187. Energy Transfer from a Lumazine (= Pteridine-2,4(1*H*,3*H*)-dione) Chromophore to Bathophenanthroline-ruthenium(II) Complexes during Hybridization Processes of DNA

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(20.IX.91)

We incorporated 6,7-dimethyllumazine chromophores and a [Ru^{II}(bathophenanthroline)] complex into different oligodeoxynucleotides, the former *via* the corresponding phosphoramidites of the 2'-deoxynucleosides and the latter specifically by the phosphonate approach at the 5'-end of synthetic oligonucleotides, directly in the course of their synthesis on solid support. Efficiency measurements of this energy-transfer system with the lumazine as energy donor and the Ru complex as energy acceptor allowed us to distinguish between the hybridized and the non-hybridized state of oligonucleotides. Thus, the approach should be of value in DNA probe technology for the detection of single-stranded DNA targets.

1. Introduction. – DNA probe technology is based on the hybridization process of an oligonucleotide onto a single-stranded target DNA and can be applied for the detection of pathogens as well as for the detection of mutations in a DNA [1] [2]. In order to distinguish between the hybridized and the unhybridized state, the reactions are mainly carried out on solid-support materials allowing the removal of unhybridized DNA by washing steps as in the well known *Southern* hybridization [3]. The detection is mainly based on radioactive labels. New developments are aiming for a replacement of the radioactive labels by nonradiative reporter systems, and another trend is the application of homogeneous assay formats eliminating elaborately washing steps. These are the prerequisites to open up a wider applicability for the DNA probe technology. One principle which combines both the nonradiative as well as a homogeneous detection has been described recently [4]. It consists of a hybridization protection of intercalating acridinium esters followed by a chemiluminescence reaction.

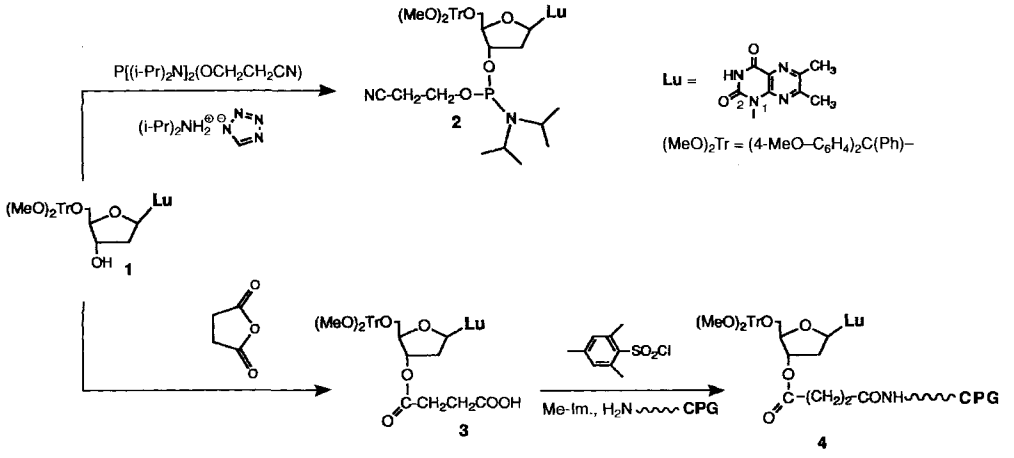
A further possibility for a homogeneous DNA detection exists with interactive label molecules. These labels can represent enzymes like in the glucose oxidase-peroxidase system [5] or donor/acceptor combinations in which an energy transfer from the donor to the acceptor is possible if the spectroscopic properties match and the acceptor is in close proximity to the donor.

Since the efficiency of the energy transfer in a given system is highly dependent on the distance between donor and acceptor, energy-transfer systems lend themselves to measurements of molecule interactions if one molecule is equipped with the donor and its counterpart with the acceptor [6] [7]. With this principle, it is possible to distinguish between a hybridized and a non-hybridized state of two DNA molecules, if one DNA molecule is equipped with a donor and the other DNA molecule carries the acceptor. Several such systems mainly based on fluorescein/rhodamine for the application in DNA probe technology have been described recently [8–10].

