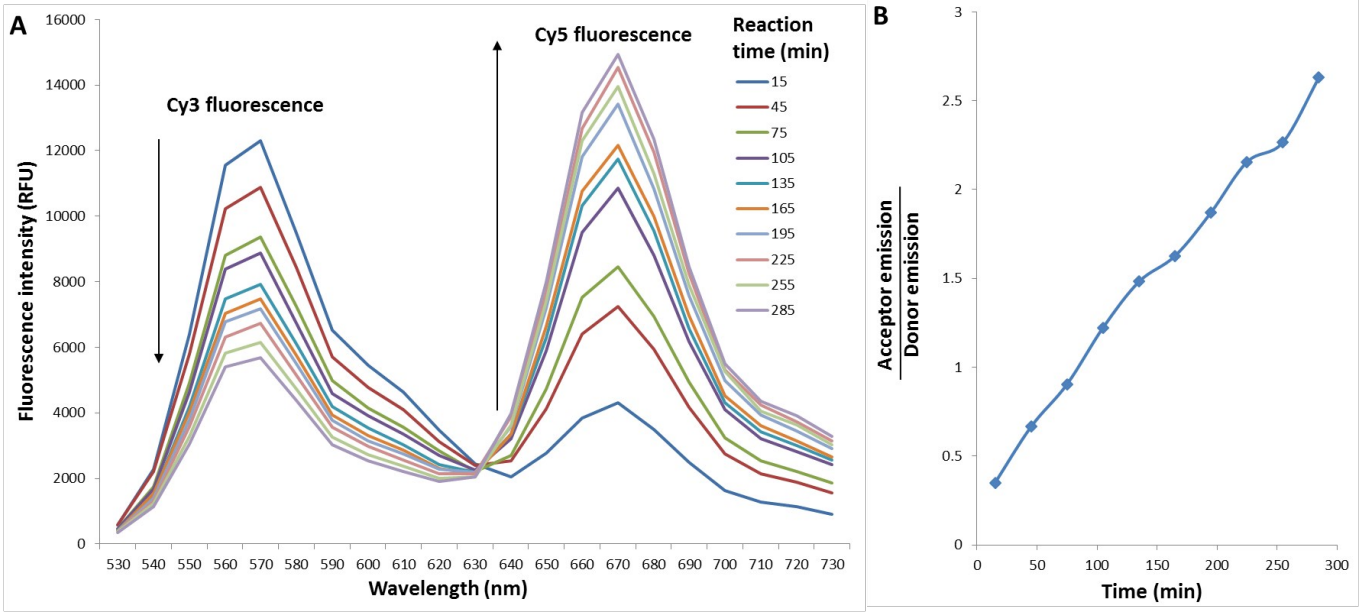


Fig. 7 (A) Changes over time in the fluorescence spectrum (λex = 460 nm) of Reaction 1 (see Table 1 for the reaction conditions). (B) Changes over time in the ratio of the emission at 670 nm (Cy5 emission) to the emission at 570 nm (Cy3 emission) in the fluorescence spectrum of Reaction 1 (see Table 1 for the reaction conditions).

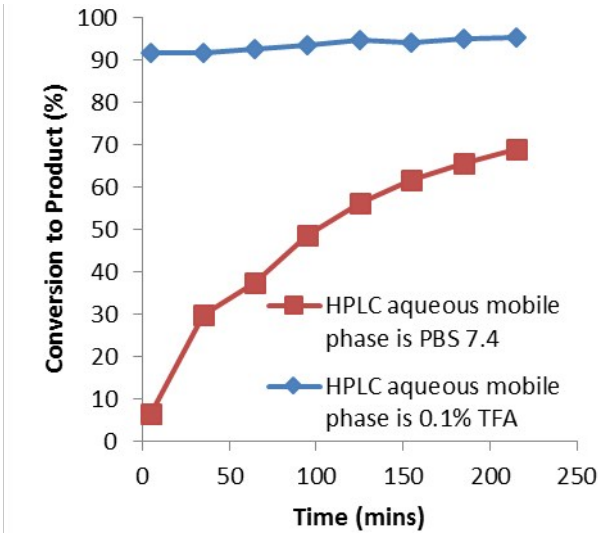


Fig. 8 The conversion to product over time in the oxime ligation Reaction 3 between compound **12** and compound **11b** (see Table 1 for the reaction-conditions), as measured by HPLC. The percent conversion to product was measured by performing integration of the absorbance signal of the product **18b** and the reactant **11b** at λ=750 nm and dividing the value obtained for the product by the sum obtained for both the product and the reactant. The separation in the case of a 0.1% (v/v) TFA – HPLC aqueous mobile phase was performed on a C-18 RP-column (gradient: 10% to 90% ACN in 0.1% (v/v) TFA aqueous solution over 20 min. The separation in the case of a PBS 7.4 – HPLC aqueous mobile phase was performed on a C-18 RP-column (gradient: 5% to 70% ACN in a 20 mM PBS 7.4 aqueous solution over 20 min).

progress. To begin with, a Pearson product-moment correlation coefficient (Pearson's r) was calculated between time and the acceptor to donor emission ratio for Reaction 3 (Fig. S12†). There was a positive correlation between the variables with r = 0.998 ($\rho < 0.001$). A linear regression equation for predicting acceptor to donor emission ratios based on given time value was calculated to be y = 0.011*x + 0.130. Based on this equation, the acceptor to donor emission ratio was predicted for 30 minutes interval values, starting at 5 minutes. A second Pearson product-moment correlation coefficient was calculated for Reaction 3 between the natural log of the predicted acceptor to donor emission ratio and the percent conversion to product that was measured by HPLC with PBS 7.4 as the aqueous mobile phase (Fig. 8). There was a positive correlation between the variables with r = 0.997 ($\rho < 0.001$, Fig. S14†). The linear regression formula to predict percent conversion to product based on the natural logarithm of the acceptor to donor fluorescence ratio was calculated to be y = 24.377 * ln (x) + 45.866. The excellent correlation between the HPLC method and the FRET-based method to monitor the oxime ligation reaction has allowed us to use the latter linear equation to estimate the conversion to product from the spectral measurements in catalyst screens.

