Application of a Highly Robust and Efficient Fluorescence-Resonance-Energy-Transfer (FRET) System in DNA

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Dedicated to Professor *Wolfgang Pfleiderer*

We report a feasibility study for the application of our newly developed highly efficient and robust fluorescence-resonance-energy-transfer (FRET) system to DNA. A 2′-oligodeoxynucleotide, **12**, equipped with a quinolinone derivative as donor and a (bathophenanthroline)ruthenium(II) complex as acceptor and having a single uridine as potential cleavage site under basic conditions revealed an intensive FRET, which almost vanished after cleavage of the oligonucleotide under basic conditions (*Fig. 7*). Furthermore, in the arrangement of a molecular beacon (MB) DNA (see **13**), a significant decrease of the FRET was observed after hybridization to a target sequence (*Fig. 9*). Due to the long decay times of the fluorescence of the Ru-complex, the system allows for highly sensitive time-gated measurements.

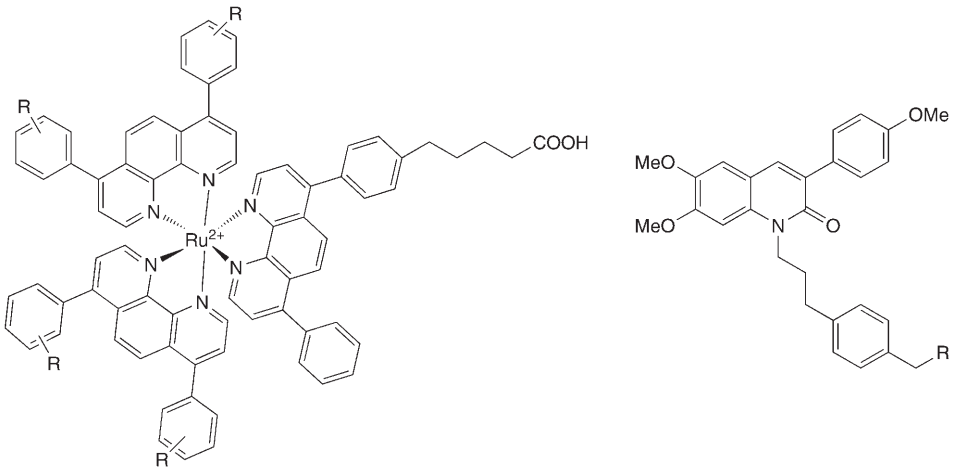
Introduction. – Fluorescence resonance energy transfer (FRET) is based on the transfer of fluorescence energy between a donor chromophore and a corresponding acceptor fluorophore. According to the *Förster* equation, the intensity of this transfer is highly dependent on the distance of the two chromophores and decreases with r^{-6}, r being the distance between the donor chromophore and the acceptor entity [1]. Furthermore, suitable spectral properties of the two dyes are required, *i.e.*, the emission wavelength of the donor should sufficiently overlap with the absorption wavelength of the acceptor. An additional requirement is the correct orientation of these two to each other.

What makes such systems especially attractive is their sensitivity towards slight changes in the distance of donor and acceptor allowing to follow biochemical events in a real time mode. Even the folding process of a protein was investigated *in vivo* by FRET measurements [2]. The myriad applications of FRET have been summarized in a number of reviews [3–6]. FRET has been used as a tool for the development of DNA-based assays, DNA probes, and DNA sequencing [7][8]. The concept of molecular beacons (MB) as DNA probe has been largely used. It consists of a fluorophore at one end of a hairpin DNA with a quencher attached to the other end [9–11]. Although advantageous, it depends on one fluorophore which has its emission at a specific wavelength. We describe the extension of our FRET system to molecular beacons. Our system has the advantage that it offers the possibility to monitor the fluorescence at two wavelengths [12]. Upon binding to a complementary sequence, the

FRET will be reduced, hence the emission of acceptor will decrease, whereas the emission of the donor will increase.

Meanwhile, a number of different donor–acceptor combinations for FRET have been reported. The most prominent ones are fluorescein–rhodamine, Cy3–Cy5, dabcyl-edans, and blue and green fluorescent protein [13]. However, despite of the number of reported systems, sensitivity and robustness still remains an issue. A possible solution to the sensitivity problem are FRET systems which can be measured in a time-resolved mode. This requires a long excited-state lifetime but yields excellent signal-to-noise ratios. Suitable candidates for this purpose are lanthanide complexes [14]. They are commonly employed as caged chelates; however, they have relatively low extinction coefficients, and relatively low stability, resulting in dissociation at low concentrations, so that leakage of the lanthanide metal can occur.

Recently, we have published an alternative donor–acceptor pair which can be measured in a time-resolved mode [15][16]. The system is based on the (bathophenanthroline)ruthenium(II) complex **1a** as acceptor entity (bathophenanthroline = 4,7-diphenyl-1,10-phenanthroline) [17] (*Fig. 1*). After its excitation at 450 nm, it reveals a metal-to-ligand charge transfer (MLCT) causing an emission at *ca.* 620 nm. Depending on the conditions, the lifetime for the excited state is in the range of 0.5–5 μs allowing for time-gated measurements with high sensitivity since interferences caused by autofluorescent compounds, color quenchers, or by scattered light from precipitated particles can be avoided. In addition, the [Ru^{II}(bathophenanthroline)] complexes are thermodynamically very stable and chemically inert. A further advantage is their high stability towards light and their large *Stokes* shift. An additional feature is that the Ru-complex as acceptor reveals an absorption minimum at the



1a R = SO_3Na
1b R = H

2a R = CH(NHFmoc)COOH
2b R = COOH

Fig. 1. *Donor–acceptor pair*

wavelength of the excitation of the donor so that direct excitation of the acceptor is minimal. Ru^{II} Complex **1a** is soluble in aqueous systems due to the sulfonyl groups and can be coupled covalently to target molecules *via* stable amide functions. As suitable donor molecule, we have identified a substituted quinolinone of which we prepared the derivative **2a** (Fmoc = (9*H*-fluoren-9-ylmethoxy)carbonyl). Building block **2a** represents a phenylalanine analogue and can be inserted during solid-phase synthesis into peptides. The donor molecule **2a** is very robust and has a high extinction coefficient. When donor **2a** and acceptor **1a** were incorporated into a peptide at abuttal sides to the recognition sequence for thrombin, a high FRET could be observed and the ratio of signal to noise was *ca.* 12.

2. Results and Discussion. – Here we demonstrate a feasibility study for the application of the newly developed system in DNA by using the [Ru^{II}(bathophenanthroline)] complex **1b** as acceptor and the quinolinone derivative **2b** as donor. Complex **1b** differs from **1a** in the absence of sulfonate groups which were used with peptides for solubility reasons. They are not necessary while working with DNA since DNA sufficiently mediates the solubility in aqueous solution. Complex **1b** was prepared like **1a** but starting from unmodified bathophenanthroline as ligand [16].