Recently we were able to show that the chromophoric system of 6,7-dimethyllumazine (= 6,7-dimethylpteridine-2,4(1*H*,3*H*)-dione) can transmit energy taken up from a light source onto bathophenanthroline-ruthenium(II) complexes *via* the long-wavelength metal-to-ligand charge-transfer (MLCT) absorption band [11]. The system represents a nonradiative donor/acceptor system with the lumazine as donor and the Ru complex as acceptor. An advantage of this combination is, on the one hand, the easy introduction of the Ru complex to the 5′-end of DNA directly in the course of its synthesis on a solid support by the phosphoramidite approach [11–13]. Here, we describe that such a specific insertion of the Ru complex can also be performed using phosphonate chemistry. As compared to our previous procedure, no functionalization of the DNA is neccessary. On the other hand, the 6,7-dimethyllumazine chromophore can be inserted into DNA in the form of the phosphoramidite of its 2′-deoxynucleoside also by standard solid-phase techniques. The 6,7-dimethyllumazine replaces in this way one or several natural bases and can be placed at will into a synthetic DNA fragment. Since the lumazine chromophore carries no reactive functional group, there is no need for an extra protecting group for this heterocycle.

2. Results and Discussion. – For an evaluation of the system lumazine/[Ru^{II}-(bathophenanthroline)] complex for studies of molecule interactions and especially hybridization processes, we synthesized the phosphoramidite **2** and the hemisuccinate **3** according to *Scheme 1*. Both compounds were obtained in reasonably good yield, comparable with that of the natural analogues. The hemisuccinate **3** was then applied to the

Scheme 1



Ru-(5'-3')d(A-T-A-A-T-C-C-A-C-C-T-A-T-C-C-C-A-G-T-A-G-G-A-G-A-A-A-T) **10**
(3'-5')d(Lu_4-T-A-T-T-A-G-G-T-G-G-A-T-A-G-G-G-T) **6**
(3'-5')d(Lu_4-A-G-G-T-G-G-A-T-A-G-G-G-T-C-A-T-C) **7**
(3'-5')d(Lu_4-G-C-C-A-C-T-T-G-T-A-T-G-C-A-A-C-T) **8**
(3'-5')d(T-A-T-T-A-G-G-T-G-G-A-T-A-G-G-G-T) **9**
} Controls

Ru- = (Ru complex)-$(CH_2)_5$-OP(O)O^- Lu = 5′-*O*-deprotected **1**

functionalization of controlled pore glass (CPG) with mesitylene-2-sulfonyl chloride/1-methyl-1*H*-imidazole as condensing reagent [14]. A loading of 27.5 μmol per g of support **4** was obtained. For all these experiments, the β-D-isomer of the 2′-deoxynucleoside was employed. Using the solid support **4** and the phosphoramidite **2**, we then prepared the oligonucleotides **6**–**8** all having 4 consecutive 6,7-dimethyllumazines at their 3′-end. The performance of the syntheses can be judged from *Fig. 1*.

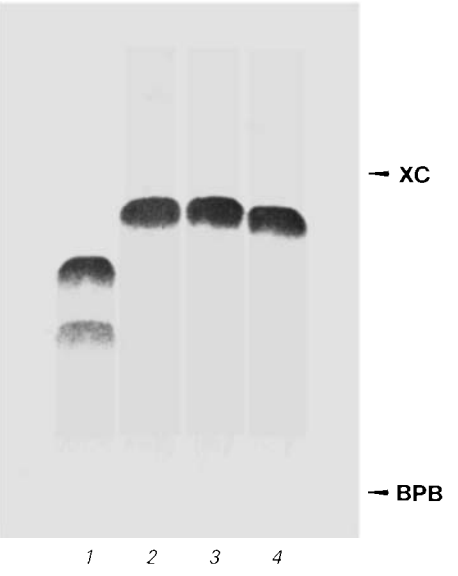
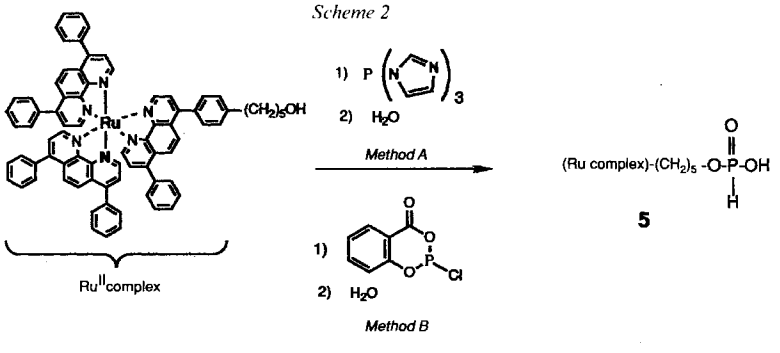