Screening for improved oxime ligation catalysts using the cyanine FRET-based probes

We show here that the probe system introduced in this work could be used to screen for catalysts of the oxime ligation (Table 2). In order to test our system's capacity for catalyst screening we included 2-amino-5-methoxybenzoic acid in our catalyst sample. This compound was previously reported to outperform aniline as an oxime ligation catalyst.[20] We show that our probe system also detects this catalyst's ability to outperform aniline in catalyzing the oxime ligation in physiological pH (42% and 27% conversion after 120 minutes, respectively). We further show that other compounds that were not tested before as oxime ligation catalysts, such as 4-aminophenol and 3-methoxy-4-phenylaniline, performed even better in the screen we conducted (47% and 62% conversion after 120 minutes, respectively). We hypothesized that thiourea derivatives could serve as catalysts for the oxime ligation and therefore tested the ability of 1-(3,5-bis(trifluoromethyl)phenyl)thiourea and 1,3-bis(3,5-bis(trifluoromethyl)phenyl)thiourea to catalyze the reaction between **11b** and **12** (Entries 3, 10). We show that the monosubstituted thiourea increases the donor to acceptor fluorescence ratio in the oxime ligation reaction between **11b** and **12** and that the addition of aniline does not enhance that ratio (entries 1-3). No significant enhancement was observed for the corresponding disubstituted or unsubstituted thiourea derivatives (Entries 10 and 11, respectively). This could be explained by the lack of an unsubstituted nitrogen in the disubstituted thiourea derivative and an unoptimal pKa in the case of the unsubstituted thiourea derivative.

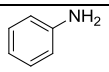
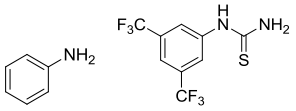
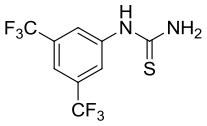
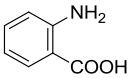
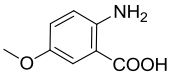
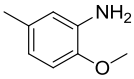
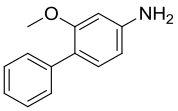
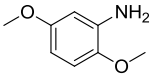
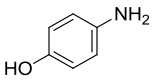
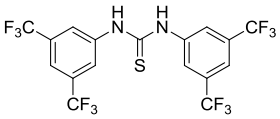
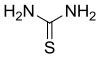
We verified our assay's ability to identify new catalysts for the oxime ligation by testing one of the identified catalysts in the reaction between alternative starting materials, 4-formylbenzoic acid (**32**) and (aminooxy)acetic acid hemihydryochloride (**33**) to form oxime product **30** (Fig. S17†). Indeed, 4-aminophenol serves as a more efficient catalysis than aniline in the reaction between the alternative starting materials (K_{obs} = 2.6938 $M^{-1}S^{-1}$, 0.9968 $M^{-1}S^{-1}$, respectively, Fig. S18, S19†) as measured in kinetic NMR experiments in deuterated PBS (pH 7.4). A similar measurement with 1-(3,5-bis(trifluoromethyl)phenyl)thiourea as a catalyst has shown no rate enhancement over the uncatalyzed reaction (data not shown).

Conclusions

A FRET-based assay for convenient monitoring, investigating and improving small-scale reactions under physiological conditions was introduced. We showed the application of this assay in the native chemical ligation reaction and in the oxime ligation. We demonstrated that this assay could shed light on our understanding of these reactions by studying parameters such as alternating functional groups, catalysts and conditions. The modular design of our FRET-based probe system could be further applied to other reactants and other ligation reactions by changing the functional groups that are attached to the fluorophore moieties.

We characterized a trimeric intermediate formed in the NCL reaction and showed the effect of changing the thiolate leaving group and the amino acid residue on the formation of this intermediate. In the catalyst screen we performed for the oxime ligation we found new catalysts, such as 3-methoxy-4-phenylaniline and 4-aminophenol, that outperform leading previously reported oxime ligation catalysts. We hope that this demonstration of the utility of our assay for the discovery of new oxime ligation catalysts will inspire the employment of this assay to search for new catalysts for the oxime ligation and for

Table 2 A screen for oxime ligation catalysts based on FRET measurements of the oxime ligation reaction between compound **11b** and compound **12.**

Entry	Catalyst	Structure	Acceptor to donor emission ratio[a]	Conversion to product (%)[b]
1	Aniline		1.00	27.1
2	Aniline + 1-(3,5-bis(trifluoromethyl)phenyl)thiourea		2.15	45.8
3	1-(3,5-bis(trifluoromethyl)phenyl)thiourea		2.27	47.1
4	anthranilic acid		0.75	20.1
5	2-amino-5-methoxybenzoic acid		1.87	42.4
6	2-methoxy-5-methylaniline		0.61	14.9
7	3-methoxy-4-phenylaniline		4.25	62.5
8	2,5-dimethoxyaniline		0.53	11.5
9	4-aminophenol		2.26	47.1
10	1,3-bis(3,5-bis(trifluoromethyl)phenyl)thiourea		0.62	15.5
11	Thiourea		0.44	6.8
12	No Catalyst	-	0.46	8.1

[a]Measurements were taken after 120 min of reaction and the results were normalized to entry 1. Acceptor emission was measured at 780 nm and donor emission was measured at 570 nm. [b]Percent conversion to product was calculated according to the acceptor to donor emission ratio of each entry after 120 min of reaction plugged into the linear regression equation y = 24.377 * ln (x) + 45.866 described above. Reaction conditions: 50 µM of compound **11b**, 50 µM of compound **12** and 1 mM of the catalyst at room temperature in PBS (pH 7.4) containing 7.5% DMSO (v/v).

other ligation reactions. Lastly, as the assay we developed is based on near-IR cyanine dyes, the direct continuation of this research should be the use of this assay to study ligation reactions *in vivo* when applicable.