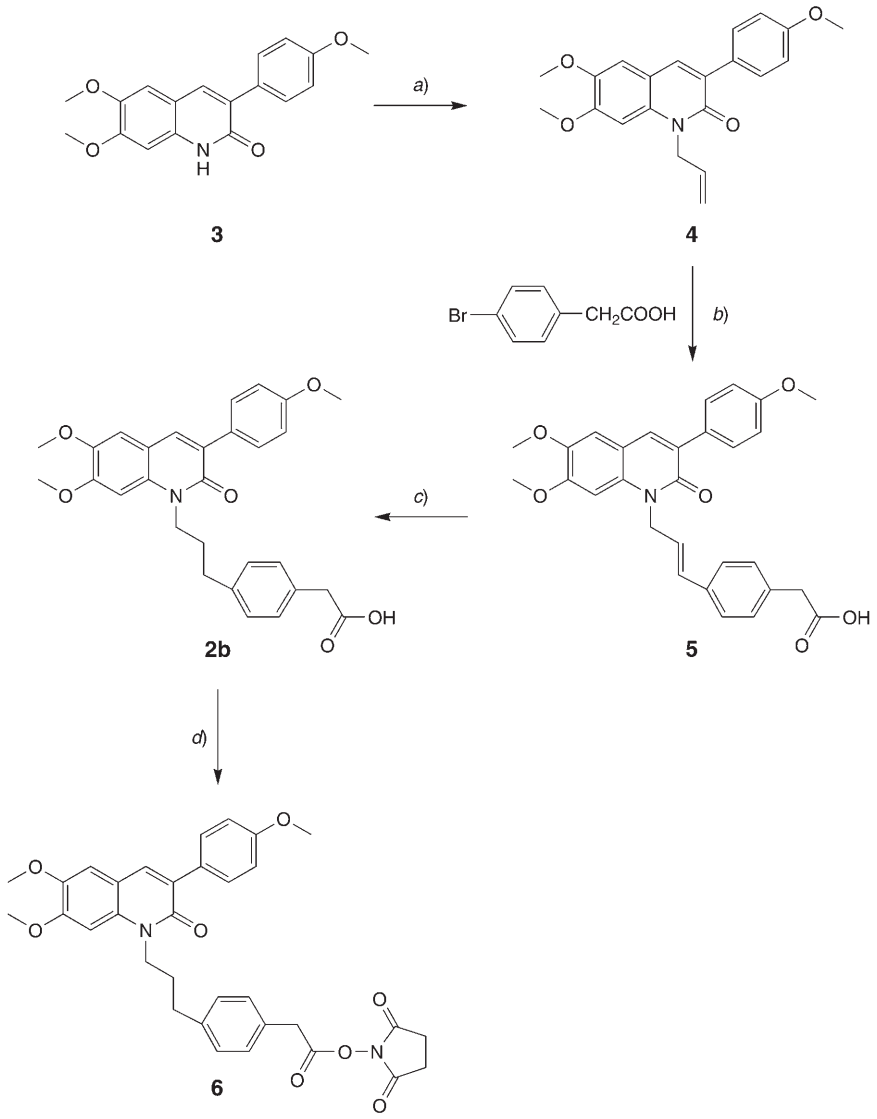
The donor entity was synthesized according to *Scheme 1*. The quinolinone derivative **3**, which showed an absorption maximum at 368 nm and an emission with a maximum at 435 nm and a high extinction coefficient (ε 20800 M^{-1} cm^{-1}), was transformed into the allyl-substituted compound **4** as previously described [15][16]. A *Heck* reaction with 4-bromobenzeneacetic acid yielded compound **5** which was hydrogenated to give **2b**. Activation of the carboxy function of **2b** with TSTU yielded the desired activated ester **6** [18] (TSTU = *N,N,N′,N′*-tetramethyl-*O*-succinimidouronium tetrafluoroborate). During this transformation, no change in the spectral properties could be observed.

The concept for a feasibility study for the FRET system in a single-stranded synthetic DNA fragment is outlined in *Fig. 2*. The activated donor **2b** (*i.e.*, **6**) was coupled to a 5-(3-aminoprop-1-ynyl)-2′-deoxyuridine (= α-(2-aminoethylidyne)thymidine) unit *via* an amide bond. The 5′-end of the oligonucleotide consisting of a modified 5′-amino-5′-deoxythymidine was coupled with the activated acceptor **1b** (as the hydroxysuccinimide ester). As potential cleavage site, we inserted a uridine unit which could be specifically cleaved under basic conditions [19][20]. In this construct, both donor and acceptor are in close proximity so that a FRET is possible, whereas after cleavage under basic conditions, the donor and acceptor are separated. As a result no FRET is observed.

To evaluate our strategy, we had to investigate first the cleavage conditions, and, therefore, we synthesized the oligonucleotide 5′-d(CCGAT)**U**d(TATCAT)-3′ (**7**) consisting of 2′-deoxynucleotide building blocks, except the uridine at the potential cleavage point. Oligonucleotide **7** was subjected to basic conditions, resulting in the expected fragments as shown by HPLC (*Fig. 3*).

For the insertion of the amino functions into DNA allowing to attach the donor and the acceptor, we needed the two modified building blocks 5-(3-aminoprop-1-ynyl)-2′-deoxyuridine phosphoramidite **8** and 5′-amino-5′-deoxythymidine phosphoramidite **9**, respectively (*Fig. 4*). Phosphoramidite **8** was synthesized by performing first a

Scheme 1. *Synthesis of Donor Molecule* **6**



a) KHMDS (potassium hexamethyldisilazanide), allyl bromide, −78°, 1.5 h, r.t., 30 min, microwave 120°, 15 min; 83%. *b*) [$Pd(OAc)_2$], PPh_3, Cs_2CO_3, H_2O, DMF, 120°, 16 h; 99%. *c*) H_2, Pd/C, MeOH, overnight; quant. *d*) TSTU, $^iPr_2EtN$, DMF, r.t., 2 h, (79%).

Sonogashira coupling between 5-iodo-2′-deoxyuridine and 2,2,2-trifluoro-*N*-(prop-2-ynyl)acetamide [21]. Under ligand-free conditions (Pd/C) and by using resin-bound Et_3N (*Amberlite IRA-67*) instead of Et_3N, the desired nucleoside was obtained after chromatographic purification (silica gel) in 79% yield [22]. This step was followed by

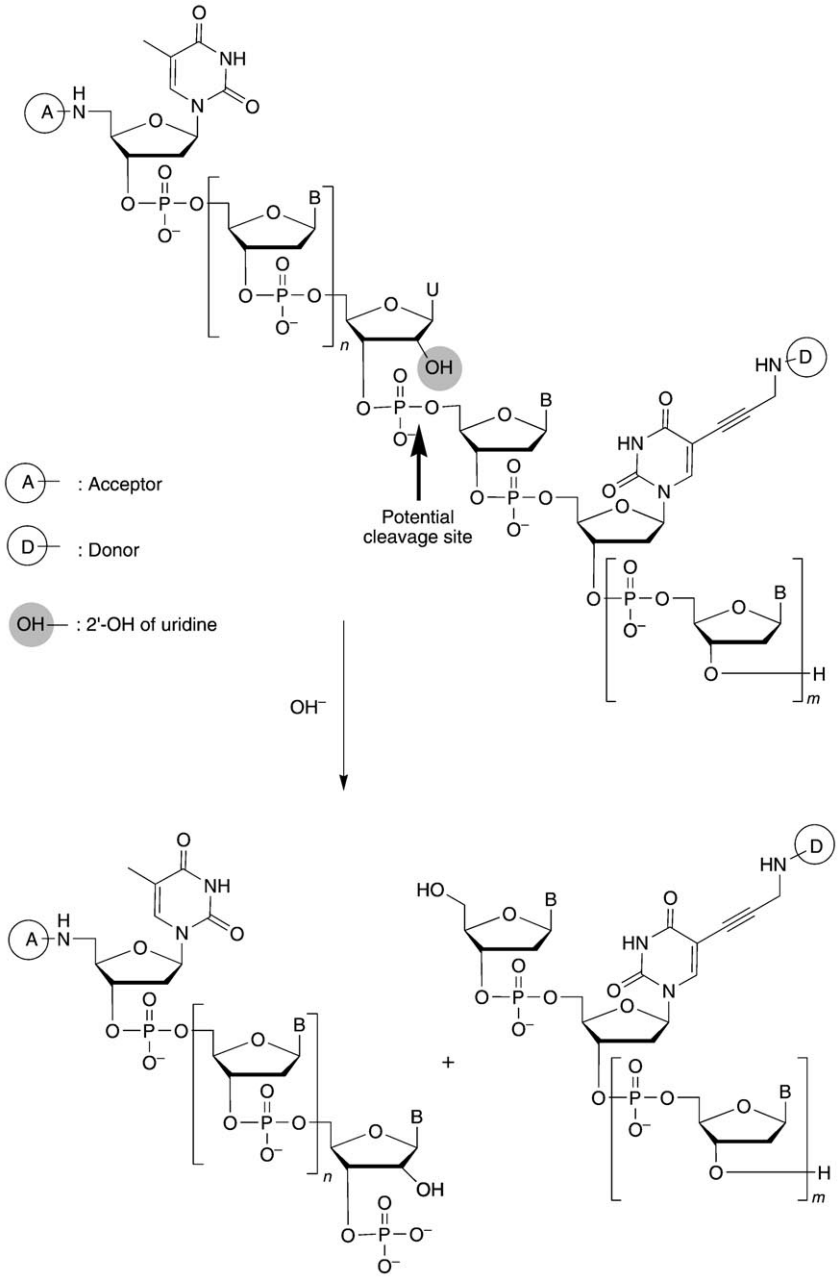


Fig. 2. *Design for the feasibility study of the FRET in single-stranded DNA*

regioselective introduction of the 4,4′-dimethoxytrityl ($(MeO)_2Tr$) group into the 5′-position and conversion to the pertinent phosphoramidite at the 3′-position with 2-

