# 186. Energy Transfer within Oligonucleotides from a Lumazine (= Pteridine-2,4(1*H*,3*H*)-dione) Chromophore to Bathophenanthroline-ruthenium(II) Complexes

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The chemical insertion of 1-(2′-deoxy-β-D-ribofuranosyl)-6,7-dimethyllumazine (**1**) and of a bathophenanthroline-ruthenium(II) complex into synthetic oligodeoxynucleotides is described. This combination represents a new energy-transfer system with the lumazine chromophore as donor and the Ru complex as acceptor within the nucleotide. The system can be measured by time-resolved fluorometry or by lifetime measurements of the lumazine fluorescence and bears the potential of a spectroscopic ruler.

**1. Introduction.** – Recently, we described bathophenanthroline-ruthenium(II) complexes (bathophenanthroline = 4,7-diphenyl-1,10-phenanthroline) as nonradioactive label molecules to be employed for DNA-probe technology [1] [2] and DNA sequencing [3]. These Ru complexes show a strong and long-lasting fluorescence after excitation with light pulses of short duration allowing their detection by time-resolved fluorometry with high sensitivity. The excitation of the Ru complexes was performed with a combination composed of a $N_2$ laser and a dye laser. The light emitted from the $N_2$ laser (337 nm) was transformed by the dye to 453 nm which in turn excited the [$Ru^{II}$(bathophenanthroline)] complexes *via* the metal-to-ligand charge-transfer band (MLCT) and resulted in a long-wavelength fluorescence emission of 618 nm. The overall efficiency of this process was in the range of 10%.

Our goal was the replacement of the dye by a suitable transmitter molecule as an energy donor which can be incorporated into Ru-complex-labelled DNA molecules. Thus, one would create a nonradiative fluorescence resonance energy-transfer system. On the one hand, this would lead to a simplification of the excitation process and, therefore, also of the apparatus used for this purpose by omitting the dye laser. On the other hand, a higher efficiency of the excitation process would also result in a higher sensitivity.

Furthermore, and perhaps most importantly, suitable *nonradiative* energy-transfer systems could be applied to the construction of spectroscopic rulers for the determination of distances within or between biomolecules since there is a strong relationship between the efficiency of the energy transfer and the distance between donor and acceptor given by *Förster*'s equation [4] [5].

In principle, there exist two possibilities for the incorporation of a transmitter molecule into Ru-complex-labelled DNA fragments. It can be either placed as a substituent into one of the bathophenathroline ligands or into the DNA part. We were aiming at the latter approach since it allows to place the transmitter in various distances from the Ru complex and thus to function possibly as a spectroscopic ruler.

**2. Results and Discussion.** – 2.1. *Synthesis.* We selected 1-(2′-deoxy-β-D-ribofuranosyl)-6,7-dimethyllumazine ( = 1-(2′-deoxy-β-D-ribofuranosyl)-6,7-dimethylpteridine-2,4-(1*H*,3*H*)-dione, **1**) as suitable fluorescent energy-transfer molecule. The spectroscopic properties of this compound as an energy donor reveal sufficient overlaps of its long-wavelength absorption band (324 nm) with the emission wavelength of the $N_2$ laser (337 nm). The lumazine chromophore **1** shows a fluorescence at 466 nm which overlaps with the MLCT band of the Ru complex as energy acceptor, resulting in the emission of the long-lived fluorescence at 618 nm (*Fig. 1*). Thus, the insertion of the lumazine chromophore into a Ru-complex-labelled oligonucleotide should provide a distance-dependent energy transfer between the lumazine moiety and the Ru complex.

