A New Robust and Highly Sensitive FRET Donor–Acceptor Pair: Synthesis, Characterization, and Application in a Thrombin Assay

by **Eva K. Kainmüller** and **Willi Bannwarth***

Institut für Organische Chemie und Biochemie, Albert-Ludwigs-Universität Freiburg, Albertstr. 21, D-79104 Freiburg
(phone: +49-761-2036073; fax: +49-761-2038705; e-mail: willi.bannwarth@organik.chemie.uni-freiburg.de)

The synthesis of a new, robust fluorescence-resonance-energy-transfer (FRET) system is described. Its donor chromophore is derived from an *N*-allyl-substituted quinolinone attached to 4-bromophenylalanine *via Heck* cross-coupling. The resulting Fmoc-protected derivative **11** was used as building block in solid-phase peptide synthesis (SPPS). As FRET acceptor, a sulfonylated ruthenium(II)–bathophenanthroline complex with a peripheral COOH function was prepared for covalent attachment to target molecules. The UV/VIS absorption and emission spectra of peptides bearing only the donor (D) or acceptor (A) dye showed a good overlap of the emission band of the donor with the absorption band of the acceptor. The fluorescence spectra of a peptide bearing both dyes revealed an additional emission after excitation of the donor, which is due to indirect excitation of the acceptor *via* FRET. The long fluorescence lifetime of the Ru^{II} complex (0.53 μs) makes it well-suited for time-resolved measurements. As a first application of this new FRET system, the peptide **18**, with the recognition sequence for the protease thrombin, flanked by the two dyes, was synthesized and successfully cleaved by the enzyme. The change in the ratio of the fluorescence intensities could be determined.

1. Introduction. – A very important task in molecular biology is the elucidation of reaction pathways and mechanisms involved in living cells and whole organisms. Research on this topic not only gives fascinating insight into the fundamental question of how life works, but also has a great impact in medicine and pharmacology. This is due to the fact that a multitude of biologically relevant molecules involved in these processes have been identified as potential drug targets against various diseases.

As analyses of these kinds of mechanisms and the identification of the molecules involved therein are difficult, the development of new methods is an ongoing task. In this context, fluorescence-resonance-energy-transfer (FRET) systems have proven to be important tools for the elucidation of distance-dependent interactions on the molecular level. With these systems, changes in distances on a nanometer scale and in real-time mode can be monitored, and are, therefore, especially well-suited for the characterization of biochemical events both *in vitro* and *in vivo*. Among these have been the binding of ligands to proteins as well as their subsequent conformational changes [1–5], DNA–protein complexation [6–10], and RNA folding and catalysis [11–13]. Another subject of current interest is the study of interactions between three different biomolecules [14], a task that is impossible to accomplish by any other biochemical method at present. Apart from this, FRET has also been applied in diagnostics and

drug research, where tools like molecular beacons [15], TaqMan probes [16], or fluorescent enzyme substrates [17–22] use the same principle.

The FRET technique is based on the transfer of fluorescence energy between a donor (D) fluorophore and an acceptor (A) fluorophore. The dyes must be chosen in a way such that the emission wavelength of the donor overlaps with the absorption wavelength of the acceptor, thereby enabling excitation energy to be transferred from the donor to the acceptor. The intensity of FRET depends on the quality of the spectral overlap of the dyes, their extinction coefficients, and their orientation to and distance from each other. According to *Förster*'s equation [23], FRET decreases with r^{-6}, when r is the distance between the two chromophores. For the detection of a meaningful signal, r should be in the range of 10–100 Å.

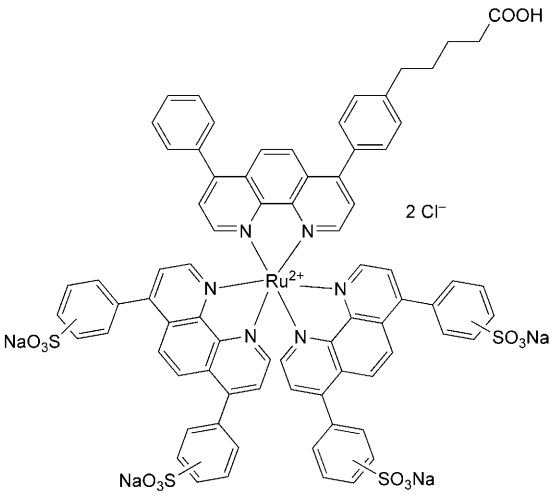
An extension of this principle is represented in ternary systems comprising a donor, an acceptor I, and an acceptor II. Energy transfer is possible to occur between the first *and* the second pair of chromophores, provided that their optical properties match the requirements described above. In this way, the measurement of two different distances can be achieved.

Another feature of a FRET system is the possibility to detect signal changes in a ratio-dependent way, *i.e.*, to monitor both the decrease in the emission of the donor and the increase in the emission of the acceptor, or *vice versa*. This enhances the sensitivity of the method in comparison to probes bearing only one reporter dye, and it is especially interesting when dealing with highly diluted samples. A number of different donor–acceptor pairs have been reported and successfully employed [24–26], whereas only very few ternary systems have been developed up to now [27][28]. In any case, the need for even more sensitive probes remains. This problem could be overcome by using chromophores that can be studied in a time-resolved mode. Among these, lanthanide-, especially Eu and Tb complexes, have been reported to be suitable candidates due to their strong fluorescence and excited-state lifetimes of up to milliseconds [29][30]. Usually, they are employed as caged chelates [31][32], but leakage of Eu can still occur. Furthermore, their very long fluorescence lifetimes make it impossible to resolve events faster than those in the range of milliseconds.

We, therefore, focused on Ru^{II} charge-transfer (CT) complexes as alternative FRET acceptors. These substances not only exhibit high thermodynamic stabilities, they are chemically inert, can be readily prepared, and exhibit excited states with a decay time in the microsecond range that allows for time-resolved measurements [33]. In a preliminary communication [34], we have described the use of the ruthenium(II)–bathophenanthroline complex **1** as a FRET acceptor in combination with the carbostyril derivative **2** as donor [34].

Herein, we report the *details* of the syntheses of the two dyes, their physical properties, their insertion into peptide substrates, and substrate optimization for a thrombin assay. Our final aim is to extend this binary FRET system into the first part of a ternary system.

2. Results and Discussion. – 2.1. *Synthesis of the Acceptor.* The Ru complex **1** was synthesized according to *Scheme 1* by adaptation of a simple known two-step procedure starting from $RuCl_3 \cdot H_2O$ [35][36]. In the first step, two ligands based on bathophenanthrolinedisulfonic acid disodium salt (bpds) were introduced to enhance solubility in



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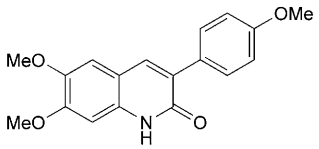

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aqueous media, affording $[Ru(bpds)_2Cl_2]$ (**3**). Next, complexation with **4** was carried out, which gave **1** in 80% overall yield over both steps. Complex **1** is equipped with a COOH function allowing for covalent attachment (*via* an amide bond) to target molecules bearing an amino group. Activation of the COOH group of **1** was performed with *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumtetrafluoroborate (TSTU), which yielded the succinyl-activated ester **5** in 73% yield [37].