Fig. 1. *UV-Shadowing electrophoresis gel of the syntheses of lumazine-chromophore-containing oligonucleotides. Lane 1*, crude **9**; *Lane 2*, crude **6**; *Lane 3*, crude **7**; *Lane 4*, crude **8**.

As oligonucleotide equipped with the Ru complex at the 5′-end, we synthesized compound **10**, firstly by employing the phosphoramidite of the Ru^{II} complex as described recently [11]. For this purpose, the phosphoramidite of the Ru complex was prepared *in situ* and used as such for the 5′-modification of synthetic DNA (attempts to purify phosphoramidites of the Ru complex resulted in poor yields).

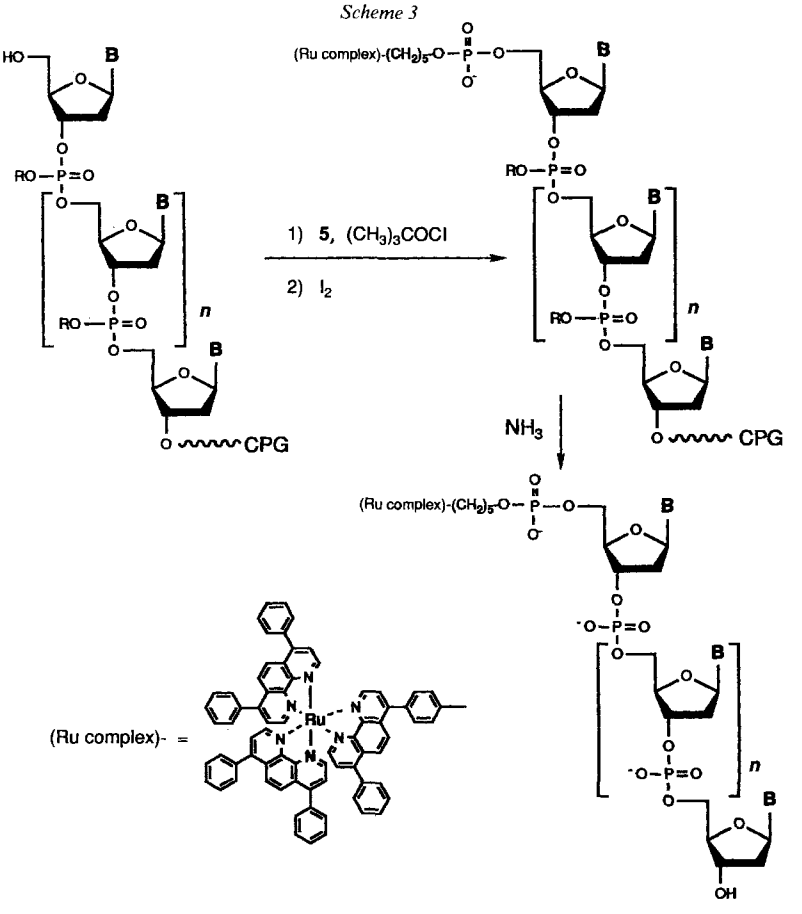
Scheme 2



Alternatively, we tethered the oligonucleotide to the Ru complex *via* the phosphonate approach [15] [16]. For this purpose, the Ru complex bearing a hydroxyalkyl group was transformed into the corresponding phosphonate **5** (*Scheme 2*), either by reaction with tri(1*H*-imidazol-1-yl)phosphine [17] or by the salicyloyl phosphite approach [18]. In both cases, a complete conversion was observed.