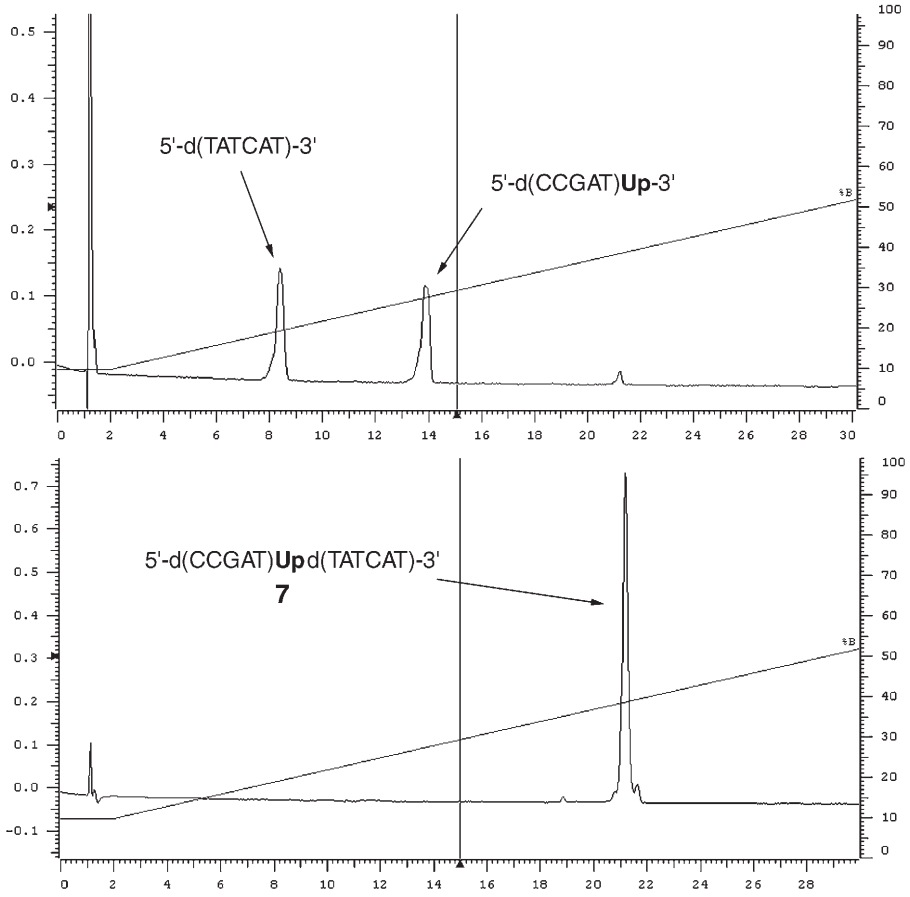


Fig. 3. *HPLC for the cleavage of oligonucleotide* **7** *under basic conditions.* Oligonucleotide **7** treated with NaOH (pH 12) overnight at r.t. Ion-exchange HPLC $0 \rightarrow 60\%$ B/A in 35 min; $A = 20$ mM KH_2PO_4 in MeCN/H_2O 1:4 (pH 6); $B = 1$M KCl 20 mM KH_2PO_4 in MeCN/H_2O 1:4 (pH 6).

cyanoethyl tetraisopropylphosphorodiamidite) in the presence of diisopropylammonium 1*H*-tetrazolide for the phosphinylation step. Phosphoramidite **9** was synthesized by a route reported previously with an overall yield of 53% [23].

To evaluate the spectral overlap between the donor and the acceptor when incorporated into single-stranded DNA, we synthesized the two DNA fragments **10** and **11** (*Fig. 5,a*). Fragment **10** was synthesized bearing a phosphate group at the 3′ terminus and corresponds to the fragment obtained after cleavage of oligonucleotide **12**. In both fragments **10** and **11**, the modified building blocks **8** and **9** were incorporated at specific sites to yield a primary amino function after deprotection for the attachment of the donor **2b** and the acceptor **1b**. The coupling of both donor as well as acceptor was performed *via* their hydroxysuccinimide-derived ester in DMF/dioxane/H_2O in the

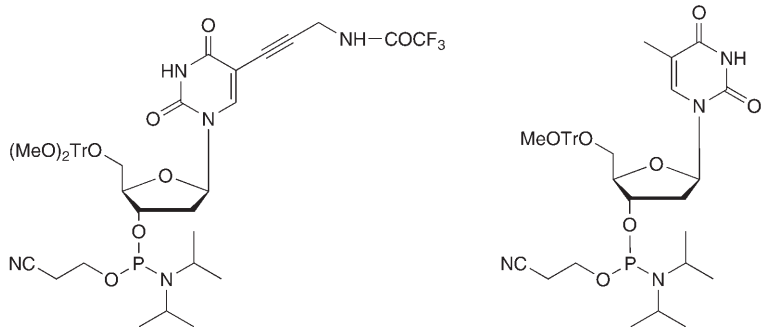


Fig. 4. *Modified phosphoramidites* **8** *and* **9** *employed for the insertion of amino functions allowing the coupling of the donor and acceptor*

presence of $^{i}Pr_2EtN$ [17]. Both oligonucleotides **10** and **11** were purified by HPLC and analyzed by polyacrylamide-gel electrophoresis (PAGE, *Fig. 5, b*). An excitation and emission spectrum of the two sequences (*Fig. 5, c*) revealed a strong overlap of the emission band of the donor with the absorption band of the acceptor which is a prerequisite for an efficient FRET.

Having confirmed the sufficient spectral overlap (*Fig. 5, c*), we synthesized oligonucleotide **12** (*Scheme 2*) which bears both the donor and the acceptor and has, furthermore, a uridine as specific cleavage point located between the donor and the acceptor. Oligonucleotide **12** was synthesized by standard phosphoramidite techniques incorporating the modified building blocks **8** and **9** in their appropriate positions. Uridine was implemented as 2′*O*-[(*tert*-butyl)dimethylsilyl]-protected phosphoramidite. After insertion of the last building block, the 4-monomethoxytrityl (MeOTr) group was not removed. After deprotection and removal from the support with base, the activated donor **2b** (*i.e.*, **6**) was coupled to the aminopropynyl group. This was followed by the removal of the MeOTr group and the attachment of the acceptor **1b** to the 5′-amino function. Finally, the (*tert*-butyl)dimethylsilyl (tbdms) group was cleaved off with a mixture of $Et_3N \cdot 3$ HF, 1-methylpyrrolidin-2-one (NMP), and Et_3N to yield crude fragment **12** which was purified by HPLC and PAGE (*Fig. 6*).

When evaluating the spectroscopic properties of **12** (*Fig. 7*), a high FRET with an emission maximum at 620 nm could be observed after excitation at 350 nm. After cleavage of **12** under basic conditions, the FRET vanished. The ratio of the FRET signal before and after cleavage was *ca.* 17:1. This weak fluorescence signal after cleavage was in the same range as that observed with an equimolar ratio of the independently synthesized fragments **10** and **11**.

In a further evaluation, the FRET donor and the FRET acceptor were extended to the MB approach. Therefore, the hairpin sequence **13** was synthesized (*Scheme 3*), carrying the quinolinone-derived FRET donor at the 3′-end, and the [Ru^{II}(bathophenanthroline)] FRET acceptor at the 5′-end. Oligonucleotide **13** was synthesized by a