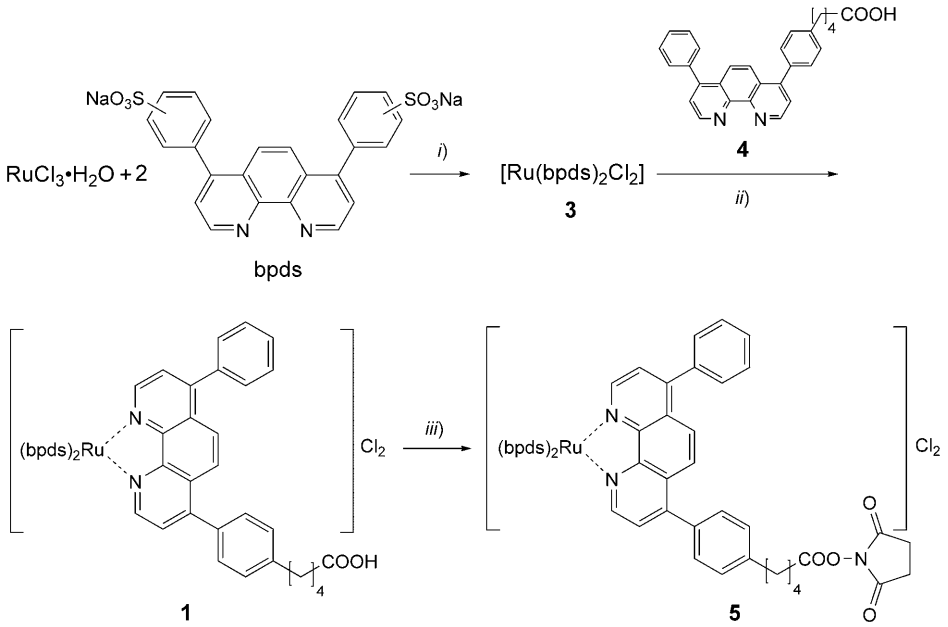
The Ru^{II} complex **1** exhibits a characteristic metal-to-ligand CT absorption band at 440–464 nm, and a strong red emission at 618 nm. It is completely stable, even under strongly acidic and basic conditions, as corroborated by exposure to 95% CF_3COOH (TFA) or 2N aq. NaOH, which resulted in no decomposition at all. Of the two different ligands in **1**, sulfonylated bathophenanthroline (bpds) is commercially available, and compound **4** was synthesized according to *Scheme 2* [38].

The key steps of the synthesis of **4** were two *Skraup* reactions: in the first one, 2-nitroaniline and β-chloropropiophenone (=3-chloro-1-phenylpropan-1-one) were reacted to **6** in 83% yield, followed by hydrogenation to afford the intermediate **7** in 77% yield. Next, **7** was combined with the *Friedel–Crafts* product **8**, obtained from **9**, which afforded a mixture of **4** (46%) and **10** (27%). The pure ligand **4** was then obtained in 72% yield by saponification.

2.2. *Synthesis of the Donor.* The carbostyril derivative **2** was envisaged as donor entity due to its chemical and spectroscopic properties. Being a lactam, it is chemically more stable than the corresponding lactones (coumarins). With an absorption maximum at 368 nm and an emission maximum at 435 nm, in combination with a high extinction coefficient ($\varepsilon = 20800\,M^{-1}\,cm^{-1}$), compound **2** is well-suited as a FRET donor [39]. For its use in solid-phase peptide synthesis (SPPS), the Fmoc-protected[1]) unnatural amino acid **11**, containing the donor as a pendant arm, was synthesized

[1]) Abbreviations: Fmoc, Boc, and Su refer to [(9*H*-fluoren-9-yl)methoxy]carbonyl, (*tert*-butoxy)carbonyl, and succinimide residues, resp.

Scheme 1. *Synthesis of the Activated Ruthenium(II)–Bathophenanthroline Complex* **5** *for Solid-Phase Peptide Synthesis*



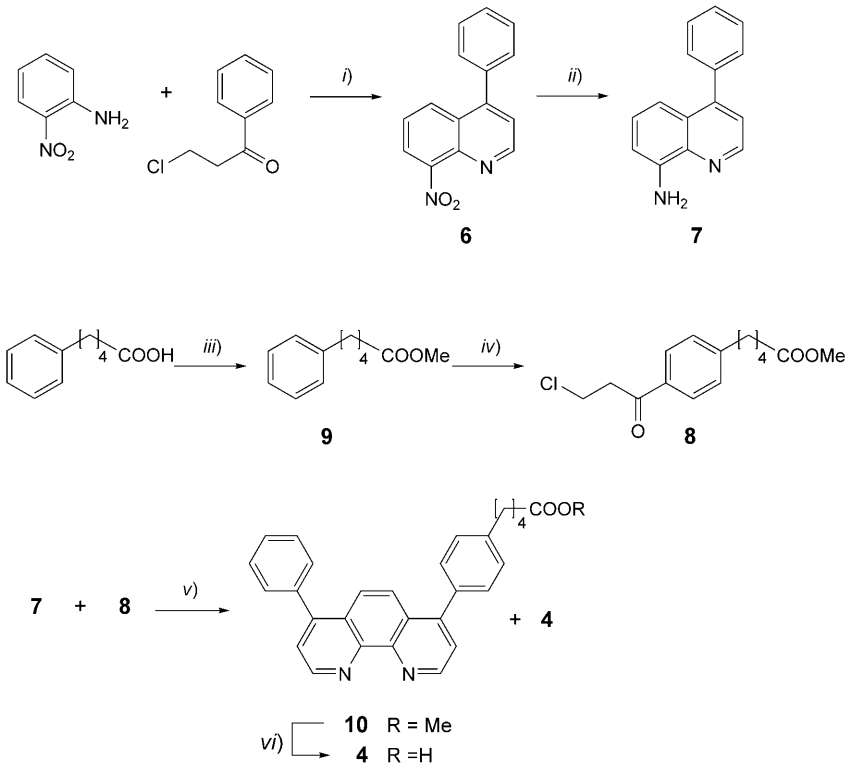
i) LiCl, DMF; quant. *ii*) MeOH/H_2O; 80%. *iii*) TSTU (= *O*-(*N*-succinimidyl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumtetrafluoroborate), i-Pr_2NEt, DMF; 73%.

according to *Scheme 3*. It is stable under the conditions employed during peptide synthesis and during cleavage and workup (95% TFA, 20% piperidine in DMF), thus being applicable directly during peptide assembly on solid support.

Briefly, the quinolinone derivative **2** [39][40] was *N*-alkylated under microwave assistance to yield the allyl intermediate **12** in 83% yield. Subsequent cross-coupling of **12** with Boc-protected[1]) racemic 4-bromophenylalanine methyl ester [41] *via Heck* reaction turned out to be the crucial step of the synthesis. Initial experiments afforded the desired product **13** in only *ca*. 40% yield. However, optimization of the reaction conditions (temperature, base, ratio of reagents) finally led to **13** in a yield of *ca*. 90%, both when performed in a microwave oven or with conventional heating. Hydrogenation of **13** over Pd/C then afforded **14** in 92% yield. This reduction step was necessary to impart both chemical stability and flexibility to the molecule. Treatment of **14** with 50% TFA led to cleavage of the Boc group, and insertion of the Fmoc group with Fmoc-OSu[1]) as reagent yielded the 'donor-labeled' target amino acid **11** in 97% yield over two steps. Compared to the starting material **2**, no change of the absorption and emission characteristics could be observed for neither **11** nor **12**, as can be seen from *Fig. 1*. The additional bands at *ca*. 300 nm in **11** are caused by the aromatic moieties of the Fmoc group.