Compound **5** was used without purification for the coupling to DNA. Thus, it was activated with pivaloyl chloride to react with the 5′-OH group of the oligonucleotide still attached in the completely protected form to the solid support (*Scheme 3*). After oxida-

Scheme 3



tion and deprotection, the Ru complex was linked *via* a phosphodiester bridge to the oligonucleotide (see **10**). This procedure has the advantage that, unlike the *in situ* prepared phosphoramidite of the Ru complex, phosphonate **5** as a powder is easy to handle and can be stored without decomposition. The overall yield of the coupling using **5** was very good: complete conversion of the oligonucleotide into the Ru-complex-labelled form was observed (*Fig. 2*). The desired labelled oligonucleotide **10** was isolated and purified either by reversed-phase HPLC or by polyacrylamide gel electrophoresis (*Fig. 3*).

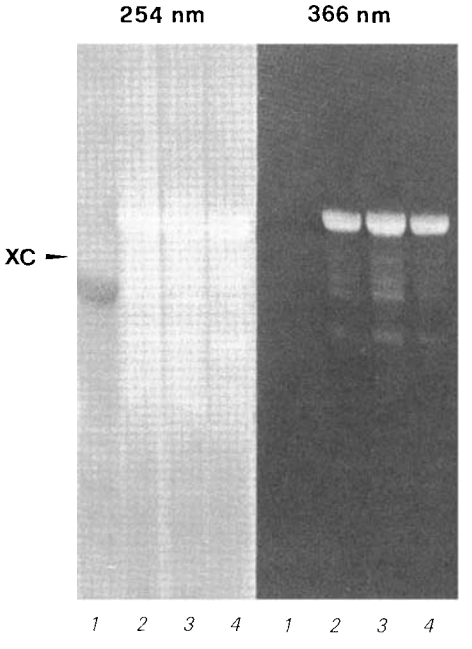


Fig. 2. *UV-Shadowing gel of the solid-phase attachment of the Ru complex by the phosphoramidite and the phosphonate approach. Lane 1*: oligonucleotide to be labelled; *Lane 2*, *Lane 3*, attachment of the Ru complex by the phosphonate approach (*Lane 2*, prepared according to [17]; *Lane 3*, prepared according to [18]), *Lane 4*, attachment of the Ru complex by the phosphoramidite approach [13].

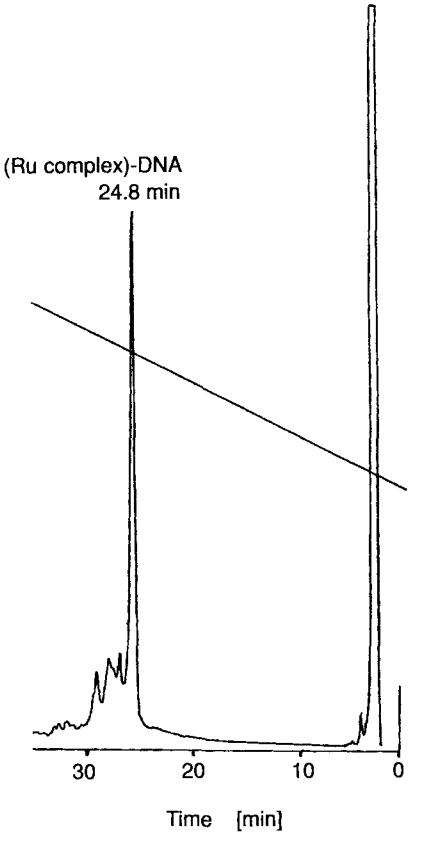
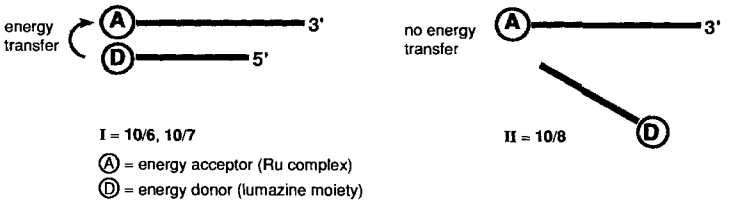


Fig. 3. *Reversed-phase HPLC of the crude Ru-complex-labelled oligonucleotide* **10**. Conditions: 30–70% MeCN in 0.1M $(Et_3NH)OAc$ (pH 7.0) with in 60 min.