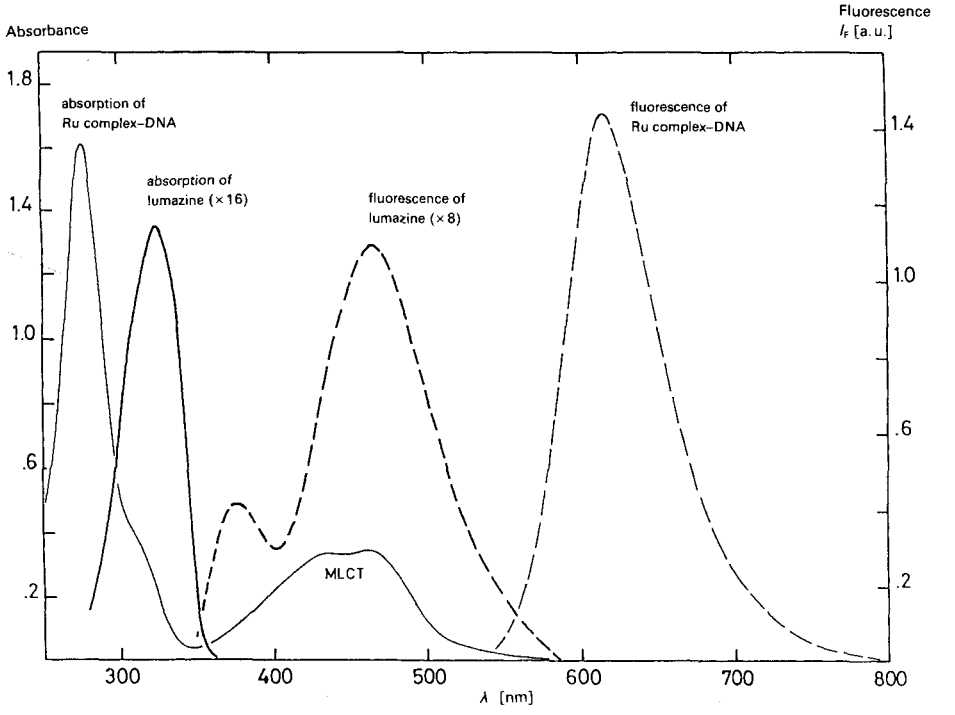
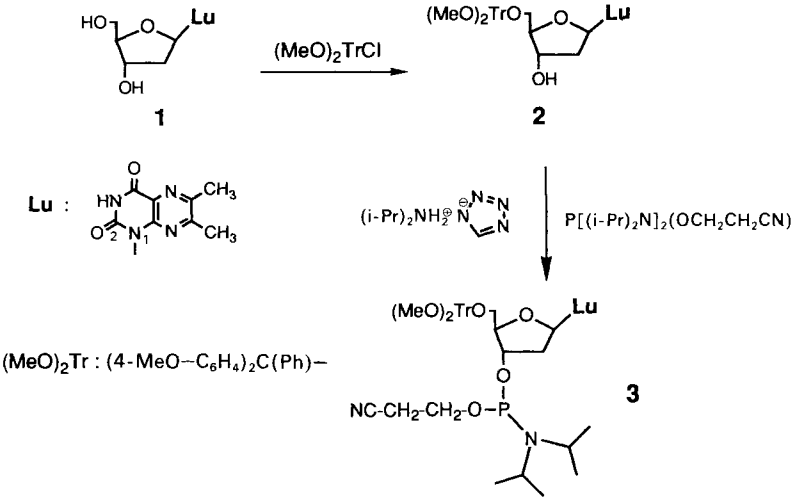



Fig. 1. *Comparison of the spectroscopic properties of the 6,7-dimethyllumazine chromophore* **1** *and the bathophenanthroline-ruthenium(II) complex* (PBS; pH 6.9). Important is the overlap between the emission band of the lumazine donor and the metal-to-ligand charge-transfer band (MLCT) of the Ru complex. Note also the amplification factors of 16 and 8 for the lumazine absorption and fluorescence, respectively.

One disadvantage of **1** is the relatively low extinction coefficient for the long-wavelength absorption of the lumazine chromophore ($\varepsilon = 8900\ M^{-1} \cdot cm^{-1}$ at 324 nm; pH 6.9). A great advantage, however, is its chemical structure and behavior which resembles very much the naturally, in DNA occurring nucleosides so that it can be placed at will into oligonucleotides in the course of their syntheses on a solid support.

In order to test these possibilities, we transformed nucleoside **1** into the corresponding phosphoramidite **3** (*Scheme 1*): protection of **1** at the 5′-end by the 4,4′-dimethoxytrityl

*Scheme 1*




((MeO)$_2$Tr) group yielded **2** which was reacted with (2-cyanoethoxy)bis(diisopropylamino)phosphine in the presence of diisopropylammonium tetrazolide to give **3** in reasonably good yield.