2.3. *Spectroscopic Properties of FRET Dyes.* To evaluate the applicability of the chromophores as FRET donor and acceptor, respectively, the model peptides **15**–**17**

Scheme 2. *Synthesis of the Ligand* **4**

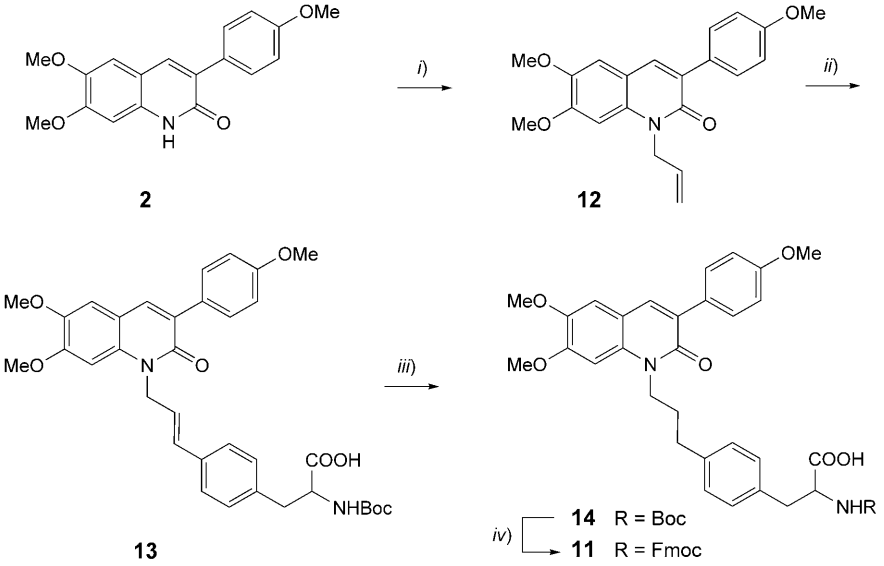


i) H_3PO_4, $As_2O_5 \cdot (H_2O)_n$; 83%. *ii*) H_2, Pd/C, MeOH, toluene; 77%. *iii*) MeOH, H_2SO_4; 93%. *iv*) 3-Chloropropanoyl chloride, $AlCl_3$, CH_2Cl_2; 83%. *v*) H_3PO_4, $As_2O_5 \cdot (H_2O)_n$; 73% (**10**+**4**). *vi*) NaOH, EtOH, H_2O; 72%.

were prepared by standard Fmoc/Boc chemistry [42]. They bear the donor and/or the acceptor dye. Incorporated into **15** was the recognition sequence and cleavage site of the protease thrombin. Peptides **16** and **17** represent the labeled fragments to be expected as cleavage products (*Scheme 4*). For the coupling of the donor, the Fmoc-protected unnatural amino acid **11** was employed as building block during SPPS, whereas **5** was used to attach the Ru complex **1** to the N-terminus of the peptides.

Excitation and emission spectra of **16** and **17** revealed a very good overlap of the emission band of the donor and the absorption band of the acceptor (*Fig. 2*), which is a crucial requirement for strong FRET. Furthermore, the excitation spectrum of the Ru complex showed a minimum at 350 nm, the wavelength at which the donor has its excitation maximum. Hence, there will be hardly any direct excitation of the acceptor. Finally, the *Stokes* shift of the N-terminally attached Ru acceptor complex is rather large: the excitation at 450 nm and the emission at *ca.* 620 nm are well-separated, allowing for undisturbed fluorescence detection, without interference of the donor emission.

Scheme 3. *Synthesis of the FRET-Donor Amino Acid* **11**



i) KHMDS (=potassium hexamethyldisilazane), allyl bromide, THF, microwave heating; 83%. *ii*) Boc-protected racemic 4-bromophenylalanine methyl ester, $[Pd(OAc)_2]$, Ph_3P, Cs_2CO_3, DMF, H_2O, microwave heating; 90%. *iii*) H_2, Pd/C, MeOH; 92%. *iv*) 1. CF_3COOH/CH_2Cl_2/i-Pr_3SiH 10:10:0.5; 2. Fmoc-OSu[1]), aq. Na_2CO_3/acetone; 97%.

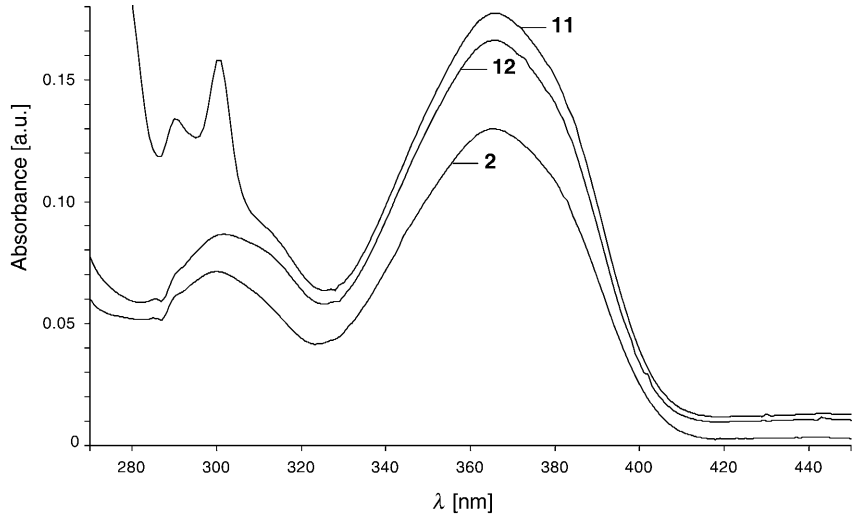
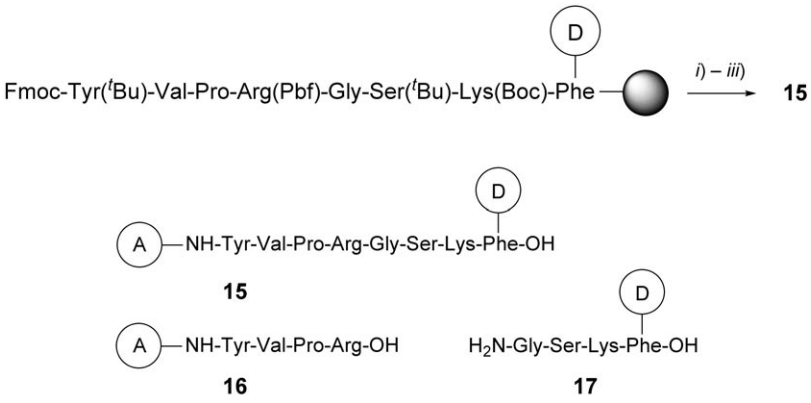


Fig. 1. *UV/VIS Absorption spectra of* **2**, **11**, *and* **12** *in DMF*

As desired, comparison of the fluorescence spectra of **15** with a mixture of **16** and **17** indicated an efficient FRET in peptide **15** (*Fig. 3*). The spectrum recorded after excitation of the donor at 350 nm displayed only the emission of the donor at 420 nm for the

Scheme 4. *Sequences of Peptides* **15**–**17**, *and Representative Synthesis of* **15**. The FRET donor and acceptor chromophores are represented by *D* and *A*, resp.



i) 20% Piperidine in DMF, 15 min. *ii*) **5**, i-Pr_2NEt, DMF, 2 d. *iii*) CF_3COOH/CH_2Cl_2/i-Pr_3SiH 95 : 3 : 2, 3 h.