Energy-transfer measurements were then carried out according to *Scheme 4*. Oligonucleotides **6** and **7** (energy donors) should show, after hybridization to **10** an energy transfer from the lumazine chromophores onto the Ru complex (energy acceptor) in **10** when excited by a light source (no significant changes of the spectroscopic properties of the Ru-complex moiety on hybridization could be detected). The energy transfer should

Scheme 4



be slightly higher for **6** as compared with **7** due to the closer distance of donor and acceptor in **I**. Oligonucleotide **8** is not complementary to sequence **10** and, thus, not able to hybridize; and although equipped with 4 lumazine chromophores, no energy transfer should be observed (see **II**). As a further control, we included in this study the oligonucleotide **9**, being able to hybridize to **10**, but since not carrying a lumazine chromophore, energy transfer is also impossible. Hybrid **10/9** allows the determination of the fluorescence intensity I_{F_2} of the Ru-complex moiety due to direct excitation.

In the preceding publication [11], we defined the fluorescence resulting from the energy transfer (I_{F_3}) as being the difference between the measured fluorescence at 620 nm (I_F) minus the fluorescence of the Ru complex through direct excitation (I_{F_2}) and the fluorescence of the incorporated lumazine chromophore at 620 nm (I_{F_1}) which can be neglected. Therefore, the formula for the intensity of the energy transfer is simplified to $I_{F_3} = I_F - I_{F_2}$.

The ratios of the measured fluorescence intensity I_F divided by I_{F_2} (see *Table*) clearly show that the energy transfer from the lumazine chromophore to the Ru complex can be

Table. *Energy-Transfer Measurements Using Fluorescence Intensities*

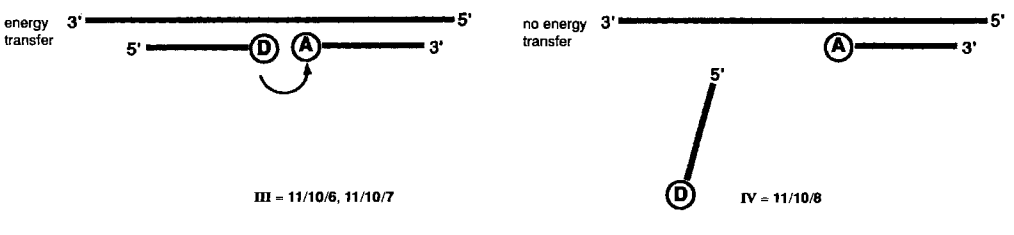
Hybrid	I_F/I_{F2}[a])	Hybrid	I_F/I_{F2}[a])
10/9	1.0	**11/10/9**	1.0
10/8	1.1	**11/10/8**	1.1
10/7	1.9	**11/10/7**	1.9
10/6	2.2	**11/10/6**	2.2

[a]) I_F = measured intensity at 620 nm; I_{F2} = intensity of the Ru complex due to direct excitation (determined on **10/9** and **11/10/9**, resp.).

observed on hybridization of the oligomers **6** and **7** to the template **10**, resulting in almost a doubling of the signal intensity, whereas this is not the case for the oligonucleotides **8** and **9**. Thus, a clear distinction between hybridization and the non-hybridized state using energy-transfer measurements according to *Scheme 4* is possible with the system 6,7-dimethyllumazine/Ru complex.

A further possibility of interactions between donor/acceptor energy-transfer systems is outlined in *Scheme 5* for the principle of so-called 'kissing probes' of oligonucleotides.

