# Synthesis of a new pair of fluorescence resonance energy transfer donor and acceptor dyes and its use in a protease assay†

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**A new, efficient and very robust fluorescence resonance energy transfer (FRET) system, which can be measured in a normal as well as in a time-resolved mode, was developed and its feasibility demonstrated in a protease assay format.**

FRET systems have emerged as powerful tools for the elucidation of distance-dependent interactions on the molecular level. As this method is able to monitor changes in distances on nanometer scale and in real time mode, it has become especially important for the characterisation of biochemical events both *in vitro* and *in vivo*. Among these have been, for example, binding of ligands to proteins and their subsequent conformational changes, DNA–DNA interactions or DNA–protein complexation.[1] Another field of application for FRET is in diagnostics and drug research, where tools like molecular beacons,[2] TaqMan probes[3] or fluorescent protease substrates, for example, use the same principle.

FRET is based on the transfer of fluorescence energy between a donor fluorophore and an acceptor fluorophore. Both dyes must correspond to each other in their spectroscopic properties, *i.e.* the emission wavelength of the donor must overlap with the absorption wavelength of the acceptor, thereby enabling excitation energy to be transferred efficiently from the donor to the acceptor. The intensity of the FRET depends largely on the spectral overlap of the two dyes, their distance from and their orientation to each other.[4]

Ratiometric detection at both emission wavelengths (from donor and acceptor) increases the sensitivity of the method in comparison to probes bearing only one reporter dye. Meanwhile, a number of different donor–acceptor combinations have been reported. Among these are fluorescein–rhodamine, cyanine dyes like Cy3–Cy5, dabcyl–edans, blue and green fluorescent protein,[5] *etc*. However, despite the large number of reported systems, sensitivity still remains an issue. This has led to the application of multivalent fluorophores.[6] Another possibility to increase sensitivity would be the insertion of an entity into the FRET-system which can be measured in a time-resolved mode. For this purpose, lanthanide-, especially Eu- and Tb-complexes, have been reported to be suitable candidates due to their strong fluorescence and long excited state lifetimes up to the range of ms. However, one major drawback is their relatively low stability, resulting in dissociation at low concentrations. To overcome this problem, they are commonly employed as EDTA-complexes and detection is carried out upon addition of an enhancer-solution. As an alternative, caged chelates can be employed,[7] but leakage of the Eu might become an issue of concern.

Therefore, we focussed on Ru(II)-charge-transfer complexes as alternative acceptor units due to their thermodynamic stability, chemical inertness, straightforward synthetic accessibility and their relatively long-lived excited states ($\tau_{em}$ = 1–10 μs) allowing for time-resolved measurements. Our aim was to establish a new FRET-system involving Ru(II)–bathophenanthroline complex **1** as acceptor (Fig. 1) and demonstrate its applicability in a protease assay. Ru-complex **1** displays a characteristic metal-to-ligand-charge-transfer (MLCT)-absorption band at 440–464 nm and a strong red emission at 618 nm with a detection limit below $10^{-14}$ mol $l^{-1}$ when measured by time-resolved fluorescence techniques.[8] Furthermore, it carries a single carboxyl function which can be used for the specific attachment to target molecules, *i.e. via* an amide bond, and two of the bathophenanthroline ligands are equipped with sulfonate groups to enhance solubility in aqueous systems. As a suitable donor with appropriate emission characteristics we have identified the carbostyril derivative **2** with