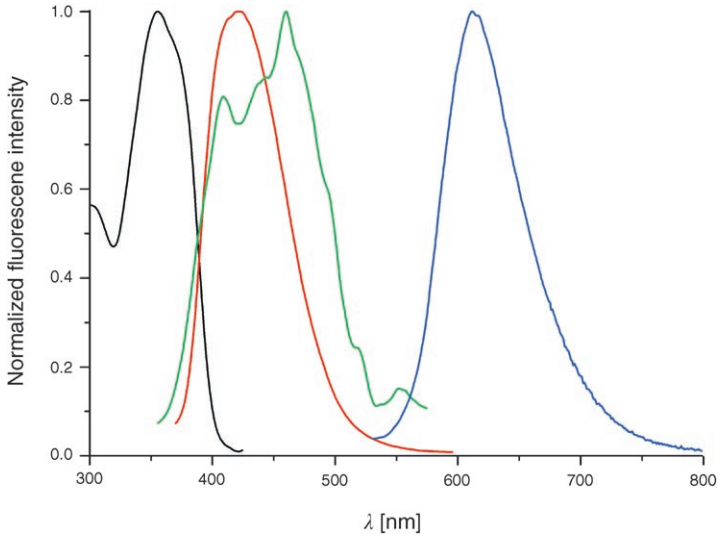


Fig. 2. *UV/VIS Excitation/emission spectra of peptides* **16** (green/blue) *and* **17** (black/red)

mixture of **16**/**17**, but an additional band at 618 nm for **15**. This emission results from the indirect excitation of the acceptor by the donor due to FRET, and not from direct excitation of the Ru complex, which has a negligible absorbance at 350 nm, as mentioned above. This indicated that the two chromophores not only possess complementary spectroscopic properties, but also that their distance and orientation in the peptide is favorable for efficient energy transfer.

The lifetime τ of the donor in the absence of FRET (peptide **17**) was found to be 0.61 ns; in the presence of FRET (peptide **15**), τ was found to be 0.54 ns (*Fig. 4*). For

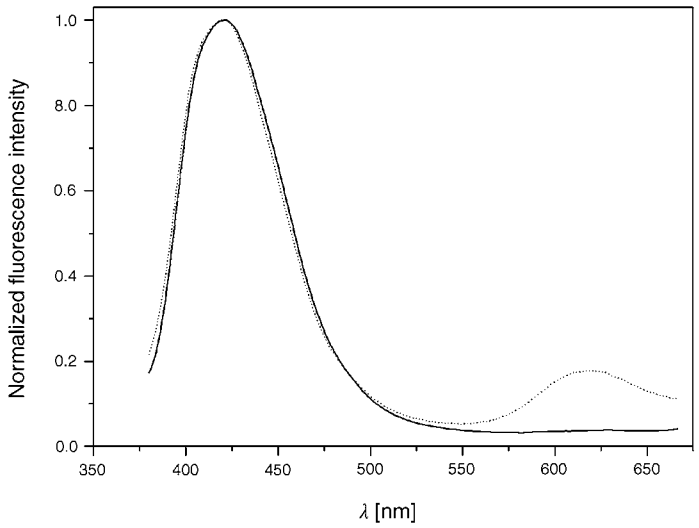


Fig. 3. *Fluorescence-emission spectra of* **15** (…) *and of a mixture of* **16**/**17** (—)

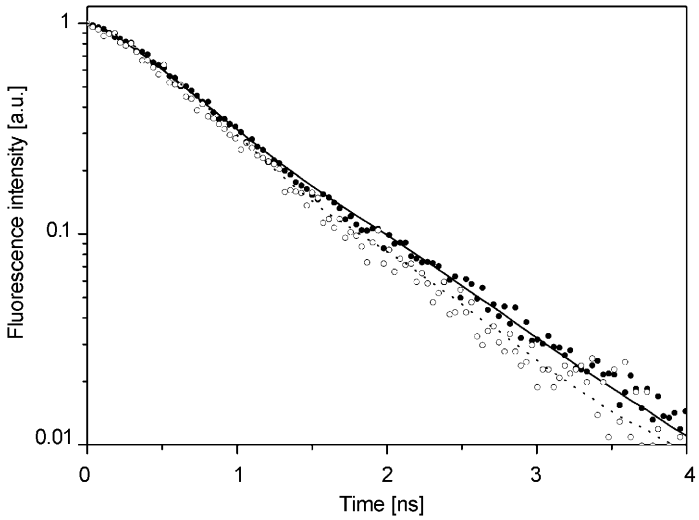


Fig. 4. *Fluorescence-relaxation curves of the donor chromophore in* **17** (filled circles) *and in* **15** (open circles) *at excitation and emission wavelengths of 381 and 420 nm, resp.*

the lifetime of the acceptor in peptide **15**, a value of 0.53 μs was obtained (*Fig. 5*). This factor of 10^3 between the lifetimes of the donor and the acceptor is well-suited for time-gated spectroscopy.

Unfortunately, peptide **15** was not soluble in the reaction buffer used for the protease reaction, nor was it accessible to cleavage by thrombin, probably due to insolubility and steric hindrance by the terminal Ru complex. Therefore, further optimization of the protease substrate was necessary.

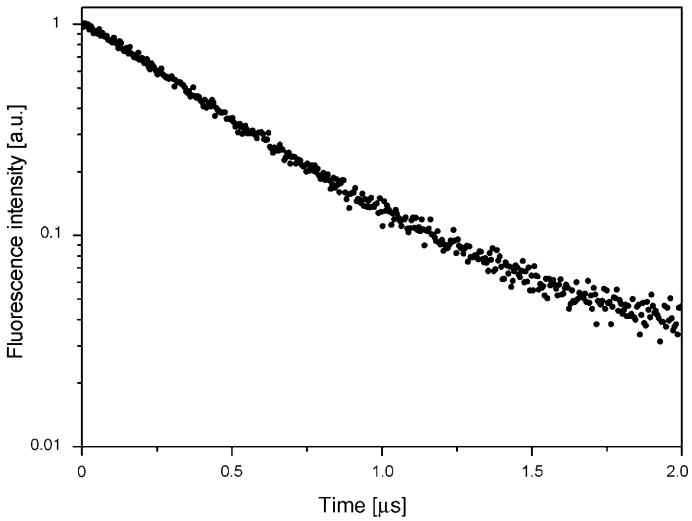
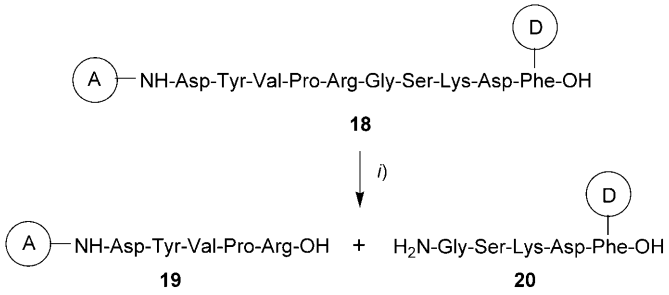


Fig. 5. *Fluorescence-relaxation curve of the acceptor chromophore in* **15** *at excitation and emission wavelengths of 358 and 620 nm, resp.*

2.4. *Optimization of the Peptide Substrate and Thrombin Assay.* To determine the optimal distance between the donor and acceptor dyes in the protease substrate regarding the intensity of the FRET and the cleavability by the enzyme, a range of peptides were synthesized. All sequences comprised the recognition sequence for thrombin, as well as the FRET donor and the FRET acceptor, but they varied in the number and nature of the amino acids between the two chromophores.

Our studies showed that the sequence of peptide **18** was the best compromise between cleavability by the enzyme and FRET intensity (*Scheme 5*, *Fig. 6*).