Experimental Section

General methods

All reactions requiring anhydrous conditions were performed under Argon atmosphere. Thin layer chromatography (TLC): silica gel plates Merck 60 F_{254}: compounds were visualized by irradiation with UV light. Flash chromatography (FC): silica gel Merck 60 (particle size 0.040-0.063 mm), eluent given in parentheses. For all column chromatography separations that involved more than 20% MeOH in the eluent mixture, the silica gel was previously mixed with MeOH, dried and eluted with DCM. High-pressure liquid chromatography (HPLC): C-18 5 µ, 250x4.6 mm, eluent given in parentheses. Unless stated otherwise, the gradient used for the HPLC separations was 10% to 90% ACN in 0.1% (v/v) TFA aqueous solution over 20 minutes. Preparative HPLC: C-18 5 µ, 250x21 mm, eluent given in parentheses. ^{1}H-NMR spectra were measured using Bruker Avance operated at 400 MHz or Bruker Ascend operated at 500 MHz, as mentioned. ^{13}C-NMR spectra were measured using Bruker Avance operated at 400 MHz as mentioned. Mass spectroscopy measurements were performed on Waters Xevo TQD mass spectrometer equipped with Acqcuity UPLC and an electronspray ion source. Unless stated otherwise, all reagents, salts and solvents were purchased from commercial sources and used without further purification. Absorption and fluorescence spectra were recorded on Infinite-M200 fluorescent spectrometer. FRET measurements were taken

after five times dilution (Fig. 3) or ten times dilution (Fig. 7, Fig. S12, Fig. S13, Fig. S16†, Table 2) of the reaction mixture into ACN to a final volume of 100 µL at the indicated time points. Deuterated PBS 7.4 was prepared from 126.1 mM NaCl, 10.3 mM Na_3PO_4, 1.5 mM K_3PO_4 and 13.6 mM DCl in D_2O. The pH was adjusted to 7.4 with a DCl solution in D_2O.

Chemical Synthesis

2-((1E,3E)-5-((E)-1-(6-((carboxymethyl)amino)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium (5a). Compound **4**[38] (111 mg, 0.18 mmol) and glycine (36.5 mg, 0.49 mmol) were dissolved in 2 mL of DMF. Et_3N (220 µL, 1.6 mmol) was added and the reaction mixture was heated to 30 °C for two hours. The reaction was monitored by TLC (DCM:MeOH:AcOH, 89:10:1). After completion the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH/AcOH 97:2:1 → DCM/MeOH/AcOH 89:10:1) to afford compound **5a** (49 mg, 50%) as a blue solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (2 H, m), 7.35 (4 H, m), 7.20-7.00 (5 H, m), 6.78 (1 H, t, *J* = 12.4 Hz), 6.63 (1 H, d, *J* = 13.7 Hz), 6.22 (1 H, d, *J* = 13.4 Hz), 3.97 (2 H, t, *J* = 7.2 Hz), 3.86 (2 H, d, *J* = 3.8 Hz), 3.80 (3 H, s), 2.25 (2 H, t, 6.7 Hz), 1.77 (4 H, m), 1.67 (12 H, s), 1.50 (2 H, m). MS (ESI+): *m/z* calc. for $C_{34}H_{42}N_3O_3^+$: 540.32 ; found: 540.5 $[M]^+$. RP-HPLC product retention time = 16.67 min.

2-((1E,3E)-5-((E)-3,3-dimethyl-1-(6-oxo-6-((2-oxo-2-(phenylthio)ethyl)amino)hexyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium (2a). Compound **5a** (49 mg, 0.091 mmol) and N,N'-Dicyclohexylcarbodiimide (DCC, 46 mg, 0.23 mmol) were dissolved in 2 mL of DCM. Thiophenol (20 µL, 0.196 mmol) was added and the reaction mixture was heated to 30 °C for two hours. The reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 21.66 min). After completion the solvent was removed under reduced pressure and the crude product was purified by preparative RP-HPLC (30-100% ACN in H_2O, 20 min) to afford compound **2a** (18.6 mg, 30%) as a blue solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.29 (1 H, br. s.), 7.81 (2 H, dt, *J* = 4.1, 12.5 Hz), 7.41-7.28 (9 H, m), 7.23-7.16 (2 H, m), 7.08 (2 H, m), 6.69 (1 H, t, *J* = 12.5 Hz), 6.31 (1 H, d, *J* = 13.7 Hz), 6.19 (1 H, d, *J* = 13.5 Hz), 4.30 (2 H, d, *J* = 6.0 Hz), 4.03 (2 H, t, *J* = 7.4 Hz), 3.54 (3 H, s), 2.47 (2 H, t, 7.1 Hz), 1.73 (4 H, m), 1.67 (12 H, s), 1.56 (2 H, m). MS (ESI+): *m/z* calc. for $C_{40}H_{46}N_3O_2S^+$: 632.33; found: 632.3 $[M]^+$.

2-((E)-3-((E)-1-(6-((2-((tert-butoxycarbonyl)amino)ethyl)amino)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3H-indol-1-ium (8). Compound **6**[29] (150 mg, 0.28 mmol), DCC (87 mg, 0.42 mmol) and NHS (48.3 mg, 0.42 mmol) were dissolved in 3 mL of DCM and the reaction mixture was stirred at room temperature for 45 minutes. The reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min). After completion, compound **7**[27] **(**134 mg, 0.84 mmol) was added and the reaction was left O.N. at R.T. The reaction was monitored by dissolving one drop of the reaction mixture in (TFA/DCM, 1:1) for 5 minutes at R.T. and then analyzing the deprotection product by RP-HPLC (10-90% ACN in H_2O, 20 min). After completion, the reaction mixture was diluted with DCM and the 1,3-dicyclohexylurea was filtrated. The solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH 95:5 → DCM/MeOH 93:7) to afford compound **8** (144 mg, 75%) as a red solid.
^{1}H NMR (400 MHz, $CDCl_3$ + 1 drop CD_3OD): δ = 8.37 (1 H, t, *J* = 13.4 Hz), 7.44-7.30 (4 H, m), 7.21 (2 H, t, *J* = 7.2 Hz), 7.11 (2 H, d, *J* = 7.6 Hz), 6.84 (1 H, d, *J* = 13.4 Hz), 6.74 (1 H, d, *J* = 13.1 Hz), 4.05 (2 H, br. t), 3.69 (3 H, s), 3.41 (2 H, m), 3.24 (2 H, m), 2.78-2.25 (6 H, m), 1.42-1.69 (16 H, m), 1.36 (9 H, s). MS (ESI+): *m/z* calc. for $C_{37}H_{51}N_4O_3^+$: 599.40; found: 599.6 $[M]^+$. RP-HPLC product retention time = 17.45 min.