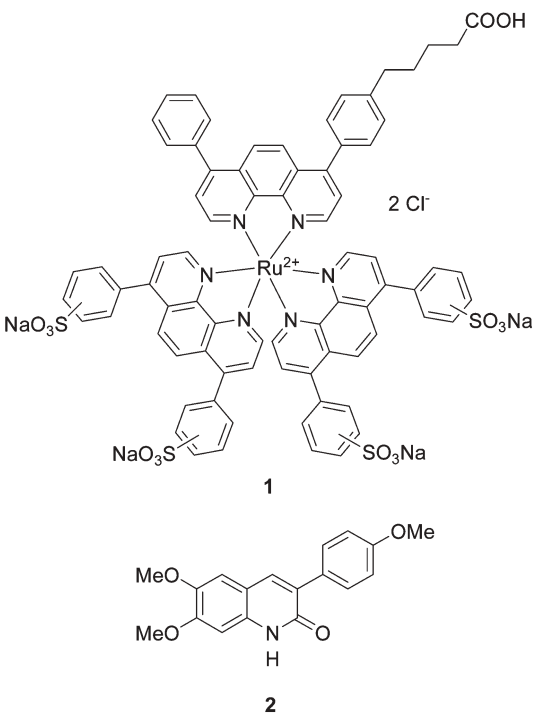


**Fig. 1** Structures of the Ru–bathophenanthroline complex **1** and the carbostyril derivative **2**.

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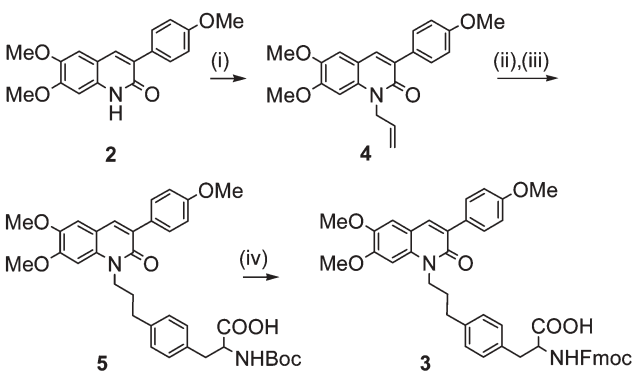
absorption and emission maxima of $\lambda_{ex}$ = 368 nm and $\lambda_{em}$ = 435 nm and a high extinction coefficient ($\varepsilon$ = 20 800 $M^{-1}$ $cm^{-1}$) (Fig. 1).[9]

As possible protease substrate, we intended to synthesise a peptide containing the recognition sequence for thrombin and carrying the donor at the C-terminal end and the acceptor at the N-terminus. Incorporation of the dyes into the peptide substrate of the protease is readily achieved for the Ru-complex by directly attaching it to the N-terminus of the peptide *via* its carboxylic acid unit after cleavage of the Fmoc-group of the N-terminal amino acid. The complex itself was synthesised according to literature[10] with minor modifications and turned out to be completely stable under both peptide synthesis and cleavage/work-up conditions (*i.e.* 20% piperidine in DMF and 95% TFA). For the coupling reaction with the N-terminal amino group, it was activated with *O*-(N-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumtetrafluoroborate (TSTU) as OSu-activated ester prior to coupling.[11]

For the covalent attachment of the donor to the peptide, however, further modifications were necessary. The most convenient way would be the creation of a suitable amino acid building block, to which the dye is attached and which can be used in automated solid phase peptide synthesis (SPPS). In order to achieve this goal, we established a route to synthesise the Fmoc-protected unnatural amino acid **3** containing the donor carbostyril dye, which can be directly used during peptide assembly on solid phase (Scheme 1).

Starting from the quinolinone derivative **2**, which was prepared *via* a reported procedure,[12] an allyl moiety at 1-*N* was introduced, yielding intermediate **4**. A Heck cross-coupling reaction with Boc-protected D,L-*p*-bromophenylalanine and subsequent hydrogenation over Pd/C gave compound **5**. Treatment of **5** with 95% TFA led to cleavage of the Boc-group, but left the chromophore unchanged. Insertion of the Fmoc-group yielded the donor-labelled amino acid **3**. During the synthesis, no changes of the absorption and emission characteristics were observed.

The next step was to verify the spectroscopic properties of the two chromophores, especially with regard to their use as a donor/acceptor pair in peptides and the establishment of the FRET. For this reason, the tetramers **6** and **7** were synthesised bearing the




**Scheme 1** Synthesis of the donor-amino acid **3**. *Reagents and conditions*: (i) KHMDS, allylbromide, THF, microwave, 83%; (ii) Boc-D,L-*p*-bromophenylalanine-OMe, $Pd(OAc)_2$, $PPh_3$, $K_2CO_3$, DMF–$H_2O$, 42%; (iii) $H_2$, Pd/C, MeOH, 92%; (iv) 1. TFA–$CH_2Cl_2$–TIS 95 : 3 : 2, 2. Fmoc-OSu, aqueous $Na_2CO_3$–acetone, 97%.