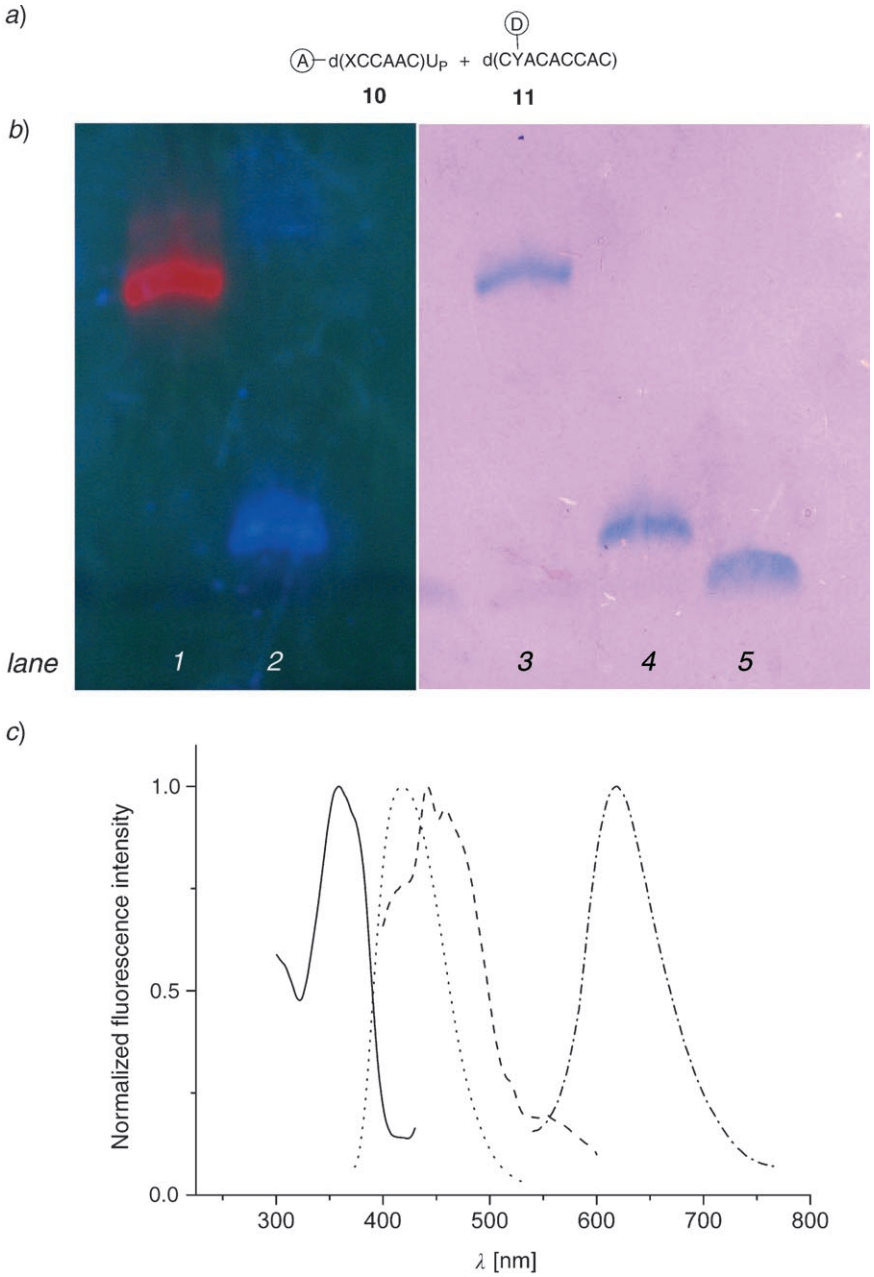
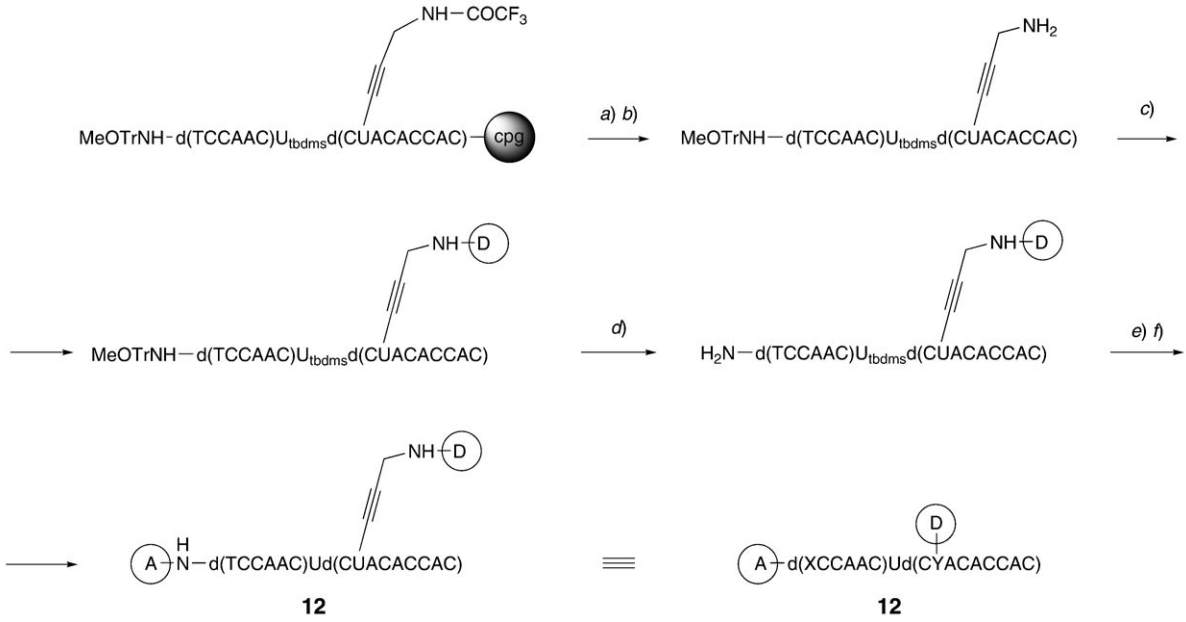


Fig. 5. a) *Oligonucleotides* **10** *and* **11** *for the evaluation of the spectral overlap* (X = 5′-amino-5′-deoxythymidine building block, Y = 5-(3-aminoprop-1-ynyl-2′-deoxyuridine building block). b) *Polyacrylamide-gel electrophoresis* (20%): *Lane 1*, fragment **10**; *Lane 2*, fragment **11** (at 366 nm); *Lane 3*, fragment **10**; *Lane 4*, fragment **11**; *Lane 5*, oligonucleotide 9-mer as reference (stained with 3,3′-diethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine bromide solution). c) *Excitation/emission spectra of oligonucleotide* **10** (- - - for excitation, – · – for emission) *and oligonucleotide* **11** (— for excitation, · · · for emission).

Scheme 2. *Synthesis of Oligonucleotide* **12**



a) 2M NH_4OH in EtOH, 30 min, 65°. *b*) 7M $MeNH_2$ in EtOH, 30 min, 65°. *c*) **6** (from **2b**), $^{i}Pr_2EtN$, DMF/dioxane/H_2O 1:1:1, 16 h, 25°. *d*) 80% AcOH. *e*) **1b** (as hydroxysuccinimide-derived ester), $^{i}Pr_2EtN$, DMF/dioxane/H_2O 1:1:1, 24 h, 25°. *f*) $Et_3N \cdot 3$ HF, 1-methylpyrrolidin-2-one (NMP), Et_3N.

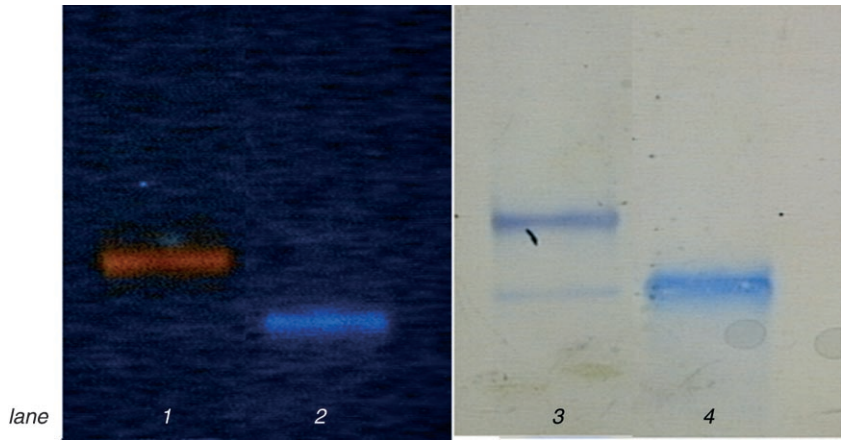


Fig. 6. *Polyacrylamide-gel electrophoresis* (20%). *Lane 1*, fragment **12**; *Lane 2*, fragment **12** labelled with donor (without acceptor, used as the reference; at 366 nm); *Lane 3*, fragment **12**; *Lane 4*, fragment **12** labelled with donor (without acceptor, used as the reference, stained with 3,3′-diethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine bromide solution).