Scheme 5. *Enzymatic Cleavage of Peptide* **18**



i) Thrombin, 25 mM *Tris*·HCl (pH 8.0), 100 mM NaCl, 30°, 24 h.

The substrate **18** was hydrolyzed to *ca.* 95% by thrombin, the ratio of its FRET intensities before and after the reaction being about 11 : 1. Such a signal change is readily detectable and can be used to monitor the reaction of the substrate with the appro-

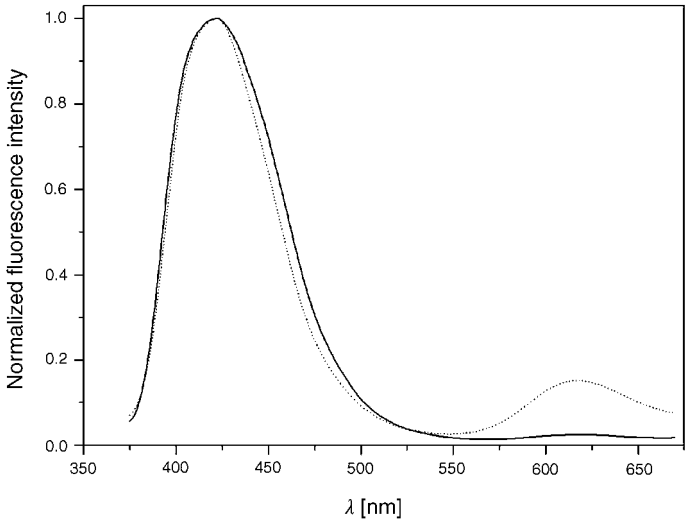


Fig. 6. *Fluorescence-emission spectra of peptide* **18** *before* (…) *and after* (—) *cleavage with thrombin*

priate protease. Furthermore, the knowledge gained during this work should be helpful to establish similar assays for other hydrolytic enzymes.

3. Conclusions. – A new pair of acceptor (**1**) and donor (**2**) dyes was developed for FRET measurements in both normal and time-resolved modes. Both dyes were readily synthesized in reasonable-to good yields over all steps, and exhibit very good chemical stability towards strongly acidic and basic conditions. They were designed in a way allowing incorporation into peptides, as verified by the synthesis of a peptide substrate for the protease thrombin. Enzymatic hydrolysis was then followed by fluorescence measurements before and after the reaction with thrombin, corroborating the excellent spectroscopic properties of the dyes. Due to the possibility of time-resolved FRET measurements, the present dye combination might serve as a very good alternative to conventional systems, especially when high sensitivity is required.

The main future goal will be to employ these chromophores as donor and acceptor-I dyes in a ternary FRET system. A suitable acceptor II has already been identified, and work in our group is currently underway to establish a system that can be applied to the measurement of two different distances and their changes relative to each other. Finally, these dyes will be used to label three biomolecules and monitor their supramolecular interactions.

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Experimental Part

General. All reagents were purchased from commercial sources and used without further purification. Amine-free DMF (*Roth*) was employed throughout peptide syntheses, water for the protease assays was purified *via* a *Direct-Q* system (*Millipore*), and THF was dried over Na/benzophenone before use. Microwave-assisted reactions were performed with a *Discover* apparatus (*CEM GmbH*). Column chromatography (CC): silica gel *60* (*Merck*). Peptide synthesis: semi-automatic *SP-4000* synthesizer (*Labortec AG*). Semi-prep. HPLC: *Agilent-1100* system, with *Nucleosil 100-5 C18 PPN* columns (*Macherey-Nagel*) for peptides or a *Source 5RPC ST 4.6/150* column (*Amersham Pharmacia Biotech*) for Ru complexes. Fluorescence-emission spectra: *Perkin-Elmer LS45* spectrometer. Fluorescence-lifetime decay: instrument from *IBH Consultants, Ltd.* (Glasgow, Scotland), with an N_2 flash lamp as excitation source (microsecond range) or a *FluoTime-200* (*Picoquant*) pulsed LED (nanosecond range). NMR Spectra: at 300 or 400 MHz (^{1}H), and at 75.5, 100.6, or 125.7 MHz (^{13}C); chemical shifts δ in ppm rel. to the respective solvent signals, J in Hz. MS: *Finnigan MAT-8200* (EI), *TSQ-7000* (ESI), or *LTQ FT* (HR-ESI) mass spectrometers; in m/z.

Synthesis of Complex **5**. Starting from bathophenanthrolinedisulfonic acid disodium salt trihydrate (bpds; 460 mg, 0.78 mmol) and $RuCl_3 \cdot H_2O$ (100 mg, 0.38 mmol), **3** was synthesized in quant. yield. Compound **3** (1.34 g, 1.07 mmol) was then reacted with **4** (607 mg, 1.4 mmol) under modified work-up conditions, *i.e.* instead of precipitation, the complex was purified by CC (SiO_2; CH_2Cl_2/MeOH 75 : 25 containing 0.05% of CF_3COOH (TFA)) to yield **1** (1.44 g, 80%). Activation of **1** as the *O*-succinimide ester with an excess of TSTU was carried out to afford **5** in 73% yield, the progress of the reaction being monitored by LC/MS.

*8-Nitro-4-phenylquinolin*e (**6**). A suspension of 2-nitroaniline (2.0 g, 14.5 mmol, 1.0 equiv.), As_2O_5 hydrate (4.0 g, 17.4 mmol, 1.2 equiv.), and 85% H_3PO_4 (14.5 ml) was heated at 100°. Then, β-chloropropiophenone (3.4 g, 20.3 mmol, 1.4 equiv.) was added within 5 min, and the mixture was stirred at 130° for 2 h. After cooling to r.t. and addition of ice-water (100 ml), the mixture was made to alkaline with conc. NH_3. The org. phase was extracted with toluene (5 × 50 ml), and the combined org. layers were washed with H_2O (100 ml), dried (Na_2SO_4), and concentrated under reduced pressure. Dissolution of the remaining black oil in CH_2Cl_2 and filtration through a short plug of SiO_2 gave a crude, which, after removal of the solvent, was recrystallized from EtOH to yield pure **6** (3.0 g, 83%). Light-yellow crystals. M.p. 126° (EtOH). ^{1}H-NMR (300 MHz, $CDCl_3$): 7.47–7.50 (*m*, 3 arom. H); 7.53–7.59 (*m*, 4 arom. H); 8.01 (*dd*, J = 7.5, 1.2, H–C(5)); 8.13 (*dd*, J = 8.6, 1.2, H–C(7)); 9.08 (*d*, J = 4.4, H–C(2)). ^{13}C-NMR (100.6 MHz, $CDCl_3$): 122.92; 123.31; 125.23; 127.87; 128.94; 129.14; 129.55; 130.11; 136.88; 140.08; 148.93; 152.08. EI-MS: 250 (100, M^+), 220 (33), 203 (35), 192 (95), 176 (71), 165 (42), 151 (26), 88 (21). Anal. calc. for $C_{15}H_{10}N_2O_2$: C 72.0, H 4.0, N 11.2; found: C 71.8, H 4.2, N 11.1.