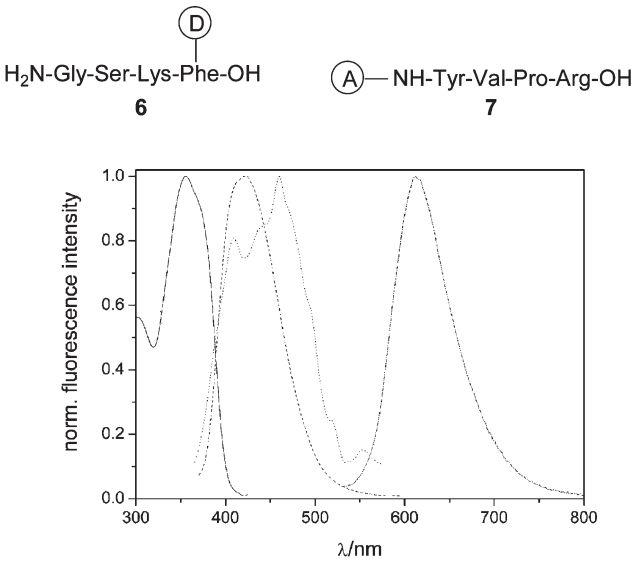



**Fig. 2** Excitation/emission spectra of peptide **6** (solid/dashed line) and peptide **7** (dotted/dot dash line) in $CH_3CN$–$H_2O$ 1 : 1 at concentrations of 5 μM. The donor chromophore is represented by D, the acceptor by A.

donor dye and the acceptor dye, respectively. Excitation and emission spectra measurements of the labelled peptides showed a very good overlap of the emission band of the donor and the

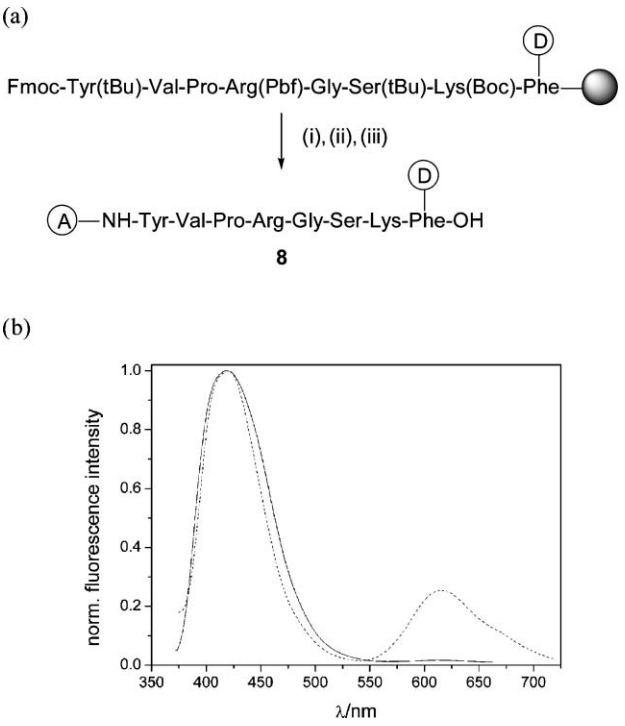
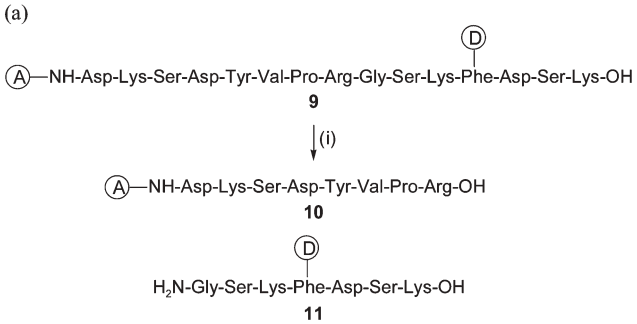