2-((E)-3-((E)-1-((R)-2,2-dimethyl-4,7,12-trioxo-6-((tritylthio)methyl)-3-oxa-5,8,11-triazaheptadecan-17-yl)-3,3-dimethylindolin-2-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3H-indol-1-ium (9). Compound **8** (100 mg, 0.15 mmol) was dissolved in a 1:1 mixture of TFA/DCM and the mixture was stirred at R.T. for 5 minutes. The reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min). After completion the solvents were removed under reduced pressure and 3 mL of DCM were added, followed by Et_3N (62 µL, 0.45 mmol), EDC (84 mg, 0.45 mmol), HOBt (68 mg, 0.45 mmol) and N-Boc-S-Trityl-L-cysteine (206 mg, 0.45 mmol) and the reaction mixture was stirred at room temperature overnight. The reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 22.14 min). After completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH 95:5 → DCM/MeOH 93:7) to afford compound **9** (49 mg, 29%) as a red solid.
^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.39 (1 H, t, *J* = 13.3 Hz), 7.90 (1 H, br. t), 7.73 (1 H, m), 7.59 (1 H, m), 7.38 (18 H, m), 7.18-7.03 (4 H, m), 6.84 (2 H, m), 4.05 (2 H, t, *J* = 7.7 Hz), 3.89 (1 H, m), 3.73 (3 H, s), 3.46-3.21 (8 H, m), 1.97 (2 H, m), 1.71 (12 H, s), 1.53 (4 H, m), 1.37 (9 H, s). MS (ESI+): *m/z* calc. for $C_{59}H_{70}N_5O_4^+$: 944.51; found: 944.8 $[M]^+$.

2-((E)-3-((E)-1-(6-((2-((R)-2-ammonio-3-mercaptopropanamido)ethyl)amino)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)prop-1-en-1-yl)-1,3,3-trimethyl-3H-indol-1-ium (1). Compound **9** (45 mg, 0.045 mmol) was dissolved in 1 mL of DCM. 0.5 mL of TFA and 0.1 mL of Et_3SiH were added and the mixture was stirred at R.T. for 5 minutes. The reaction was monitored by TLC (DCM:MeOH, 90:10). After completion the solvents were removed under reduced pressure and the crude product was purified by preparative RP-HPLC (10-90% ACN in H_2O, 20 min) to afford compound **1** (4.75 mg, 13%) as a red solid.
^{1}H NMR (400 MHz, $CDCl_3$ + 1 drop CD_3OD): δ = 8.38 (1 H, t, *J* = 13.5 Hz), 7.5-7.15 (9 H, m), 6.33 (2 H, m), 4.13 (1 H, br. t), 4.05 (2 H, br. t), 3.62 (3 H, s), 3.48 (2 H, m), 3.39 (2 H, m), 3.29 (2 H,

m), 3.03 (2 H, m), 1.71 (14 H, m), 1.46 (2 H, m). MS (ESI+): *m/z* calc. for $C_{70}H_{94}N_{10}O_4S_2^{2+}$: 601.34; found: 601.3 $[M]^+$. RP-HPLC product retention time = 13.72 min.

2-((1E,3E)-5-((E)-1-(6-((2-((2-ethoxy-2-oxoethyl)thio)-2-oxoethyl)amino)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium (2b). Compound **5a** (33 mg, 0.061 mmol) and DCC (46 mg, 0.17 mmol) were dissolved in 1 mL of DMF. Ethyl-2-mercaptoacetate (20 µL, 0.18 mmol) was added and the reaction mixture and was stirred at room temperature overnight, protected from light by aluminum foil.[39] The reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 18.51 min). After completion the solvent was removed under reduced pressure and the crude product was purified by preparative RP-HPLC (30-100% ACN in H_2O, 20 min) to afford compound **2b** (34 mg, 82%) as a blue solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.42 (1 H, br. t), 7.81 (2 H, dt, *J* = 5.1, 13.2 Hz), 7.38 (4 H, m), 7.24 (2 H, m), 7.12 (1 H, d, 7.6 Hz), 7.07 (1 H, d, 8 Hz), 6.73 (1 H, t, *J* = 12.1, 15.2 Hz), 6.35 (1 H, d, *J* = 13.6 Hz), 6.25 (1 H, d, *J* = 13.2 Hz), 4.22 (2 H, d, *J* = 6.8 Hz), 4.05 (2 H, t, *J* = 7.2 Hz), 3.66 (2 H, s), 3.59 (3 H, s), 3.46 (2 H, m), 2.43 (2 H, t, 6.74 Hz), 1.79 (4 H, m), 1.69 (12 H, s), 1.57 (2 H, m), 1.35 (3 H, m). MS (ESI+): *m/z* calc. for $C_{38}H_{48}N_3O_4S^+$: 642.34; found: 642.5 $[M]^+$.

2-((1E,3E)-5-((E)-1-(6-(((2S,3S)-1-((2-ethoxy-2-oxoethyl)thio)-3-methyl-1-oxopentan-2-yl)amino)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium (2c). Compound **4** (40 mg, 0.065 mmol) and L-isoleucine (26 mg, 0.19 mmol) were dissolved in 1 mL of DMF. Et_3N (90 µL, 0.65 mmol) was added and the reaction mixture was heated to 40 °C for three hours. The reaction was monitored by HPLC (10-90% ACN in H_2O, 20 min; product retention time = 20.10 min). After completion the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH/AcOH 97:2:1 → DCM/MeOH/AcOH 91:8:1) to afford compound **5b** (11.8 mg, 30%) as a blue solid. The latter (11.8 mg, 0.02 mmol) and DCC (12.4 mg, 0.06 mmol) were dissolved in 1 mL of DMF. Ethyl-2-mercaptoacetate (6.5 µL, 0.06 mmol) was added and the reaction mixture and was stirred at room temperature overnight, protected from light by aluminum foil.[39] The reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 20.09 min). After completion the solvent was removed under reduced pressure and the crude product was purified by preparative RP-HPLC (30-100% ACN in H_2O, 20 min) to afford compound **2c** (4.5 mg, 32%) as a blue solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.97 (2 H, m), 7.3-7.4 (6 H, m), 7.24-7.20 (2 H, m), 7.20-7.00 (5 H, m), 6.53 (1 H, d, *J* = 13.6 Hz), 6.39 (1 H, d, *J* = 13.5 Hz), 4.13 (8 H, m), 3.66 (5 H, m), 3.48 (4 H, m), 2.51 (2 H, m), 1.77 (12 H, s) 1.67 (2 H, m). MS (ESI+): *m/z* calc. for $C_{42}H_{56}N_3O_4^+$: 698.40; found: 698.6 $[M]^+$.