After having established that **3** can be coupled with the same high yield as standard phosphoramidites to a growing DNA fragment on a solid support, we synthesized several oligonucleotides where the 6,7-dimethyllumazine nucleoside is incorporated into various positions of a parent oligonucleotide **4**. Thus, the 6,7-dimethyllumazine moiety was placed at the 5′-end or within the oligonucleotide replacing the bases at position 3, 6, and 9 from the 5′-end of **4**. Furthermore, a series of 5 consecutive lumazine moieties was placed at the 5′-end. Part of the material after each synthesis was removed and depro-

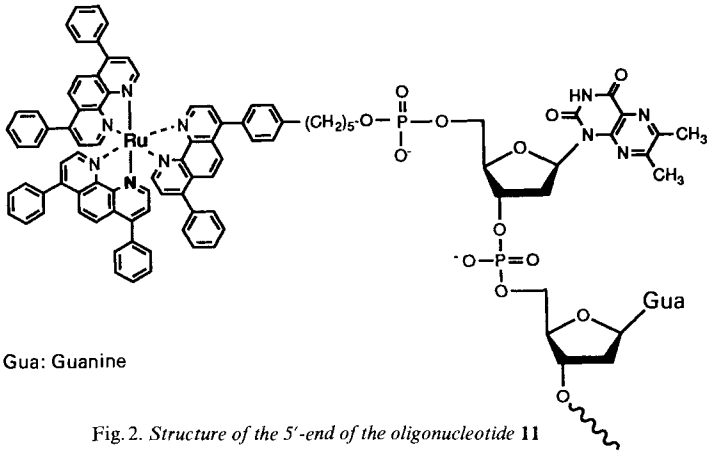
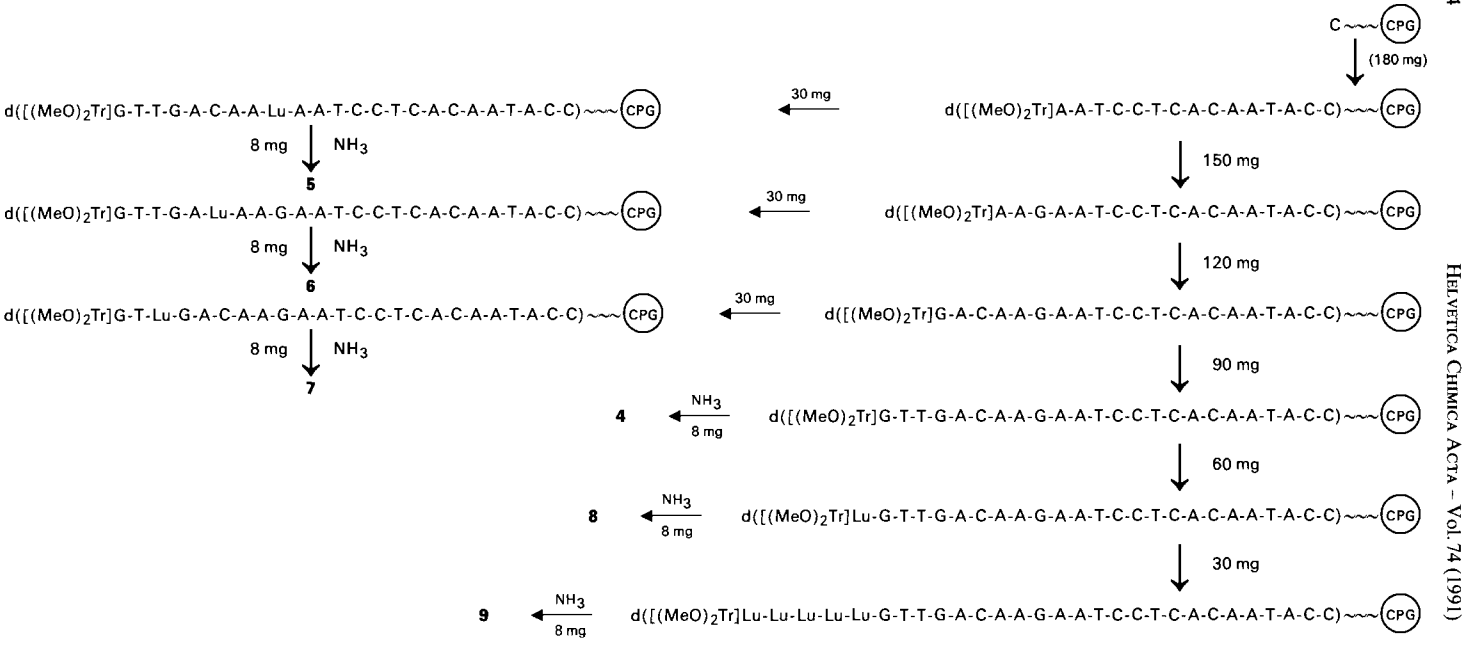