Scheme 5



(3'-5')d(A-C-C-C-T-A-T-C-C-A-C-C-T-A-A-T-A-A-A-A-A-T-A-T-T-A-G-G-T-G-----) **11**

(5'-3')d(T-G-G-G-A-T-A-G-G-T-G-G-A-T-T-A-T-Lu$_4$) Ru-(5'-3')d(A-T-A-A-T-C-C----)

6 **10**

In these systems, one oligonucleotide is labelled with the donor while the other one carries the acceptor. During hybridization they are arranged in such a way that they are sitting side by side at the single-stranded DNA they hybridize too (see **III**). In this situation, an energy transfer is possible. If the corresponding template is not present, no energy transfer occurs since the side-by-side arrangement of donor and acceptor due to the hybridization cannot be reached. The same applies to **IV** in which the donor-bearing sequence cannot hybridize to the template. System **III** is, therefore, of special value for the detection of a DNA of interest by interactive label molecules.

To test this possibility for the lumazine/Ru complex system, we hybridized the oligonucleotide **6** and also **7** as well as the Ru-complex-labelled oligonucleotide **10** to a synthetic template **11** and estimated the energy transfer. From the *Table* it can be seen that the energy transfer in **11**/**10**/**6** and **11**/**10**/**7** is of the same order of magnitude as for the hybridizations according to *Scheme 4*, doubling of the signal intensities being observed. The control oligonucleotides **8** and **9** show no energy transfer. Thus, a clearcut decision can be taken from energy-transfer measurements with our system to decide whether hybridization has occurred or not or whether a DNA allowing the side-by-side hybridization of the donor/acceptor pair is present or not.

3. Conclusion. – We investigated the energy-transfer system 6,7-dimethyllumazine/bathophenanthroline-ruthenium(II) in hybridization processes of oligonucleotides to select between a hybridized and a non-hybridized state by the efficiency of the energy transfer.

For these investigations, we inserted four consecutive 6,7-dimethyllumazine chromophores as energy donors into the 3′-end of several oligonucleotides by standard solid-phase technology for DNA synthesis. Another oligonucleotide was equipped at its 5′-end with a [Ru^{II}(bathophenanthroline)] complex as energy acceptor. For this, the Ru complex was transformed into the corresponding phosphoramidite as recently described [11]. Alternatively, we prepared the phosphonate of the Ru complex by two different ways and demonstrated that it can be coupled after activation with pivaloyl chloride specifically and with high efficiency to the 5′-end of oligonucleotides in the course of their synthesis on a solid support.

We showed that, using efficiency measurements of the energy transfer of the 6,7-dimethyllumazine/[Ru^{II}(bathophenanthroline)] system, a clearcut differentiation is possible between the hybridized and the non-hybridized state of two complementary or noncomplementary oligonucleotides. A doubling of the fluorescence intensity is obtained due to the energy transfer in those cases where hybridization-mediated energy transfer is possible.

Furthermore, we demonstrated that an arrangement in which two oligomers hybridize side by side to a single stranded DNA ('kissing probes'), the efficiency of the energy transfer is in the same order of magnitude as in the former approach. If this side-by-side arrangement is not possible, *e.g.* in the absence of the corresponding template DNA, no energy transfer is observed. This method, therefore, lends itself to the detection of DNA or RNA by energy-transfer measurements in the DNA probe technology, especially since the preparation of the lumazine-chromophore-labelled oligonucleotides and the 5′-(Ru complex)-tethered oligomers can be achieved by standard solid-phase DNA chemistry. Since the employed Ru complexes can be measured with high

sensitivity by time-resolved fluorometry, the system should also be applicable at very low concentrations [12].

In a more general way, the donor/acceptor system 6,7-dimethyllumazine/[Ru^{II}-(bathophenanthroline)] complex can be applied to study molecule interactions by measuring the efficiency of the energy transfer.

We would like to thank *B. Galko, P. Iaiza, E. Küng,* and *S. Weyermann* for excellent technical assistance. Furthermore, we thank our colleagues from Pharma Research New Technologies for C,H,N analysis (Dr. *S. Müller*), ^{1}H- and ^{31}P-NMR spectra (Dr. *G. Englert*, Dr. *W. Arnold*), MS (Dr. *W. Vetter, W. Meister*), and CD (Dr. *K. Noack*).