3-(2-((1E,3E)-5-((E)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-1-yl)propane-1-sulfonate (21). Compound **19**[40] (200 mg, 0.71 mmol) and compound **13**[41] (219 mg, 0.85 mmol) were dissolved in a 1:1 solvent mixture of AcOH and Ac_2O (2 mL of each). The mixture was refluxed for 20 minutes and monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 11.80 min, product λ_{max} = 437 nm). After cooling down to room temperature, the solvents were removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH 90:10 → DCM/MeOH 80:20) to afford compound **20** (197 mg, 61%) as a dark yellow solid, that was directly used without further characterization. Compound **20** (197 mg, 0.435 mmol) and compound **14**[38] (154 mg, 0.435 mmol) were dissolved in 20 mL of EtOH and NaOAc (43 mg, 0.52 mmol) was added. The reaction mixture was refluxed for 20 minutes and monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 20.86 min). After cooling down to room temperature the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH 90:10 → DCM/MeOH 80:20) to afford compound **21** (192 mg, 75%) as a blue solid.

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.86 (2 H, m), 7.38-7.28 (4 H, m), 7.22-7.12 (4 H, m), 7.03 (1 H, d, *J* = 6.8 Hz), 6.31 (2 H, m), 4.49 (2 H, br. t), 4.01 (2 H, br. t), 3.10 (2 H, m), 2.39 (2 H, m), 2.32(2 H, m), 2.22 (2 H, t, *J* = 8.0 Hz), 2.01 (2 H, m), 1.79 (6 H, m), 1.69 (6 H, s), 1.65 (6 H, s), 1.53 (2 H, m). MS (ESI+): *m/z* calc. for $C_{34}H_{42}N_2NaO_5S^+$: 613.27; found: 613.5 $[M + Na]^+$.

3-(2-((1E,3E)-5-((E)-1-(6-((2-((tert-butoxycarbonyl)amino)ethyl)amino)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-1-yl)propane-1-sulfonate (22). Compound **21** (192 mg, 0.32 mmol), DCC (72 mg, 0.35 mmol) and NHS (40.2 mg, 0.35 mmol) were dissolved in 3 mL of DMF and the reaction mixture was stirred at room temperature for 2 hours. The reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min). After completion, compound **7 (**160 mg, 1 mmol) was added and the reaction was stirred overnight at room temperature. The reaction was monitored by dissolving one drop of the reaction mixture in (TFA/DCM, 1:1) for 5 minutes at R.T. and then analyzing the deprotection product by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 16.60 min). Upon completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH 90:10 → DCM/MeOH 80:20) to afford compound **22** (166 mg, 71%) as a red solid.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.35 (2 H, t, *J* = 11.8 Hz), 7.79 (1 H, br. t), 7.61 (2 H, d, *J* = 7.4 Hz), 7.49-7.34 (4 H, m), 7.29-7.19 (2 H, m), 6.77 (1 H, br. t), 6.57 (1 H, t, *J* = 12.3 Hz), 6.46 (1 H, d, *J* = 13.8 Hz), 6.32 (1 H, d, *J* = 13.8 Hz), 4.58 (2 H, t, *J* = 5.5 Hz), 4.28 (2 H, t, *J* = 6.6 Hz), 4.09 (2 H, t, *J* = 7.3 Hz), 3.51 (2 H, m), 3.18 (2 H, m), 3.04 (2 H, m), 2.94 (2 H, m), 2.82 (2 H, t, 6.5 Hz), 2.58 (2 H, m), 2.04 (2 H, m), 1.69 (6 H, s), 1.68 (6 H, s), 1.36 (9

H, s). MS (ESI+): *m/z* calc. for $C_{41}H_{56}N_4NaO_6S^+$: 755.38; found: 755.6 $[M + Na]^+$.

3-(2-((1E,3E)-5-((E)-1-(2,2-dimethyl-4,8,13-trioxo-3,6-dioxa-5,9,12-triazaoctadecan-18-yl)-3,3-dimethylindolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium-1-yl)propane-1-sulfonate (23). Compound **22** (152 mg, 0.21 mmol) was dissolved in a 1 mL solvent mixture of 1:1 TFA/DCM and the reaction mixture was stirred at room temperature for 5 minutes. The reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min). After completion the solvents were removed under reduced pressure and 3 mL of DMF were added, followed by Et_3N (172 µL, 1.2 mmol), EDC (120 mg, 0.62 mmol), HOBt (64 mg, 0.2 mmol) and compound **16**[30] (120 mg, 0.62 mmol) and the reaction mixture was stirred at 40 °C overnight. The reaction progress was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 15.84 min), and upon completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH 90:10 → DCM/MeOH 85:15) to afford compound **23** (101 mg, 60%) as a blue solid.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.33 (2 H, t, *J* = 12.9 Hz), 7.99 (2 H, br. s), 7.60 (2 H, m), 7.46 (2 H, d, *J* = 8.0 Hz), 7.37 (2 H, m), 7.21 (2 H, t, *J* = 6.3 Hz), 6.54 (1 H, t, *J* = 12.2 Hz), 6.43 (1 H, d, *J* = 13.9 Hz), 6.29 (1 H, d, *J* = 13.7 Hz), 4.26 (2 H, br. t), 4.12 (2 H, s), 4.07 (2 H, br. t), 3.14 (4 H, m), 2.53 (2 H, m), 2.05-1.95 (4 H, m), 1.66 (12 H, s), 1.56 (4 H, m), 1.37 (9 H, s), 1.32 (2 H, m). MS (ESI+): *m/z* calc. for $C_{43}H_{59}N_5NaO_8S^+$: 828.40; found: 828.7 $[M + Na]^+$.