**Fig. 3** (a) Representative peptide synthesis of **8**. *Reagents and conditions*: Peptide synthesis was carried out using Wang-resin, Fmoc-protected amino acids, TBTU, DIPEA, DMF, 1 h. (i) 20% piperidine in DMF, 15 min; (ii) Ru-complex-OSu, DIPEA, DMF, 2 d; (iii) TFA–$CH_2Cl_2$–TIS 95 : 3 : 2, 3 h. The peptides were precipitated with $Et_2O$, centrifuged, washed with $Et_2O$, purified by reversed-phase HPLC and characterised by MS. (b) Fluorescence emission spectra of a mixture of **6** and **7** (solid line) and **8** (dashed line) upon excitation at 340 nm in $CH_3CN$–$H_2O$ 1 : 1 at concentrations of 5 μM.

(a)




(b)

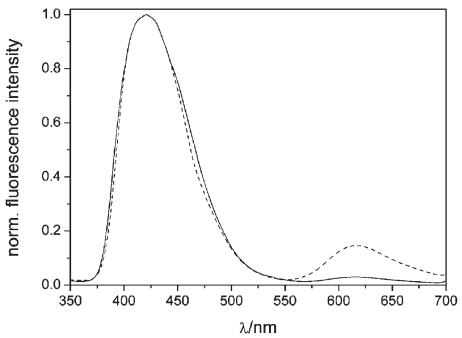



**Fig. 4** (a) Cleavage of peptide substrate **9**. *Reagents and conditions*: (i) 25 μM TRIS/HCl pH = 8.0, 100 μM NaCl, 30 °C, 24 h. The peptide concentration was 0.25 mM, the amount of thrombin used was 20 U. The cleavage reaction was monitored by reversed-phase HPLC and LC-MS. (b) Fluorescence emission spectra of **9** before (dashed line) and after (solid line) cleavage with thrombin upon excitation at 340 nm in $CH_3CN$–$H_2O$ 1 : 1 at a concentration of 2 μM.

absorption band of the acceptor, a feature crucial for a strong and effective FRET (Fig. 2).

Having confirmed the spectroscopic properties of the FRET dyes, peptide **8** was synthesised bearing the donor-amino acid at the C- and the acceptor at the N-terminus (Fig. 3a). Incorporated into this peptide was the recognition sequence and cleavage site of the protease thrombin, which was chosen due to its availability, low cost and easy handling. Comparison of the fluorescence spectra of a mixture of **6** and **7** with **8** showed an efficient FRET in peptide **8** (Fig. 3b).

Spectra recorded after excitation of the donor at 340 nm displayed only the emission of the donor at 420 nm for the mixture, but an additional band at 618 nm for **8** bearing both dyes. This emission cannot result from a direct excitation of the acceptor, because its absorbance at 340 nm is negligible. Instead, it is caused by indirect excitation due to FRET *via* the donor, indicating that the distance and orientation of the two dyes allowed effective energy transfer. Unfortunately, peptide **8** was not prone to cleavage by thrombin, possibly due to steric hindrance by the Ru-complex. In order to circumvent this problem, peptide **9** was synthesised, having a larger distance between donor and acceptor. With this substrate, the enzymatic cleavage could be carried out with an efficiency of 95% (Fig. 4a). The ratio of the fluorescence intensities of the cleavage mixture and **9** at 618 nm was about 1 : 5 and can be used to monitor the reaction of the substrate with the appropriate enzyme (Fig. 4b).

In conclusion, we report the synthesis and application of a new donor–acceptor pair for FRET. Both dyes can be synthesised in a very straightforward way with decent yields in most steps. Additionally, they have shown to be very stable under various conditions including strong acids and basic environments indicating their robustness. Due to their easy introduction into any peptide at any position, the modified chromophores can be regarded as a modular system and can be used along with the standard building blocks in SPPS. Their excellent spectroscopic properties and the possibility to be measurable both in normal and time-resolved mode makes them a very good alternative to conventional FRET pairs, especially when high sensitivity is required. At the moment, investigations are underway to optimise the peptide substrate and the conditions for the FRET assay and to extend its scope to other proteases. In future, these dyes might also be useful as labels to measure supramolecular interactions, for example protein–protein interactions.

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