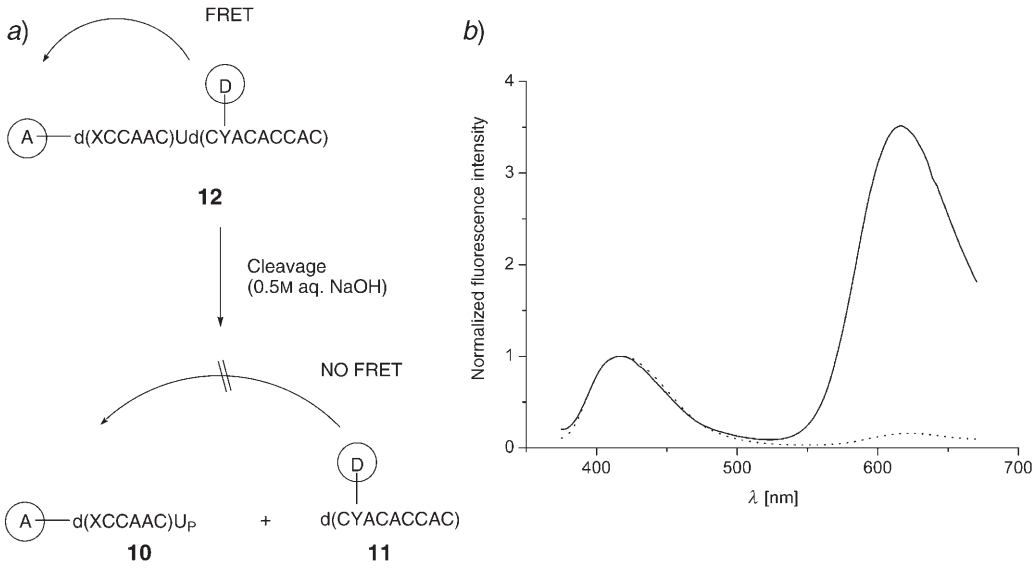


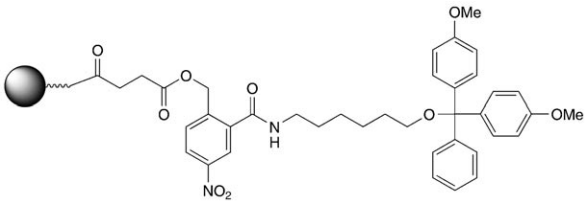
Fig. 7. a) *Cleavage of fragment* **12** *under basic conditions.* b) *Fluorescence emission spectrum of oligonucleotide* **12** (solid line), *and of fragments* **10** *and* **11** *after cleavage* (dotted line).

standard phosphoramidite protocol, however, by using the amino-ON CPG[1]) to introduce a primary amino group at the 3′-end and modified phosphoramidite **9** at the 5′-end. After insertion of the last building block, the MeOTr group was not removed. After deprotection and removal from the support with base, the donor **2b** *(i.e.*, **6**) was coupled to the amino group at the 3′-end. This was followed by the removal of the MeOTr group and the attachment of the acceptor **1b** to the 5′-amino function to yield crude fragment **13** which was purified by HPLC and analyzed by PAGE (*Fig. 8*).

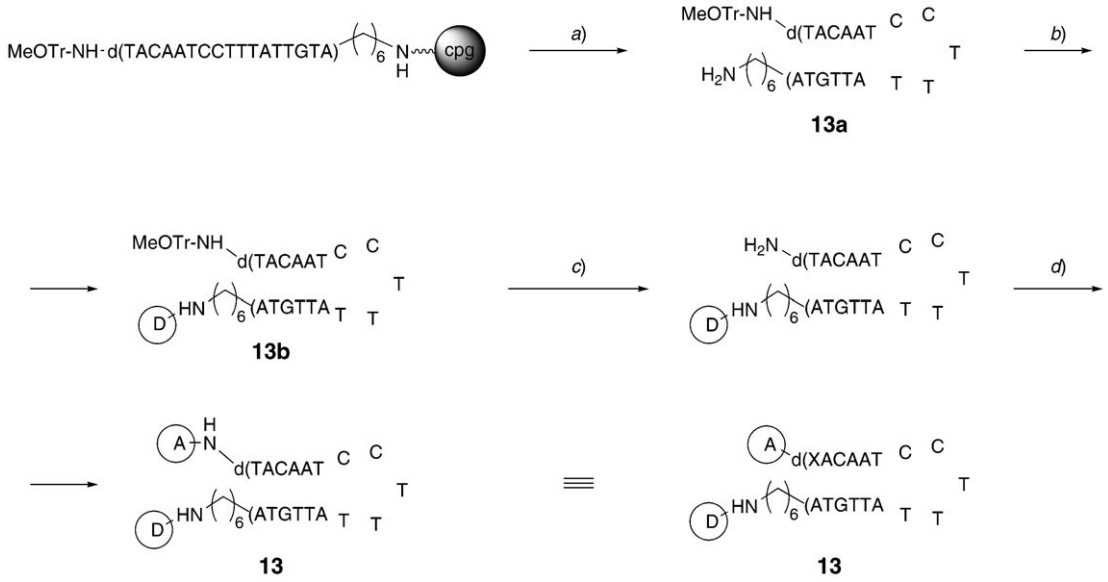
In the backfolding situation, sequence **13** revealed a strong FRET as can be seen from *Fig. 9*,*b*. The addition of a noncomplementary sequence had no influence on the FRET, but hybridization to the complementary oligonucleotide sequence **14** led to a reduced FRET due to the increase of the distance between the donor and the acceptor.

The excitation lifetime (τ) of the donor in fragment **11** was estimated to be 0.52 ns, whereas the one for the acceptor in fragment **10** was 0.40 μs (*Fig. 10*). This huge difference in the lifetime is the basis for the possibility to measure the fluorescence of

[1])



Scheme 3. *Synthesis of Oligonucleotide* **13**



a) NH_4OH. *b*) **6** (from **2b**), $^{i}Pr_2EtN$, DMF/dioxane/H_2O 1:1:1, 16 h, 25°. *c*) 80% AcOH. *d*) **1b** (as hydroxysuccinimide-derived ester), $^{i}Pr_2EtN$, DMF/dioxane/H_2O 1:1:1, 24 h, 25°; hybridization: 5 mM phosphate buffer, 0.1 mM EDTA, 100 mM NaCl.

the system in a time-gated mode with high sensitivity due to the possibility to eliminate the background fluorescence.

Conclusions. – We described the application of a new, robust, and highly sensitive FRET system in DNA. The system is based on the [Ru^{II}(bathophenanthroline)] complex **1b** as FRET acceptor and the quinolinone derivative **2b** as FRET donor. Both dyes were synthesized by straightforward routes in high yields.

Absorption and emission spectra of oligonucleotides bearing only the donor or the acceptor dye showed a good spectral overlap of the emission band of the donor with the absorption band of the acceptor which is a crucial requirement for a strong FRET. In addition, at the excitation wavelength of the donor (350 nm), the absorption spectrum of the Ru-complex reveals a minimum, thereby preventing direct excitation of the acceptor at 350 nm almost completely. The FRET was demonstrated in a single-stranded oligonucleotide bearing donor, acceptor, and a uridine moiety as the specific cleavage site. A remarkable intense FRET was observed in the intact DNA but vanished almost completely after cleavage under basic conditions.

A molecular-beacon DNA revealed also an intense FRET due to the close proximity between donor and acceptor, but it decreased significantly after hybridization to a complementary sequence. Compared to donor-quencher systems, the change of two fluorescence intensities can be monitored. The reported system allows for measurements in normal as well as in a time-resolved mode because of the long

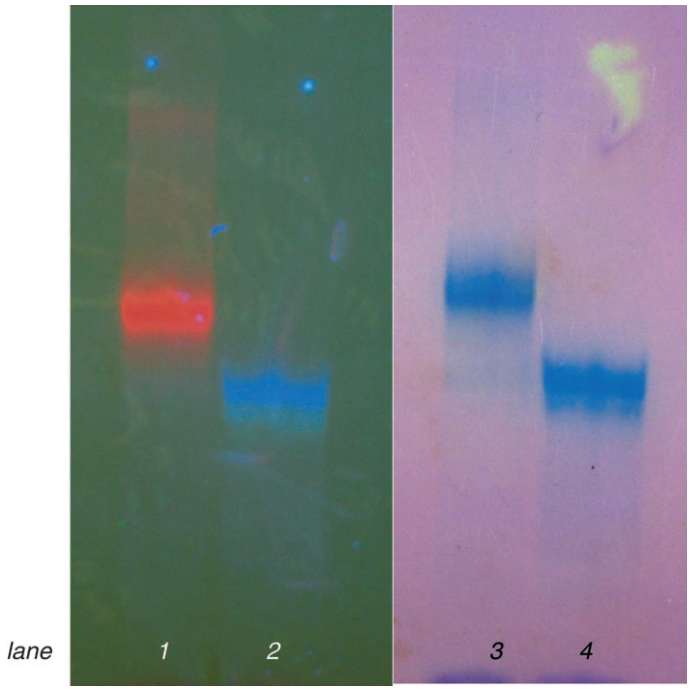


Fig. 8. *Polyacrylamide-gel electrophoresis* (20%). *Lane 1*, fragment **13**; *Lane 2*, fragment **13b** (at 366 nm); *Lane 3*, oligonucleotide **13**; *Lane 4*, fragment **13b** (stained with 3,3′-diethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine bromide solution).

fluorescence lifetime of the Ru-complex, thereby increasing the sensitivity due to the elimination of background fluorescence. We intend to apply the FRET system to the investigation of supramolecular interactions and focus furthermore on extensions to multi-FRET systems.