4-Phenylquinolin-8-amine (**7**). Compound **6** (10.0 g, 40.0 mmol) was dissolved in MeOH/toluene 1 : 1 (120 ml), and 10% Pd on carbon (250 mg) was added. The suspension was agitated under H_2 atmosphere (1 bar) for 24 h. The crude mixture was adsorbed on SiO_2, and then subjected to CC (SiO_2; cyclohexane/AcOEt 5 : 1) to afford **7** (6.8 g, 77%). Yellow solid. ^{1}H-NMR (300 MHz, $CDCl_3$): 5.08 (br. *s*, NH_2); 6.94 (*dd*, J = 7.3, 1.4, H–C(7)); 7.20 (*dd*, J = 8.5, 1.3, H–C(5)); 7.28–7.30 (*m*, 2 arom. H); 7.48–7.51 (*m*, 5 arom. H); 8.78 (*d*, J = 4.2, H–C(2)). ^{13}C-NMR (100.6 MHz, $CDCl_3$): 109.85; 114.09; 121.64; 127.28; 128.13; 128.36; 129.48; 138.59; 138.77; 144.26; 146.85; 148.28. EI-MS: 220 (100, M^+), 191 (15), 165 (16), 110 (11), 96 (13). Anal. calc. for $C_{15}H_{12}N_2$: C 81.8, H 5.5, N 12.7; found: C 81.6, H 5.8, N 12.5.

Methyl 5-[4-(3-Chloropropanoyl)phenyl]pentanoate (**8**). To a mixture of $AlCl_3$ (5.15 g, 0.04 mol, 3.7 equiv.) in CH_2Cl_2 (5.5 ml), 3-chloropropanoyl chloride (1.1 ml, 11.4 mmol, 1.1 equiv.) was added. Then, after cooling with a water-bath, methyl 5-phenylpentanoate (**9**; 2.0 g, 10.4 mmol, 1.0 equiv.) was added over 10 min. The mixture was stirred at r.t. for 1 h. The reaction was quenched with a mixture of H_2O (35 ml), ice (30 g), and CH_2Cl_2 (30 ml), and the aq. layer was separated and extracted with CH_2Cl_2 (3 × 30 ml). The combined org. phases were washed with H_2O (30 ml), aq. 5% $NaHCO_3$ soln. (30 ml), and H_2O (30 ml), and the solvent was removed under reduced pressure. The remaining solid was recrystallized at 0° from Et_2O to afford **8** (2.43 g, 83%) as colorless crystals. M.p. 41° (Et_2O). ^{1}H-NMR (300 MHz, $CDCl_3$): 1.68 (*m*, CH_2CH_2); 2.34 (*m*, CH_2COOMe); 2.70 (*m*, CH_2Ph); 3.43 (*t*, J = 6.8, CH_2COPh); 3.66 (*s*, COOMe); 3.92 (*t*, J = 6.9, $ClCH_2$); 7.28 (*d*, J = 8.5, 2 arom. H); 7.88 (*d*, J = 8.2, 2 arom. H). ^{13}C-

NMR (100.6 MHz, $CDCl_3$): 24.51; 30.43; 33.82; 35.63; 38.84; 41.22; 51.55; 128.31; 128.79; 134.36; 148.52; 173.89; 196.33. EI-MS: 282 (42, M^+), 219 (100), 195 (32), 187 (41), 159 (19), 131 (37), 91 (54). Anal. calc. for $C_{15}H_{19}ClO_3$: C 63.7, H 6.8; found: C 63.6, H 6.9.

5-[4-(7-Phenyl-1,10-phenanthrolin-4-yl)phenyl]pentanoic Acid (**4**) *and Methyl 5-[4-(7-Phenyl-1,10-phenanthrolin-4-yl)phenyl]pentanoate* (**10**). Compound **7** (2.8 g, 12.7 mmol, 1.0 equiv.) and As_2O_5 hydrate (3.5 g, 15.23 mmol, 1.2 equiv.) were suspended in 85% H_3PO_4 (14.5 ml), and then heated at 110°. To the resulting black soln. was added **8** (5.0 g, 17.7 mmol, 1.4 equiv.) within 5 min, and the mixture was stirred at 130° for another 1.5 h. After cooling to r.t., ice-water (150 ml) was added, the pH was adjusted to 5.0 with 4N aq. NaOH soln., and the aq. phase was extracted with CH_2Cl_2 (5 × 100 ml). The combined org. layers were washed with brine (200 ml), dried (Na_2SO_4) and concentrated under reduced pressure. The residue was adsorbed on SiO_2 and purified by CC (SiO_2; CH_2Cl_2/MeOH 300:1 → 30:1) to afford a crude, which was recrystallized from MeOH to yield 2.51 g (46%) of pure **4**, together with **10** (1.54 g, 27%), which contained a trace amount of an nonpolar impurity, but was hydrolyzed without further purification. Thus, **10** (1.5 g, 3.36 mmol, 1.0 equiv.) in EtOH (9 ml) was treated with NaOH (0.45 g, 11.25 mmol, 3.3 equiv.) in H_2O (2.8 ml) and heated at reflux for 2 h. After concentration under reduced pressure, the residue was taken up in a mixture of CH_2Cl_2 (30 ml) and H_2O (40 ml). The aq. layer was adjusted to pH 3 with H_3PO_4, and extracted with CH_2Cl_2 (30 ml). The combined org. phases were washed with brine (20 ml), dried (Na_2SO_4), and concentrated under reduced pressure. The crude product was adsorbed on SiO_2 and subjected to CC (SiO_2; CH_2Cl_2/MeOH 50:1) to afford **4** (1.04 g, 72%).

Data of **4**. Pale-yellow solid. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.77 (*m*, CH_2CH_2); 2.45 (*m*, CH_2COOH); 2.76 (*m*, CH_2Ph); 7.35 (*d*, *J* = 8.1, 2 arom. H); 7.45 (*d*, *J* = 8.1, 2 arom. H); 7.53 (*m*, 5 arom. H); 7.63 (*d*, *J* = 4.5, H–C(3)); 7.64 (*d*, *J* = 4.5, H–C(8)); 7.88 (*d*, *J* = 9.4, H–C(6)); 7.92 (*d*, *J* = 9.5, H–C(5)); 9.30 (*d*, *J* = 4.7, H–C(2)); 9.31 (*d*, *J* = 4.5, H–C(9)); 10.9 (br. *s*, COOH). ^{13}C-NMR (100.6 MHz, $CDCl_3$): 24.41; 30.75; 33.89; 35.35; 123.53; 123.57; 123.93; 124.19; 126.42; 126.51; 128.49; 128.62; 128.68; 129.68; 129.71; 135.44; 137.96; 142.63; 146.74; 146.76; 148.51; 148.54; 149.69; 149.73; 178.58. EI-MS: 432 (100, M^+), 388 (8), 345 (46), 331 (8). Anal. calc. for $C_{29}H_{24}N_2O_2$: C 80.5, H 5.6, N 6.5; found: C 80.0, H 5.7, N 6.4.

Data of **10**. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.70–1.76 (*m*, CH_2CH_2); 2.38 (*m*, CH_2COOMe); 2.68–2.77 (*m*, CH_2Ph); 3.68 (*s*, COOMe); 7.35 (*d*, *J* = 8.1, 2 arom. H); 7.45 (*d*, *J* = 8.2, 2 arom. H); 7.53 (*m*, 5 arom. H); 7.61 (*d*, *J* = 4.5, H–C(3)); 7.62 (*d*, *J* = 4.5, H–C(8)); 7.87 (*d*, *J* = 9.5, H–C(6)); 7.91 (*d*, *J* = 9.4, H–C(5)); 9.28 (*d*, *J* = 4.5, H–C(2)); 9.29 (*d*, *J* = 4.7, H–C(9)).