Experimental Part

1. *General.* All solvents were of highest purity available. The DNA fragments **9** and **10** were prepared on controlled pore glass as solid support [19] applying 2-cyanoethyl phosphoramidites [20] and our standard technologies [14]. The phosphoramidite of the [Ru^{II}(bathophenanthroline)] complex was prepared *in situ* as described earlier [13]. The 1-[2′-deoxy-5′-*O*-(dimethoxytrityl)-β-D-ribofuranosyl]-6,7-dimethylpteridine-2,4(1*H*,3*H*)-dione (**1**) was obtained according to [11] and salicyloyl chlorophosphite according to [22]. All hybridizations were carried out in 1:1 molar ratios of the oligonucleotides (10^{-6} M) in 1M NaCl; 10 mM p_i at pH 7 in a volume of 500 µl. The probes were heated at 70° for 4 min and cooled down slowly to r.t. before fluorescence measurements. Short column chromatography (CC) [21]: silica gel *60* (0.063–0.040 mm, *Merck*). TLC: HPTLC, silica-gel plates (*Merck*). ^{1}H-NMR (250 MHz): chemical shifts in δ (ppm) rel. to TMS. Fluorometry: *SLM* spectrofluorometer, model *4048 S*; excitation at 337 nm, fluorescence measurement at 460 nm (lumazine) and 620 nm (Ru complex).

2. *1-[2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-succinyl-β-*D*-ribofuranosyl]-6,7-dimethylpteridine-2,4-(1*H*,3*H*)-dione* (**3**). Compound **1** (0.29 mmol, 175 mg) was twice taken up in anh. pyridine and evaporated. It was dissolved in 5 ml of anh. CH_2Cl_2, and succinic anhydride (0.91 mmol, 91 mg) and 4-(dimethylamino)pyridine (0.33 mmol, 41 mg) were added as well as Et_3N (0.91 mmol, 126 µl). The mixture was stirred at r.t. for 5 h, then poured into 10 ml of 1% AcOH/H_2O and extracted with CH_2Cl_2 (3 × 50 ml). The combined org. layers were dried and evaporated. The residue was purified by CC (20 g of silica gel, CH_2Cl_2 containing 1% of Et_3N and an increasing amount of EtOH (1, 3, and 5%)). The residue of the pure fractions (TLC (CH_2Cl_2/MeOH 9:1): R_f 0.32) was dissolved in 5 ml of 1% Et_3N/CH_2Cl_2 and precipitated into 350 ml of pentane. The precipitate was collected and dried: 165 mg (79%) of pure **3**·Et_3N. ^{1}H-NMR ($CDCl_3$): 1.44 (*t*, 3 C*H*$_3$CH$_2$); 2.22–2.37 (1 H–C(2′)); 2.50–2.68 (*m*, 1 H–C(2′)); 2.36 (*s*, Me); 2.60 (*s*, Me, CH_2CH_2); 3.06 (*q*, 3 CH_3CH_2); 3.29 (*dd*, 1 H–C(5′)); 3.50 (*dd*, 1 H–C(5′)); 3.76 (*s*, 2 MeO); 4.22–4.35 (*m*, H–C(4′)); 4.43–4.60 (*m*, H–C(3′)); 6.66–6.83 (*m*, H–C(1′), 4 arom. H (C_6H_4)); 7.05–7.41 (*m*, 9 arom. H).

3. *Functionalization of the CPG-Support with* **3**. The CPG support (2.5 g) and **3** (0.18 mmol, 130 mg) were suspended together in 10 ml of anh. pyridine and evaporated. This was repeated twice. The residue was taken up in 10 ml of anh. pyridine, and mesitylene-2-sulfonyl chloride (4.6 mmol, 1.0 g) and 1-methyl-1*H*-imidazole (4 mmol, 0.4 ml) were added. The mixture was left for 18 h, occasionally shaken, then filtered, and washed with pyridine and Et_2O. Unreacted amino functions were capped by addition of 15 ml of a soln. containing 1 g of 4-(dimethylamino)pyridine, 2 ml of Ac_2O, and 2 ml of 2,6-dimethylpyridine. After 30 min, the soln. was filtered and the functionalized support washed with pyridine and Et_2O and dried. The functionalization as determined by UV (499 nm) of the cleaved $(MeO)_2$Tr group was 27 µmol/g of support.