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Experimental Part

General: Oligonucleotide synthesis was carried out on an *Expedit-6800* DNA synthesizer on a 1 μmol scale by means of standard phosphoramidite technique. Modified CPG[1]) was purchased from *Proligo*. Replacement of NH_4^+ with K^+ was done by co-evaporation (3 ×) of the oligonucleotide with KCl (10 mg) in H_2O (200 μl). Oligonucleotide samples were desalted on *NAP-10* columns. Column chromatography (CC): short column; *Merck* silica gel *60*. HPLC: *Merck/Hitachi* system; reversed phase: *SP-250/10-Nucleosil-100-5-C18 or EC-125/4-Nucleosil-100-5-C18* columns, $A = 0.1$M $Et_3NH(OAc)$ buffer at pH 7.0, B = MeCN; ion-exchange: *250/4 Dionex DNAPac-PA-100* column, $A = 20$ mM KH_2PO_4 in MeCN/H_2O 1:4 at pH 6.0, $B = 1$M KCl, 20 mM KH_2PO_4 in MeCN/H_2O 1:4 at pH 6.0. PAGE: polyacrylamide gels were stained with a soln. of 3,3′-diethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine bromide ('Stains-

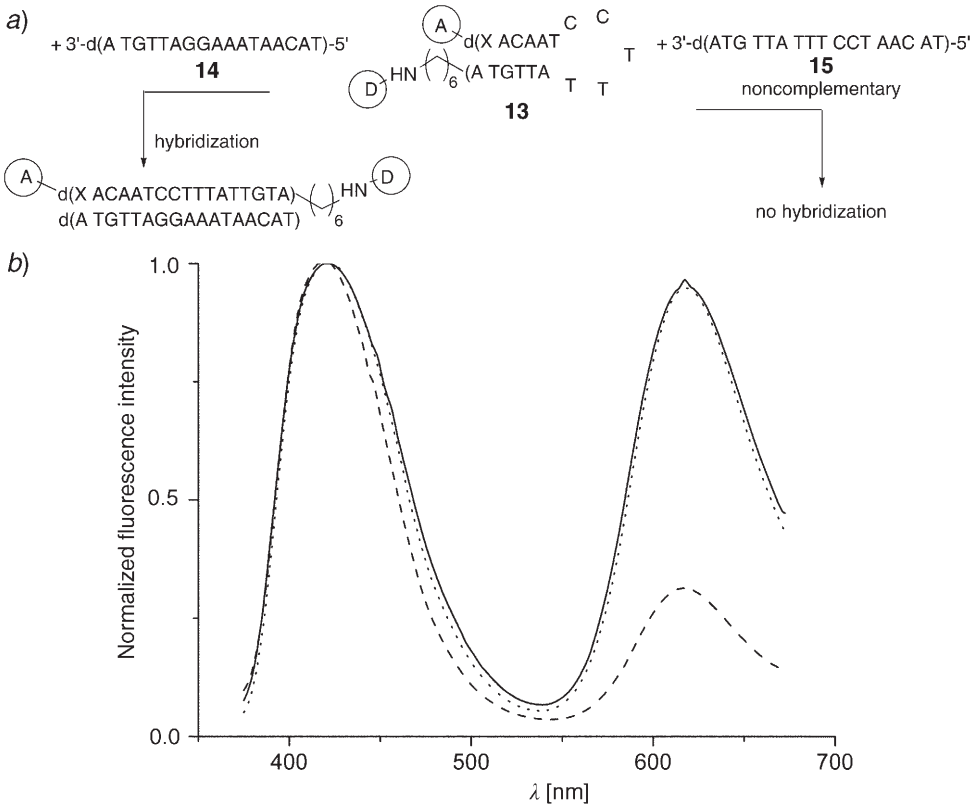

Fig. 9. a) *Structure of the molecular beacon and hybridization to the complimentary target sequence* (hybridization conditions: 5 mM phosphate buffer, 0.1 mM EDTA, 100 mM NaCl). b) *Fluorescence emission spectrum of MB* (—), *MB + noncomplementary oligonucleotide* **15** (· · ·), *and MB* **13** *+ target oligonucleotide* **14** (- - -), *excitation at 350 nm.*

all'; *Fluka*). UV Spectra: *Perkin-Elmer-Lambda-35*-UV/VIS spectrometer. Fluorescence spectra: *Perkin-Elmer LS45* spectrometer. Fluorescence lifetime decay: *IBH Consultants Ltd.* (Glasgow, Scotland), N_2 flash lamp as excitation source (microsecond range); *FluoTime 200* from *Picoquant* with a pulsed LED (nanosecond range). ^{1}H- (300 MHz) and ^{13}C-NMR (125 MHz) spectra: *Varian Mercury-VX-300*, and *Bruker DRX-500* spectrometers, resp.; chemical shifts δ in ppm rel. to Me_4Si, referenced to residual solvent signals; *J* in Hz. MS: *LCQ Advantage* (ESI) mass spectrometer.

Analytical Gel Electrophoresis. Polyacrylamide gels (20%) of 0.4 mm thickness were used. Pre-electrophoresis was performed for 2 h at 500 V with *Tris* borate running buffer. Oligonucleotide (1 µl, 0.1 *OD* units) and bromophenol blue/xylenecyanol soln. (3 µl) were heated to 90° for 2 min and rapidly cooled to 0°. Electrophoretic separation was performed for 2 h at 500 V and 4 mA. Oligonucleotide bands were visualized at 366 nm or stained.

4-{3-[1,2-Dihydro-6,7-dimethoxy-3-(4-methoxyphenyl)-2-oxoquinolin-1-yl]prop-1-enyl}benzeneacetic Acid (**5**). A mixture of 6,7-dimethoxy-3-(4-methoxyphenyl)-1-(prop-2-enyl)-1*H*-quinolin-2-one (**4**; 500 mg, 1.42 mmol), 4-bromobenzeneacetic acid (458 mg, 2.13 mmol), Cs_2CO_3 (1.38 g, 4.26 mmol), PPh_3, (55.8 mg, 0.23 mmol), and $[Pd(OAc)_2]$ (15.9 mg, 0.07 mmol) was suspended in DMF/H_2O 2:1 (17 ml). After heating for 16 h at 120°, the solvent was evaporated and the residue dissolved in H_2O (50 ml) and extracted with Et_2O. The aq. soln. was adjusted to pH 2.0 with 2N HCl and extracted with