*6,7-Dimethoxy-3-(4-methoxyphenyl)-1-(prop-2-en-1-yl)-1*H*-quinolin-2-one* (**12**). To a suspension of **2** (883 mg, 2.84 mmol, 1.0 equiv.) in THF (10 ml), a 0.5M soln. of potassium hexamethyldisilazane (KHMDS; 6.8 ml, 3.4 mmol, 1.2 equiv.) in toluene was added at −78°. The mixture was stirred for 30 min at this temp., and then allowed to reach r.t. Allyl bromide (0.75 ml, 8.5 mmol, 3.0 equiv.) was added, and the white suspension was placed for 15 min in a microwave oven at 120° (max. 15 bar, 200 W). Then, H_2O (70 ml) and $CHCl_3$ (70 ml) were added, the phases were separated, and the aq. layer was extracted with $CHCl_3$ (2 × 70 ml). The combined org. layers were dried ($MgSO_4$), filtered, and the solvent was removed under reduced pressure. The residue was purified by CC (SiO_2; cyclohexane/AcOEt 2:1) to yield **12** (828 mg, 83%). Light-yellow solid. ^{1}H-NMR (400 MHz, $CDCl_3$): 3.84 (*s*, MeO); 3.94 (*s*, MeO); 3.96 (*s*, MeO); 5.02 (*dt*, *J* = 4.8, 1.5, CH_2); 5.20 (*dd*, *J* = 17.2, 1.1, 1 H of CH=CH_2); 5.26 (*dd*, *J* = 10.4, 1.1, 1 H of CH=CH_2); 6.00 (*ddt*, *J* = 17.3, 10.4, 5.2, CH=CH_2); 6.80 (*s*, H–C(5)); 6.95 (*m*, H–C(3′,5′)); 6.99 (*s*, H–C(8)); 7.70 (*m*, H–C(2′,6′)); 7.71 (*s*, H–C(4)). ^{13}C-NMR (100.6 MHz, $CDCl_3$): 45.66; 55.40; 56.15; 56.28; 97.98; 109.36; 113.62; 114.43; 117.23; 129.27; 129.54; 130.17; 132.35; 134.47; 135.60; 145.23; 151.68; 159.40; 161.18. EI-MS: 351 (97, M^+), 336 (100), 320 (11), 310 (11), 292 (9). Anal. calc. for $C_{21}H_{21}NO_4$: C 71.8, H 6.0, N 4.0; found: C 71.6, H 6.4, N 3.9.

*4-[(1*E*)-3-[6,7-Dimethoxy-3-(4-methoxyphenyl)-2-oxoquinolin-1(2*H*)-yl]prop-1-enyl]-*N*-[(1,1-dimethylethoxy)carbonyl]phenylalanine* (**13**). A mixture of **12** (150 mg, 0.42 mmol, 1.0 equiv.), Boc-protected, racemic 4-bromophenylalanine methyl ester (229 mg, 0.66 mmol, 1.5 equiv.), [$Pd(OAc)_2$] (7.2 mg, 0.04 mmol, 0.09 equiv.), Ph_3P (25.2 mg, 0.1 mmol, 0.24 equiv.), and Cs_2CO_3 (416 mg, 1.26 mmol, 3.0 equiv.) was suspended in DMF (3 ml) and H_2O (1.5 ml). The mixture was heated at 80° for 10 h in a microwave

oven (30 W)[2]), the solvent was removed under reduced pressure, and the remaining solid was taken up in a mixture of CH_2Cl_2 (10 ml) and 2N aq. HCl (10 ml). The aq. phase was extracted with CH_2Cl_2 (5×5 ml), and the combined org. layers were dried (Na_2SO_4). After removal of the solvent under reduced pressure, the crude product was purified by CC (SiO_2; CH_2Cl_2/MeOH 100:1 → 95:5) and subsequently precipitated with pentane to afford **13** (232 g, 90%). Light-yellow powder. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.39 (*s*, Me_3C); 2.94–3.17 (*m*, $PhCH_2CH$); 3.84 (*s*, MeO); 3.94 (*s*, MeO); 3.95 (*s*, MeO); 4.47–4.56 (*m*, NHC*H*COOH); 4.96 (br. *d*, *J*=7.0, NH); 5.17 (*d*, *J*=5.3, NCH_2CH); 6.30 (*dt*, *J*=16.1, 5.8, CH_2CH=CH); 6.57 (*d*, *J*=16.0, CH=C*H*Ph); 6.90 (*s*, H–C(5)); 6.95 (*d*, *J*=8.8, H–C(3′,5′)); 7.01 (*s*, H–C(8)); 7.07 (*d*, *J*=8.1, 2 arom. H); 7.22 (*d*, *J*=8.1, 2 arom. H); 7.69 (*d*, *J*=8.8, H–C(2′,6′)); 7.74 (*s*, H–C(4)). ^{13}C-NMR (75.5 MHz, $CDCl_3$): 28.49; 37.77; 45.62; 54.63; 55.54; 56.38; 56.45; 80.19; 98.11; 109.60; 113.81; 114.78; 123.64; 126.64; 128.60; 129.40; 129.64; 129.91; 130.35; 132.72; 134.60; 135.03; 136.10; 145.56; 152.02; 155.80; 159.57; 161.52; 176.33. HR-MS: 615.2700 ($[M+H]^+$, $C_{35}H_{39}N_2O_8^+$; calc. 615.2706).

*4-{(1*E*)-3-[6,7-Dimethoxy-3-(4-methoxyphenyl)-2-oxoquinolin-1(2*H*)-yl]propyl}*-N-*[(1,1-dimethylethoxy)carbonyl]phenylalanine* (**14**). Compound **13** (2.11 g, 3.4 mmol) was dissolved in MeOH (60 ml), and 10% Pd on carbon (318 mg) was added to the soln. The mixture was agitated under H_2 atmosphere (1 bar) for 16 h. The catalyst was removed by filtration over *Kieselguhr*, and the solvent was distilled off at reduced pressure. The residue was purified by CC (SiO_2; CH_2Cl_2/MeOH 100:1 → 80:20) to yield **14** (1.96 g, 92%). Light-yellow powder. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.40 (*s*, Me_3C); 2.10 (*tt*, *J*=7.5, $CH_2CH_2CH_2$); 2.78 (*t*, *J*=7.3, CH_2CH_2Ph); 3.03–3.17 (*m*, $PhCH_2CH$); 3.79 (*s*, MeO); 3.84 (*s*, MeO); 3.91 (*s*, MeO); 4.31 (*t*, *J*=7.9, NCH_2CH_2); 4.49–4.60 (*m*, NHC*H*COOH); 5.02 (br. *d*, *J*=4.8, NH); 6.53 (*s*, H–C(5)); 6.92–6.96 (*m*, H–C(3′,5′), H–C(8)); 7.12 (*d*, *J*=8.1, 2 arom. H); 7.18 (*d*, *J*=8.1, 2 arom. H); 7.66 (*m*, H–C(2′,6′)); 7.67 (*s*, H–C(4)). ^{13}C-NMR (100.6 MHz, $CDCl_3$): 28.38; 28.58; 32.98; 37.58; 42.87; 54.47; 55.42; 56.19; 56.32; 80.28; 97.28; 109.63; 113.70; 114.75; 128.75; 129.30; 129.52; 129.69; 130.21; 134.04; 134.08; 135.67; 140.02; 145.37; 151.96; 155.51; 159.47; 161.42; 174.33. HR-MS: 617.28575 ($[M+H]^+$; $C_{35}H_{41}N_2O_8^+$; calc. 617.28574).