4. *Lumazine-Chromophore-Containing Oligonucleotides* **6–8**. The syntheses were started with 40 mg of support **4** (1.08 µmol). Condensations with **2** were carried out with 20 mg (24 µmol) of **2** and 17 mg (240 µmol) of tetrazole in 0.5 ml of anh. MeCN in a standard reaction cycle. After the incorporation of the lumazine chromophores at the 3′-end, syntheses were continued using standard phosphoramidites and our standard protocols. After deprotections, gel analysis showed high coupling efficiency for the incorporation of **2**. Isolation of **6–8** was performed by electroelution after polyacrylamide gel electrophoresis (*Fig. 1*).

5. *Phosphonate* **5** *of the Bathophenanthroline-ruthenium(II) Complex. Method A:* To a soln. of 1*H*-imidazole (2.7 mmol, 180 mg) and Et_3N (2.8 mmol, 380 µl) in 10 ml of anh. MeCN was added under Ar with a syringe within 5 min PCl_3 (0.8 mmol, 70 µl). The mixture was stirred at r.t. for 30 min. The Ru complex (0.1 mmol, 126 mg) was

twice evaporated from anh. MeCN, then taken up in 10 ml of anh. MeCN, added to the mixture containing the tri(1*H*-imidazol-1-yl)phosphine, and stirred for 2 h at r.t. (TLC: complete conversion into **5**). The mixture was poured into 100 ml of 0.1M $(Et_3NH)OAc$ pH 7.0 and extracted with CH_2Cl_2 (3 × 50 ml). The combined org. layers were dried (Na_2SO_4) and evaporated. The residue was digerated in Et_2O, washed with Et_2O and dried whereby we obtained 120 mg (86%) of **5**·Et_3N, which was used as such for the coupling to the DNA. Part of the material was purified by CC (CH_2Cl_2/MeOH containing 2% pyridine). ^{31}P-NMR (rel. to H_3PO_4): 4.25 ppm (*s*). FAB-MS: 1399.5 ($[M + H]^+$).

Method B: The Ru complex (0.1 mmol, 126 mg) was evaporated 3 times from anh. MeCN and then taken up in 5 ml of anh. MeCN and 1 ml of anh. pyridine. To this was added salicyloyl chlorophosphine (0.5 mmol, 101 mg) in 2 ml of anh. MeCN. The mixture was stirred at r.t. for 1.5 h (TLC: complete conversion to **5**). The mixture was poured into 50 ml of 0.1M $(Et_3NH)OAc$, and workup as above yielded 120 mg (86%) of **5** which was used as such for the coupling to DNA.

6. *Coupling of* **5** *to Oligonucleotides.* Compound **5** (12 μmol, 36 mg) was evaporated twice from anh. MeCN and taken up in 1 ml of anh. pyridine. From this soln., 0.5 ml were added, at the same time as 7.3 μl of pivaloyl chloride in 0.5 ml of anh. MeCN, to 0.4 μmol of the sequence d(A-T-A-A-T-C-C-A-C-C-T-A-T-C-C-C-A-G-T-A-G-G-A-G-A-A-A-T), which was still attached in the completely protected form (but lacking the 5′-$(MeO)_2$Tr group) to the solid support (10 mg of support). Condensation time was 4 min, and after removal of excess **5** and pivaloyl chloride, the condensation process was repeated in the same way and with the same amounts. This was followed by oxidation with 1 ml of 0.2M I_2/THF and 1 ml of Et_3N/H_2O/THF 1:1:8 (*v*/*v*) and washing steps with MeCN and Et_2O. For deprotection, 10 mg of the support were treated with 700 μl of conc. ammonia for 2 h at 67°. Polyacrylamide gel electrophoresis showed a complete conversion of the starting oligonucleotide into the desired Ru-complex-labelled DNA fragment (*Figs. 2* and *3*).

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