1-(6-((2-((tert-butoxycarbonyl)amino)ethyl)amino)-6-oxohexyl)-2,3,3-trimethyl-3H-indol-1-ium (24). Compound **14** (800 mg, 2.26 mmol), DCC (840 mg, 4.05 mmol) and NHS (390 mg, 3.4 mmol) were dissolved in 4 mL of DMF and the reaction mixture was heated to 40 °C for 6 hours and afterwards kept at room temperature overnight. The reaction was monitored by TLC (DCM:MeOH, 90:10). After completion, compound **7** (1084 mg, 6.8 mmol) was added and the reaction was stirred for 2 hours at room temperature. The reaction was monitored by dissolving one drop of the reaction mixture in (TFA/DCM, 1:1) for 5 minutes at R.T. and then analyzing the deprotection product by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 12.45 min). Upon completion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH 90:10 → DCM/MeOH 80:20) to afford compound **24** (815 mg, 86%) as a beige solid.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.76 (1 H, t, *J* = 5.3 Hz), 7.12 (1 H, d, *J* = 7.2 Hz), 7.07 (1 H, dt, *J* = 1.12, 7.6 Hz), 6.75 (1 H, t, 5.34), 6.69 (1 H, t, *J* = 7.47 Hz), 6.62 (1 H, d, *J* = 7.8 Hz), 5.58 (2 H, d, *J* = 7.86 Hz), 3.87 (1 H, d, *J* = 1.4 Hz), 3.83 (1 H, d, *J* = 1.4 Hz), 3.48 (2 H, t, *J* = 7.2 Hz), 3.04 (2 H, m), 2.96 (2 H, m), 2.04 (2 H, d, *J* = 7.4 Hz), 1.52 (4 H, m), 1.38 (9 H, s), 1.26 (6 H, s). MS (ESI+): *m/z* calc. for $C_{24}H_{36}N_3O_3^+$: 416.29; found: 416.4 $[M]^+$.

3-(2-((1E,3E,5E)-7-((E)-1-(6-((2-((tert-butoxycarbonyl)amino)ethyl)amino)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)hepta-1,3,5-trien-1-yl)-3,3-dimethyl-3H-indol-1-ium-1-yl)propane-1-sulfonate (26). Compound **19** (400 mg, 1.4 mmol) and Glutaconaldehydedianil hydrochloride (483 mg, 1.7 mmol) were dissolved in a 1:1 solvent mixture of AcOH and Ac_2O (4 mL each). The mixture was refluxed for 30 minutes and the reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 13.24 min, product λ_{max} = 482 nm). After cooling to room temperature the solvents were removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH 90:10 → DCM/MeOH 85:15) to afford compound **25** (185 mg, 28%) as a dark red solid, that was directly used without further characterization. Compound **25** (155 mg, 0.33 mmol) and compound **24** (135 mg, 0.33 mmol) were dissolved in 3 mL of pyridine. The reaction mixture was heated to 50 °C for 30 minutes and the reaction progress was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 17.80 min). Upon completion the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH 90:10 → DCM/MeOH 85:15) to afford compound **26** (131 mg, 52%) as a blue solid.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.35 (2 H, t, *J* = 13.1 Hz), 7.78 (1 H, t, 5.4 Hz), 7.62 (2 H, d, *J* = 7.5 Hz), 7.41 (4 H, m), 7.26 (2 H, m), 6.77 (1 H, t, *J* = 5.4 Hz), 6.57 (1 H, t, *J* = 12.4 Hz), 6.28 (2 H, m), 4.58 (2 H, t, *J* = 5.5 Hz), 4.09 (2 H, t, *J* = 7.4 Hz), 3.51 (2 H, m), 3.42 (2 H, m), 3.31 (2 H, m), 3.02 (2 H, m), 2.95 (2 H, m), 2.06 (2 H, m), 1.69 (12 H, s), 1.54 (4 H, m), 1.36 (9 H, s). MS (ESI+): *m/z* calc. for $C_{43}H_{58}N_4NaO_6S^+$: 781.39; found: 781.6 $[M + Na]^+$.

3-(2-((1E,3E,5E)-7-((E)-1-(2,2-dimethyl-4,8,13-trioxo-3,6-dioxa-5,9,12-triazaoctadecan-18-yl)-3,3-dimethylindolin-2-ylidene)hepta-1,3,5-trien-1-yl)-3,3-dimethyl-3H-indol-1-ium-1-yl)propane-1-sulfonate (27). Compound **26** (84 mg, 0.11 mmol) was dissolved in a 1 mL solvent mixture of 1:1 TFA/DCM and the reaction mixture was stirred at room temperature for 5 minutes. The reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min). After completion the solvents were removed under reduced pressure and 2 mL of DMF were added, followed by Et_3N (46 µL, 0.33 mmol), EDC (43 mg, 0.22 mmol), HOBt (34 mg, 0.22 mmol) and compound **16** (43 mg, 0.22 mmol) and the reaction mixture was stirred at room temperature for two hours. The reaction progress was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 17.07 min), and upon completion, the solvent was removed under reduced pressure and the crude product was purified by preparative RP-HPLC (10-90% ACN in H_2O, 20 min) to afford compound **27** (20 mg, 22%) as a blue solid.

^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.27 (1 H, s), 8.02 (1 H, t, 5.14), 7.84 (3 H, m), 7.58 (2 H, t, *J* = 6.9 Hz), 7.48 (1 H, d, *J* = 7.8 Hz), 7.38 (3 H, m), 7.23 (2 H, m), 6.54 (3 H, t, *J* = 12.7 Hz), 6.35 (1 H, d, *J* = 13.6 Hz), 4.25 (2 H, t, 7.0 Hz), 4.15 (2 H, s), 4.05 (2 H, t, *J* = 6.5 Hz), 3.13 (4 H, m), 2.54 (2 H, m), 2.07 (2 H, t, *J* = 7.2 Hz), 2.00 (2 H, m), 1.64 (6 H, s), 1.63 (6 H, s), 1.56 (4 H, m), 1.40 (9 H, s), 1.35 (2 H, m). MS (ESI+): *m/z* calc. for $C_{45}H_{61}N_5NaO_8S^+$: 854.41; found: 854.7 $[M + Na]^+$.