*4-{(1*E*)-3-[6,7-Dimethoxy-3-(4-methoxyphenyl)-2-oxoquinolin-1(2*H*)-yl]propyl}*-N-*{[(9*H*-fluoren-9-yl)methoxy]carbonyl}phenylalanine* (**11**). *a*) To a soln. of **14** (0.70 g, 1.14 mmol) in CH_2Cl_2 (10 ml), CF_3COOH (10 ml) and i-Pr_3SiH (0.5 ml) were added, and the mixture was stirred at r.t. TLC Examination (SiO_2; CH_2Cl_2/MeOH 4:1) showed complete conversion after 2 h. The mixture was co-evaporated with MeCN (3×30 ml) to dryness under reduced pressure, the residual solid was suspended in H_2O (30 ml), and extracted with Et_2O (5×20 ml). The org. phase was extracted with H_2O (20 ml), and the combined *aq*. layers were evaporated to dryness by azeotropic distillation with MeCN under reduced pressure. The resulting crude intermediate, *4-{(1*E*)-3-[6,7-dimethoxy-3-(4-methoxyphenyl)-2-oxoquinolin-1(2*H*)-yl]propyl}phenylalanine*, was used in the next step without further purification.

b) The *N*-deprotected amino acid (1.14 mmol, 1.0 equiv.) was suspended in aq. 9% Na_2CO_3 soln. (20 ml) and cooled in an ice bath. To this mixture, a suspension of Fmoc-OSu[3]) (0.58 g, 1.70 mmol, 1.5 equiv.) in acetone (10 ml) was added dropwise. After 1 h of stirring, the ice-bath was removed, more acetone (20 ml) was added, and the suspension was stirred for a further 3 h. Then, conc. HCl was added to neutralize the mixture, and the solvent was removed under reduced pressure. The residue was suspended in a NaCl soln. containing 2N HCl (60 ml), and extracted with CH_2Cl_2 (5×40 ml). The combined org. layers were re-extracted with brine (40 ml), dried (Na_2SO_4), warmed to 40°, and filtered. The residue was purified by CC (SiO_2; CH_2Cl_2/MeOH 100:1 → 90:10) to afford **11** (0.82 g, 97%). Light-yellow powder. ^{1}H-NMR (300 MHz, $CDCl_3$): 2.08 (*tt*, *J*=7.5, 7.3, $CH_2CH_2CH_2$); 2.78 (*t*, *J*=7.0, CH_2CH_2Ph); 3.13 (*m*, $PhCH_2CH$); 3.79 (*s*, MeO); 3.83 (*s*, MeO); 3.90 (*s*, MeO); 4.15 (*dd*, *J*=6.7, H–C(9) of Fmoc); 4.24–4.33 (*m*, 1 H of CH_2 of Fmoc, NCH_2CH_2); 4.44 (*dd*, *J*=10.3, 7.0, 1 H of CH_2 of Fmoc); 4.66 (*m*, NHC*H*COOH); 5.34 (*d*, *J*=8.1, NH); 6.51 (*s*, H–C(5)); 6.93–6.96 (*m*, H–C(3′,5′), H–C(8)); 7.10 (*d*, *J*=7.5, 2 arom. H); 7.17 (*d*, *J*=7.5, 2 arom. H); 7.24–7.29 (*m*, 2 arom. H of Fmoc); 7.36 (*t*, *J*=7.4, 2 arom. H of Fmoc); 7.51–7.54 (*m*, 2 arom. H of Fmoc); 7.63–7.66 (*m*, H–C(2′,6′), H–C(4)); 7.71 (*d*, *J*=7.5, 2 arom. H of

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[2]) The same result was obtained when the mixture was heated in a sealed vessel for 21 h at 90° with an oil bath.

[3]) Systematic name: 1-({[(9*H*-fluoren-9-yl)methoxy)]carbonyl}oxy)pyrrolidine-2,5-dione.

Fmoc). ^{13}C-NMR (125.7 MHz, $CDCl_3$): 28.41; 32.89; 37.54; 42.89; 47.13; 54.76; 55.33; 56.02; 56.16; 67.03; 97.01; 109.32; 113.64; 114.68; 119.93; 125.03; 127.02; 127.68; 128.70; 129.41; 129.52; 129.68; 130.17; 133.86; 133.88; 135.84; 139.91; 141.25; 143.72; 145.29; 151.88; 155.80; 159.38; 161.39; 174.10. HR-MS: 739.3014 ($[M+H]^+$, $C_{45}H_{43}N_2O_8^+$; calc. 739.3019).

Peptide Synthesis. The syntheses were carried out on a 0.02-mmol scale using Fmoc protocol and *Wang* resin (loading 0.75 mmol/g), with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluroniumtetrafluoroborate (TBTU) as coupling reagent. Standard Boc and *t*-Bu side-chain-protected amino acids were employed. As building block for the incorporation of the donor, compound **11** (30 mg, 0.04 mmol, 2.0 equiv.) was used. For the coupling of the Ru complex to the solid-phase-bound peptide at the N-terminus, a soln. of **5** (40 mg, 0.022 mmol, 1.1 equiv.) in DMF (1 ml) and i-Pr_2NEt (70 µl, 0.40 mmol, 20 equiv.) were added to the resin, which was agitated for 2 d. The peptides were deprotected and cleaved from the solid support by exposure to CF_3COOH/CH_2Cl_2/i-Pr_3SiH 95 : 3 : 2, and then purified by RP-HPLC and analyzed by LC/MS.

Data of **15**. ESI-MS: 1402 (100, $[M-4\,Na+4\,H-2\,Cl]^{2+}$), 935 (26, $[M-4\,Na+5\,H-2\,Cl]^{3+}$).

Data of **16**. ESI-MS: 1017 (100, $[M-4\,Na+4\,H-2\,Cl]^{2+}$).

Data of **17**. ESI-MS: 789 (100, $[M+H]^+$), 395 (12, $[M+2H]^{2+}$).

Data of **18**. ESI-MS: 1582 (100, $[M-4\,Na+4\,H-2\,Cl]^{2+}$), 1055 (30, $[M-4\,Na+5\,H-2\,Cl]^{3+}$).

Thrombin Assay. To a soln. of the peptide (0.125 µmol) in a mixture of 330 µl of H_2O and 50 µl of buffer (0.2M *Tris*·HCl (pH 8), 0.8M NaCl), 20 µl of human thrombin (20 U) was added. Before and after incubation of the soln. at 30° for 24 h, aliquots were taken, deactivated at 100° for 30 s, and diluted with the same volume of MeCN. The samples were analyzed by RP-HPLC to determine the cleavage efficiency, and by LC/MS to identify the cleavage fragments. For the measurement of fluorescence-emission spectra, the samples were further diluted with H_2O/MeCN 1:1 to give a final peptide concentration of *ca.* 0.4 µM. Excitation was performed at 350 nm.

Spectroscopic Measurements. Excitation/emission spectra were recorded at a peptide concentration of 5 µM in H_2O/MeCN 1 : 1. The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were 520 and 615 nm for peptide **16**, resp., and 350 and 440 nm for peptide **17**, resp. Fluorescence-emission spectra were recorded at a peptide concentration of 0.4 µM in H_2O/MeCN 1 : 1 at λ_{ex} 350 nm. Fluorescence-relaxation curves were established at a peptide concentration of 0.4 µM in H_2O/MeCN 1 : 1 at λ_{ex} 381 and λ_{em} 420 nm for the donor in both peptides **15** and **17**, and at λ_{ex} 358 and λ_{em} 620 nm, resp., for the acceptor in peptide **15**.

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