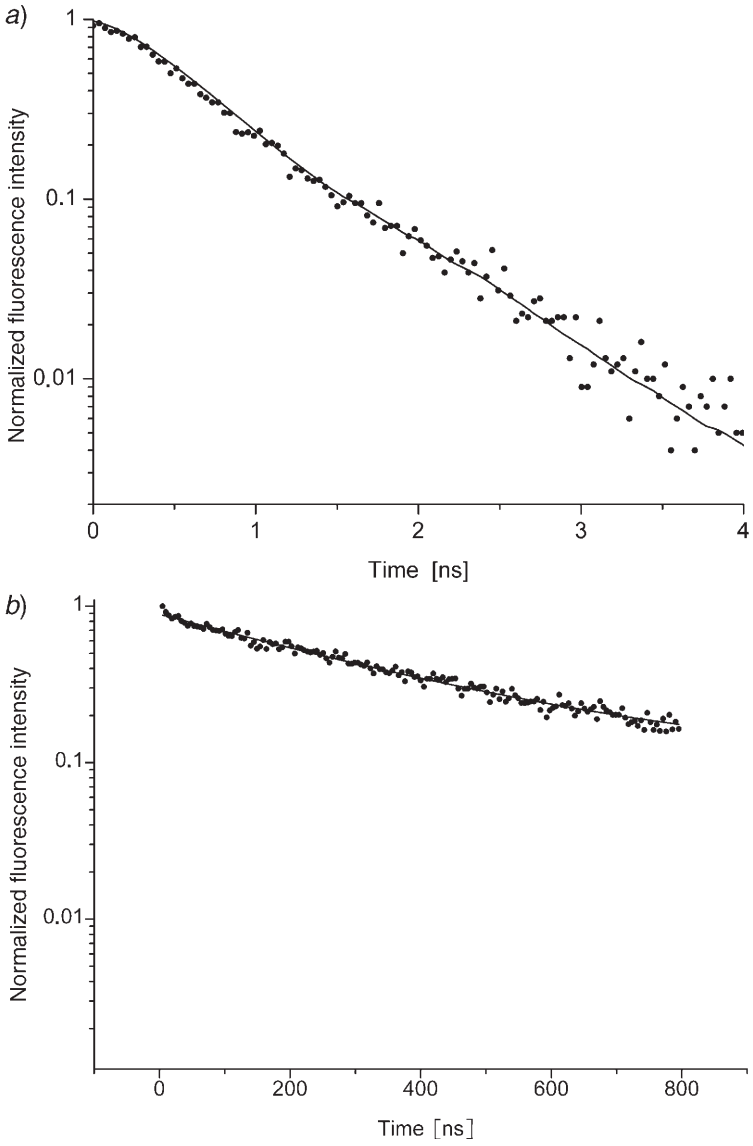

Fig. 10. a) *Normalized fluorescence decay of the donor in fragment* **11** (5 mM) *in* H_2O *at 20°* (pulse excitation, 381 nm; detection, 420 nm). b) *Normalized fluorescence decay of the acceptor in fragment* **10** (5 mM) *in* H_2O *at 20°* (pulse excitation, 358 nm; detection, 620 nm).

AcOEt (3 × 30 ml). The org. layer was dried (Na_2SO_4) and concentrated and the crude product purified by CC (silica gel, 2 → 9% MeOH in CH_2Cl_2): 663 mg (99%) of **5**. ^{1}H-NMR (300 MHz, $CDCl_3$): 3.50 (*s*, CH_2CO); 3.77 (*s*, MeO); 3.86 (*s*, MeO); 3.87 (*s*, MeO); 5.10 (*d*, *J* = 5.0, CH_2N); 6.21–6.28 (*m*, C*H*=C*H*$C_6H_4CH_2$); 6.48–6.53 (*m*, C*H*=C*H*$C_6H_4CH_2$); 6.82 (*s*, H–C(5)); 6.88 (*d*, *J* = 9.0, H–C(3′), H–C(5′)); 6.93 (*s*, H–C(8)); 7.08–7.18 (*m*, arom. H); 7.63 (*d*, *J* = 9.0, H–C(2′); H–C(6′)); 7.66 (*s*, H–C(4)). ^{13}C-NMR (125 MHz, $CDCl_3$): 39.96; 40.29; 55.08; 55.73; 55.91; 110.01; 113.27; 113.70; 124.18;

126.12; 129.31; 129.61; 129.83; 131.71; 133.93; 134.46; 134.56; 144.72; 151.52; 158.72; 160.08; 172.51. ESI-MS: 486.0 (100, $[M+H]^+$).

4-{3-[1,2-Dihydro-6,7-dimethoxy-3-(4-methoxyphenyl)-2-oxoquinolin-1-yl]propyl}benzeneacetic Acid (**2b**). Compound **5** (578 mg, 1.18 mmol) was hydrogenated in CH_2Cl_2/MeOH 1.5:1 (35 ml), over 10% Pd/C (204 mg). After 20 h, more MeOH was added, and the catalyst was removed by filtration over *Celite*. The solvent was evaporated: 580 mg (quant.) of **2b**. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.97–2.07 (*m*, CH_2); 2.73 (*t*, *J* = 7.0, $CH_2C_6H_4CH_2CO$); 3.54 (*s*, CH_2CO); 3.62 (*s*, MeO); 3.77 (*s*, MeO); 3.84 (*s*, MeO); 4.21–4.26 (*m*, CH_2N); 6.34 (*s*, H–C(5)); 6.86–6.89 (*m*, H–C(3′), H–C(5′), H–C(8)); 7.15 (*s*, arom. H); 7.57–7.61 (*m*, H–C(2′), H–C(6′), H–C(4)). ^{13}C-NMR (125 MHz, (D_6)DMSO): 28.24; 32.02; 39.96; 40.31; 55.08; 55.70; 97.05; 110.10; 113.25; 126.50; 127.29; 128.23; 129.33; 129.80; 132.59; 133.63; 135.38; 139.49; 144.58; 151.62; 158.68; 160.06; 172.73. ESI-MS: 488.2 (100, $[M+H]^+$).

4-{3-[1,2-Dihydro-6,7-dimethoxy-3-(4-methoxyphenyl)-2-oxoquinolin-1-yl]propyl}benzeneacetic Acid 2,5-Dioxopyrrolidin-1-yl Ester (**6**). To the soln. of **2b** (130 mg, 0.26 mmol) in dry DMF (5 ml) under Ar, TSTU (128.24 mg, 0.42 mmol) and ^{i}Pr$_2$EtN (114 µl, 0.66 mmol) were added under stirring. The mixture was stirred for 3 h at r.t. After removal of DMF, the residue was purified by CC (silica gel, 1 → 5% MeOH/CH_2Cl_2): 118 mg (79%) of **6**. ^{1}H-NMR (300 MHz, $CDCl_3$): 2.03–2.08 (*m*, CH_2); 2.73–2.78 (*m*, 3 CH_2); 3.54 (*s*, CH_2CO, 2 H); 3.62 (*s*, MeO); 3.77 (*s*, MeO); 3.84 (*s*, MeO); 4.21–4.26 (*m*, NCH_2); 6.34 (*s*, H–C(5)); 6.86–6.89 (*m*, H–C(3′), H–C(5′), H–C(8)); 7.15 (*s*, arom. H); 7.57–7.61 (*m*, H–C(2′), H–C(6′), H–C(4)).

5′-Amino-5′-deoxythymidylyl-(3′ → 5′)-2′-deoxycytidylyl-(3′ → 5′)-2′-deoxycytidylyl-(3′ → 5′)-2′-deoxyadenylyl-(3′ → 5′)-2′-deoxyadenylyl-(3′ → 5′)-2′-deoxycytidylyl-(3′ → 5′)-uridylic Acid 5′-Terminal Amide with Bis(1,7-diphenyl-1,10-phenanthroline-κN^{1},κN^{10}*)[4-(7-phenyl-1,10-phenanthrolin-4-yl-*κN^{1},κN^{10}*)benzenepentanoic Acid]ruthenium(II)* (**10**). The synthesis of **10** was performed on a 1 µmol scale by using CPG (controlled-pore glass) carrying a phosphate linker[2]) [24]. Modified building block **9** was incorporated manually (60 mg, 0.07 mmol) by using 0.3M of 5-(benzyl(thio)-1*H*-tetrazole (BMT, 700 µl) in MeCN as coupling reagent, and the coupling time was 6 min. In the last cycle, the MeOTr protecting group at the 5′-end was cleaved under acidic conditions (3% $CHCl_2COOH$ in CH_2Cl_2). Removal from the solid support and cleavage of the protecting groups was performed with dithioerythrol (30 mg), 2M NH_4OH in EtOH (500 µl), and 7M $MeNH_2$ in EtOH (500 µl), at 65° for 30 min. The solvent was removed by decantation, and the CPG was washed with EtOH/H_2O 1:1. The combined solns. were lyophilized in an *Eppendorf* concentrator. After replacement of NH_4^+ with K^+ by co-evaporation with KCl in H_2O and desalting with a *NAP-10* column, the crude oligonucleotide was lyophilized. The oligonucleotide (6.2 *OD*, 87 nmol) was dissolved in DMF/dioxane/H_2O 1:1:1 (261 µl), and ^{i}Pr$_2$EtN (3 µl, 17 µmol) and the activated (as hydroxysuccinimid-derived ester) acceptor **1b** (2.9 mg, 2.18 µmol) were added. The mixture was incubated at 25° in the dark for 24 h. After removal of the solvents, the pellets were treated with $CHCl_3$ (500 µl), vortexed, and centrifuged, and the $CHCl_3$ was removed by decantation (3×). Part of the crude product was purified by reversed-phase HPLC using (*EC 125/4 Nucleosil 100–5 C18*, 0 → 60% *B* in 30 min; t_R 15.23 min). The desired sequence **10** was analyzed by PAGE (*Fig. 5*, *b*) and MS. ESI-MS: 3299.0 (100, $[M-2\ Cl]^{2+}$).