3-(2-((E)-3-((E)-1-(5-carboxypentyl)-3,3-dimethylindolin-2-ylidene)prop-1-en-1-yl)-3,3-dimethyl-3H-indol-1-ium-1-yl)propane-1-sulfonate (28). Compound **19** (400 mg, 1.41 mmol) and compound **15**[42] (277 mg, 1.41 mmol) were dissolved in a 3 mL of Ac_2O. The mixture was heated to 120 °C for 30 minutes and the reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 14.88 min). After cooling down to room temperature compound **14** in 3 mL of pyridine was added and the reaction mixture was stirred at room temperature for 40 minutes and at 40 °C for 40 additional minutes. Upon completion the solvents were removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH 95:5 → DCM/MeOH 90:10) to afford compound **28** (493 mg, 62%) as a red solid.
^{1}H NMR (400 MHz, DMSO-d_6): δ = 12.04 (1 H, br. s, 8.36 (1 H, t, *J* = 13.5 Hz), 7.64 (2 H, dd, *J* = 2.3, 7.3 Hz), 7.58 (1 H, d, *J* = 8.0 Hz), 7.45 (3 H, m), 7.30 (2 H, m), 6.62 (1 H, d, *J* = 13.4 Hz), 6.49 (1 H, d, *J* = 13.4 Hz), 4.29 (2 H, t, *J* = 7.4 Hz), 4.12 (2 H, t, *J* = 7.2 Hz), 2.59 (2 H, t, *J* = 7.2 Hz), 2.23 (2 H, t, *J* = 7.2 Hz), 2.06 (2 H, quintet, *J* = 7.2 Hz), 1.78 (2 H, m), 1.71 (6 H, s), 1.70 (6 H, s), 1.58 (2 H, quintet, *J* = 7.4 Hz), 1.43 (2 H, m). MS (ESI+): *m/z* calc. for $C_{32}H_{41}N_2NaO_5S^+$: 587.25; found: 587.4 $[M + Na]^+$.

3-(2-((E)-3-((E)-1-(6-((2-((tert-butoxycarbonyl)amino)ethyl)amino)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)prop-1-en-1-yl)-3,3-dimethyl-3H-indol-1-ium-1-yl)propane-1-sulfonate (29). Compound **28** (290 mg, 0.51 mmol), DCC (159 mg, 0.77 mmol) and NHS (89 mg, 0.77 mmol) were dissolved in 3 mL of DMF and the reaction mixture was stirred overnight at room temperature. This step was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min). After completion, compound **7 (**160 mg, 1 mmol) was added and the reaction was stirred for 30 minutes at room temperature. This step was monitored by TLC (DCM:MeOH, 90:10). Upon complete conversion, the solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (DCM/MeOH 95:5 → DCM/MeOH 90:10) to afford compound **29** (300 mg, 83%) as a red solid.
^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.36 (1 H, t, *J* = 13.5 Hz), 7.87 (1 H, t, *J* = 5.6 Hz), 7.64 (2 H, t, *J* = 7.4 Hz), 7.58 (1 H, d, *J* = 8.0 Hz), 7.45 (3 H, m), 7.29 (2 H, m), 6.78 (1 H, t, *J* = 5.5 Hz), 6.66 (1 H, d, *J* = 13.4 Hz), 6.46 (1 H, d, *J* = 13.4 Hz), 4.30 (2 H, t, *J* = 7.1 Hz), 4.11 (2 H, m), 3.17 (4 H, m), 3.02 (2 H, m), 2.93 (2 H, m), 2.84 (2 H, t, *J* = 6.4 Hz), 2.09 (2 H, m), 1.71 (6 H, s), 1.70 (6 H, s), 1.58 (2 H, m), 1.38 (2 H, m), 1.36 (9 H, s). MS (ESI+): *m/z* calc. for $C_{39}H_{54}N_4NaO_6S^+$: 729.36; found: 729.6 $[M + Na]^+$. RP-HPLC product retention time = 15.95 min.

General procedure for obtaining compounds 11a-b. Compounds **23** and **27** were used as the Boc-protected precursors for compounds **11a** and **11b**, respectively. The appropriate Boc protected derivative (5 mg, 0.006 mmol) was dissolved in 0.5 mL of TFA and the reaction mixture was stirred at R.T. for 5 minutes. The reaction progress was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min). The solvent was removed under reduced pressure and compounds **11a-b** were used without further purification to prepare a 10 mM stock solution in DMSO that was kept at -20 °C for immediate use. RP-HPLC retention times for products **11a**, **11b** = 13.83, 14.41 min, respectively.

3-(2-((E)-3-((E)-1-(6-((2-(4-formylbenzamido)ethyl)amino)-6-oxohexyl)-3,3-dimethylindolin-2-ylidene)prop-1-en-1-yl)-3,3-dimethyl-3H-indol-1-ium-1-yl)propane-1-sulfonate (12). 4-carboxybenzaldehyde (156 mg, 1.04 mmol), DCC (247 mg, 1.2 mmol) and NHS (138 mg, 1.2 mmol) were dissolved in 4 mL of DCM and the reaction mixture was heated to 40 °C for 3 hours. The reaction was monitored by TLC (EtOAc:Hex, 40:60). Upon completion, the reaction mixture was diluted with DCM and the 1,3-dicyclohexylurea was filtrated out. The solvent was removed under reduced pressure and the crude product **17** immediately used without further purification. Compound **29** (100 mg, 0.14 mmol) was dissolved in a 1 mL solvent mixture of 1:1 TFA/DCM and the reaction mixture was stirred at room temperature for 5 minutes. The reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min). Upon completion the solvents were removed under reduced pressure and 3 mL of DCM were added, followed by Et_3N (100 µL, 0.71 mmol) and compound **17** (0.7 mmol). The reaction mixture was stirred at room temperature overnight. The reaction progress was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min; product retention time = 15.31 min), and upon completion, the solvent was removed under reduced pressure and the crude product was purified by preparative RP-HPLC (10-90% ACN in H_2O, 20 min) to afford compound **12** (35 mg, 33%) as a red solid**.**
^{1}H NMR (400 MHz, DMSO-d_6): δ = 10.05 (1 H, s), 8.74 (1 H, t, *J* = 5.5 Hz), 8.34 (1 H, t, *J* = 13.4 Hz), 8.10-7.90 (5 H, m), 7.63 (2 H, d, *J* = 7.3 Hz), 7.55 (1 H, d, *J* = 8.0 Hz), 7.44 (3 H, m), 7.29 (2 H, m), 6.67 (1 H, d, *J* = 13.4 Hz), 6.42 (1 H, d, *J* = 13.4 Hz), 4.30 (2 H, t, *J* = 7.3 Hz), 4.06 (2 H, t, *J* = 7.1 Hz), 3.32 (2 H, m), 3.23 (2 H, m), 2.61 (2 H, t, 7.0 Hz), 2.08 (4 H, m), 1.69 (12 H, s), 1.64 (2 H, m), 1.58 (2 H, m), 1.38 (2 H, m). ^{13}C NMR (400 MHz, DMSO-d_6): δ = 194.20, 175.32, 175.00, 173.67, 168.81, 151.12, 143.25, 143.20, 141.96, 140.82, 139.03, 131.17, 131.00, 130.68, 130.00, 129.25, 126.57, 126.48, 123.83, 113.01, 112.80, 104.41, 103.90, 56.24, 50.25, 50.20, 49.24, 45.04, 44.21, 39.43, 36.49, 28.83, 28.77, 28.05, 27.07, 26.16, 24.87. MS (ESI+): *m/z* calc. for $C_{42}H_{50}N_4NaO_6S^+$: 761.34; found: 761.6 $[M + Na]^+$.