2′-Deoxythymidylyl-(3′ → 5′)-2′-deoxy-{α-{2-{{{4-{3-[1,2-dihydro-6,7-dimethoxy-3-(4-methoxyphenyl)-2-oxoquinolin-1-yl]propyl}phenyl}acetyl}amino}ethylidyne}}thymidylyl-(3′ → 5′)-2′-deoxyadenylyl-(3′ → 5′)-2′-deoxycytidylyl-(3′ → 5′)-2′-deoxyadenylyl-(3′ → 5′)-2′-deoxycytidylyl-(3′ → 5′)-2′-deoxycytidylyl-(3′ → 5′)-2′-deoxyadenylyl-(3′ → 5′)-2′-deoxycytidine (**11**). The synthesis of **11** was carried out on a

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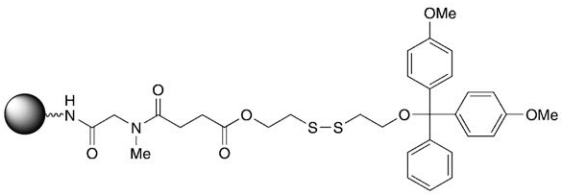
[2])



1 μmol scale. Modified building block **8** was incorporated manually (50 mg, 70 μmol) by using the method described for **10**. Removal from the solid support and cleavage of the protecting groups were performed with 25% NH_3 soln. at 55° overnight. After replacement of NH_4^+ with K^+ and desalting, the crude oligonucleotide (21.8 *OD*, 0.23 μmol) was dissolved in DMF/dioxane/H_2O 1:1:1 (716 μl), and $^iPr_2EtN$ (8 μl, 47.7 μmol) and activated (as **6**) donor **2b** (3.5 mg, 5.9 μmol) were added. The mixture was incubated at 25° in the dark for 16 h. After removal of the solvents, the residue was washed with EtOH (3 × 500 μl) (as described for **10** with $CHCl_3$) to remove the excess of the donor. Part of the crude product was purified by reversed-phase HPLC (*EC 125/4 Nucleosil 100–5 C18*, 0 → 100% *B* in 45 min; t_R 35.63 min). The desired sequence **11** was analyzed by PAGE (*Fig. 5,b*) and MS. ESI-MS: 3135.0 (100, $[M+1]^+$).

DNA d{(5′-Amino-5′-deoxy)T-C-C-A-A-C-(2′-hydroxy)U-C-{α-{2-{{{4-{3-[1,2-dihydro-6,7-dimethoxy-3-(4-methoxyphenyl)-2-oxoquinolin-1-yl]propyl}phenyl}acetyl}amino}ethylidyne}}T-A-C-A-C-C-A-C} 5′-Terminal Amide with Bis(1,7-diphenyl-1,10-phenanthroline-κN^{1}*,κN*^{10}*)[4-(7-phenyl-1,10-phenanthrolin-4-yl-κN*^{1}*,κN*^{10}*)benzenepentanoic Acid]ruthenium(II)* (**12**). The synthesis of oligonucleotide **12** was carried out on a 1 μmol scale. Modified building blocks **8** and **9** were incorporated manually at specific sites by using the method described for **10** and **11**. In the last cycle, the MeOTr protecting group at the 5′-end was not cleaved. After cleavage from the solid support with 2M NH_4OH in EtOH (500 μl) for 30 min at 65°, the oligonucleotide was treated with 7M $MeNH_2$ in EtOH (500 μl) for 30 min at 65°. After replacement of NH_4^+ with K^+ and desalting, the oligonucleotide (33 *OD*, 2.06 nmol) was dissolved in DMF/dioxane/H_2O 1:1:1 (618 μl), and $^iPr_2EtN$ (7 μl, 41 μmol) and the activated (as **6**) donor **2b** (3 mg, 5.1 μmol), were added. The mixture was incubated at 25° in the dark for 16 h. After removal of the solvents, the residue was washed with EtOH (3 × 500 μl) (as described for **10** with $CHCl_3$). The product was purified by reversed-phase HPLC (*SP 250/10 Nucleosil 100-5 C18*, 0 → 60% *B* in 45 min; t_R 23.68 min). The sequence was analyzed by PAGE. Afterwards, it was treated with 80% AcOH (320 μl) for 20 min to remove the MeOTr protecting group, and the product was precipitated with 50 μl of 3M AcONa and 1 ml of $^iPr_2OH$ and centrifuged at 11 000 r.p.m. for 20 min at 4°. The solvent was removed and the pellets dried in an *Eppendorf* concentrator at 45°. The product (6.5 *OD*, 0.4 nmol) was dissolved in DMF/dioxane/H_2O 1:1:1 (121 μl), and $^iPr_2EtN$ (1.38 μl, 8 μmol) as well as activated (as hydroxysuccinimide-derived ester) acceptor **1b** (1.3 mg, 1.0 μmol) were added. The mixture was incubated at 25° in the dark for 24 h. After removal of the solvents, the residue was washed with $CHCl_3$ (3 × 500 μl; by using the method described for **10**) to remove the excess of the acceptor. Part of the crude oligonucleotide **12** was purified by reversed-phase HPLC (*EC 125/4 Nucleosil 100-5 C18,* 0 → 100% *B* in 45 min; t_R 33.04 min). The product was analyzed by PAGE (*Fig. 6*).

DNA d{5′-Amino-5′-deoxy)T-A-C-A-A-T-C-C-T-T-T-A-T-T-G-T-{3′-O-{6-{{{4-{3-[1,2-dihydro-6,7-dimethoxy-3-(4-methoxyphenyl)-2-oxoquinolin-1-yl]propyl}phenyl}acetyl}amino}hexyl}}A} 5′-Terminal Amide with Bis(1,7-diphenyl-1,10-phenanthroline-κN^{1}*,κN*^{10}*)[4-(7-phenyl-1,10-phenanthrolin-4-yl-κN*^{1}*,κN*^{10}*)benzenepentanoic Acid]ruthenium(II)* (**13**). Oligonucleotide **13** was synthesized in a similar manner as **12** on a 1 μmol scale by using amino-ON CPG[1]). Modified building block **9** was incorporated manually by using the method described for **10**. The MeOTr protecting group at the 5′-end was not cleaved. Removal from the solid support and cleavage of the protecting groups were performed with 25% NH_3 soln. at 55° for 2 h. After replacement of NH_4^+ with K^+ and desalting, the resulting oligonucleotide (40 *OD*, 0.22 μmol) was dissolved in DMF/dioxane/H_2O 1:1:1 (669 μl), and $^iPr_2EtN$ (8 μl, 44 μmol) and activated (as **6**) donor **2b** (3.2 mg, 5.5 μmol) were added. The mixture was incubated at 25° in the dark for 16 h. After removal of the solvents, the residue was washed with EtOH (3 × 500 μl) to remove the excess of the donor. The product was analyzed by reversed-phase HPLC (*EC 125/4 Nucleosil 100-5 C18*, 0 → 60% *B* in 45 min; t_R 27.67 min) and PAGE (*Fig. 8*). Afterwards, it was treated with 80% of AcOH (320 μl) for 20 min to remove the MeOTr protecting group and the product precipitated by using the method described for oligonucleotide **12**. The pellets (39 *OD*, 0.2 μmol) were dissolved in DMF/dioxane/H_2O 1:1:1 (652 μl), and $^iPr_2EtN$ (8 μl, 43 μmol) and activated (as hydroxysuccinimide-derived ester) acceptor **1b** (7.4 mg, 5.4 μmol) were added. The mixture was incubated at 25° in the dark for 24 h. After removal of the solvents, the residue was washed with $CHCl_3$ (3 × 500 μl) to remove the excess of **1b**. Part of the crude **13** was purified by reversed-phase HPLC (*EC 125/4 Nucleosil 100-5 C18,* 5 min 100% *A*, then 0 → 90% *B* in 45 min; t_R 29.5 min). The product was analyzed by PAGE (*Fig. 8*).

Hybridization was carried out with **13**/**14** 1:2 in 300 µl of 5 mM phosphate buffer pH 7.0, 100 mM NaCl, and 0.1M EDTA. The probe was heated at 70° for 4 min and cooled down slowly to r.t.

Cleavage of Oligonucleotide **12**. Oligonucleotide **12** (1.2 *OD*) was treated with 0.5M NaOH (50 µl) and stirred for 5 h at r.t. The mixture was neutralized with AcOH (100 µl) and evaporated.

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