Dicyanine oxime conjugate 18a. Compound **23** (28 mg, 0.038 mmol) was dissolved in a 1 mL of TFA and the reaction mixture was stirred at room temperature for 5 minutes. The reaction was monitored by RP-HPLC (10-90% ACN in H_2O, 20 min). Upon completion, TFA was removed under reduced pressure and compound **12** (17 mg, 0.023 mmol) dissolved in 1.5 mL of pyridine was added. The reaction mixture was stirred at room temperature for 2 hours. Upon completion, the solvent was removed under reduced pressure and the crude product was purified by preparative RP-HPLC (10-90% ACN in H_2O, 20 min) to afford compound **18a** (11.6 mg, 23%) as a purple solid**.**
^{1}H NMR (400 MHz, DMSO-d_6): δ = 8.62 (1 H, t, *J* = 5.5 Hz), 8.34 (4 H, m), 8.02 (1 H, *J* = 5.3 Hz), 7.93 (1 H, t, *J* = 5.3 Hz), 7.86 (3 H, m), 7.68-7.54 (8 H, m), 7.50-7.34 (8 H, m), 7.32-7.17 (4 H, m), 6.66 (1 H, d, *J* = 13.5 Hz), 6.52 (1 H, d, *J* = 12.5 Hz), 6.45 (2 H, m),

6.28 (1 H, d, *J* = 13.5 Hz), 4.52 (2 H, s), 4.30 (4 H, m), 4.05 (4 H, m), 3.30 (4 H, m), 3.23-3.07 (8 H, m), 2.63 (4 H, m), 2.04 (8 H, m), 1.69 (12 H, s), 1.67 (12 H, s), 1.59 (2 H, m), 1.49 (2 H, m), 1.38 (2 H, m), 1.30 (2 H, m). MS (ESI+): *m/z* calc. for $C_{80}H_{99}N_9Na_2O_{11}S_2^{2+}$: 735.83; found: 735.9 $[M + 2Na]^{2+}$. RP-HPLC product retention time = 16.12 min.

4-(((2-methoxy-2-oxoethoxy)imino)methyl)benzoic acid (31). 4-formylbenzoic acid (110 mg, 0.73 mmol) and (Aminooxy)acetic acid hemihydrochloride (73 mg, 0.67 mmol) were dissolved in 2 mL methanol. Acetic acid (210 µL, 3.4 mmol) was added dropwise and the reaction was stirred at room temperature. After 7 hours a precipitate formed, 2 more mL of methanol were added and the reaction was left overnight. The reaction was monitored by TLC (EtOAc:Hex:AcOH, 80:19:1). Upon completion the solvent was removed under reduced pressure and the crude product was purified by column chromatography (EtOAc/Hex/AcOH, 69:30:1) to afford compound **31** (108 mg, 68%) as a white solid.
^{1}H-NMR (500 MHz, DMSO-d_6): δ = 13.05 (1 H, br. s), 8.46 (1 H, s), 7.98 (2 H, d, *J* = 8.3 Hz), 7.74 (2 H, d, *J* = 8.3 Hz), 4.81 (2 H, s), 3.69 (3 H, s). MS (ESI+): *m/z* calc. for $C_{11}H_{11}NNaO_5^+$: 260.05; found: 260.038 [M + Na]+; MS (ESI-): *m/z* calc. for $C_{11}H_{10}NO_5^-$: 236.06; found: 236.06 $[M - H]^-$.

4-(((carboxymethoxy)imino)methyl)benzoic acid (30). Compound **31** (50 mg, 0.21 mmol) was dissolved in an NaOH solution in H_2O (1 N) and the reaction was stirred at room temperature for 90 minutes. The reaction was monitored by TLC (EtOAc:Hex:AcOH, 80:19:1). Upon completion the reaction was placed in an ice bath (0° c) and an HCl solution in H_2O (6 N) was added dropwise until the solution pH reached 4. Et_2O (25 mL) was then added, followed by 3 mL of sat. NaCl solution in H_2O (3 mL). A precipitate formed and was filtered. The filtrate was placed again at 0°c and Et_2O (20 mL) was added. A precipitate formed and was filtered. The organic phase of the filtrate was extracted and then the aqueous phase was washed with additional Et_2O (25 mL). The combined organic phase was dried with $MgSO_4$ and the solvent was removed under reduced pressure to yield compound **30** (35 mg, 75%) as a white solid.
^{1}H-NMR (500 MHz, DMSO-d_6): δ = 12.95 (2 H, br. s), 8.43 (1 H, s), 7.97 (2 H, d, *J* = 8.4 Hz), 7.73 (2 H, d, *J* = 8.4 Hz), 4.69, (2 H, s). MS (ESI-): *m/z* calc. for $C_{10}H_8NO_5^-$: 222.04; found: 221.949 $[M - H]^-$.

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