Fig. 2. *Structure of the 5′-end of the oligonucleotide* **11**

*Scheme 2*




d(G-T-T-G-A-C-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **4**

d(G-T-Lu-G-A-C-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **7**

d(G-T-T-G-A-Lu-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **6**

d(G-T-T-G-A-C-A-A-Lu-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **5**

d(Lu-G-T-T-G-A-C-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **8**

d(Lu-Lu-Lu-Lu-Lu-G-T-T-G-A-C-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **9**

(Ru complex)-d(G-T-T-G-A-C-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **10**

(Ru complex)-d(G-T-Lu-G-A-C-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **12**

(Ru complex)-d(G-T-T-G-A-Lu-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **13**

(Ru complex)-d(G-T-T-G-A-C-A-A-Lu-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **14**

(Ru complex)-d(Lu-G-T-T-G-A-C-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **11**

(Ru complex)-d(Lu-Lu-Lu-Lu-Lu-G-T-T-G-A-C-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C) **15**

tected with $NH_3$ leading to the crude products **5–9** (*Scheme 2*). They were checked by polyacrylamide gel electrophoresis showing the good coupling performance of the phosphoramidite **3** of the 6,7-dimethyllumazine deoxyriboside. From the residual material of the synthesis of each of these fragments, the dimethoxytrityl group was removed, and the syntheses were continued by coupling the [$Ru^{II}$(bathophenanthroline)] complex in the form of its *in situ* prepared phosphoramidite as we described recently [6]. A single cleavage step with $NH_3$ led then directly to the crude compounds **10–15** (*Scheme 2*), since the $NH_3$ treatment does not harm the Ru complex due to its great chemical stability. In these compounds, the Ru complex is attached *via* a very stable phosphodiester linkage to the DNA (see *Fig. 2*).

The performance of the syntheses of compounds **4–15** was judged from the UV-shadowing gel of the crude materials (*Fig. 3*). The insertion of phosphoramidite **3** proceeded

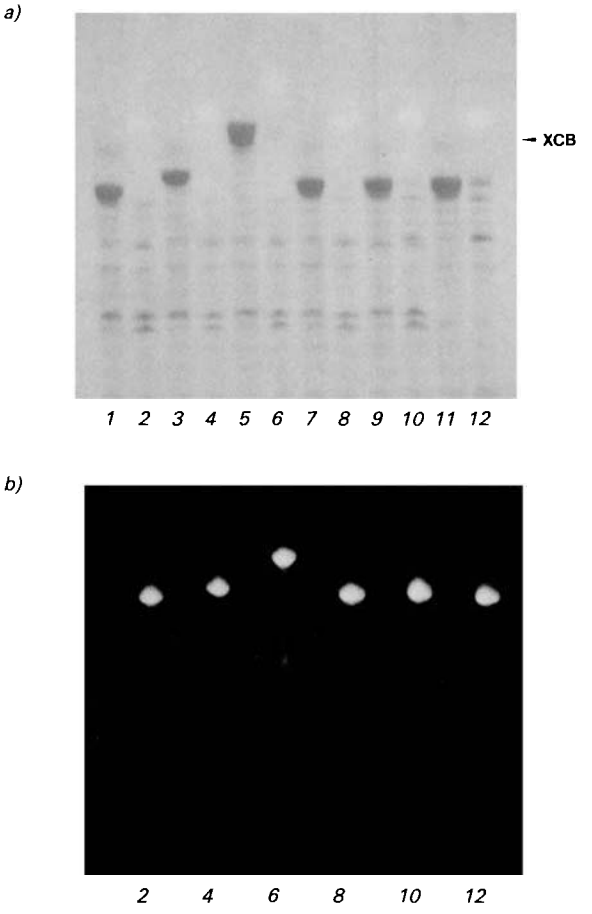



Fig. 3. *UV-Shadowing gel of the crude oligonucleotides after deprotection,* a) *at 256 nm and* b) *at 366 nm. Lane 1,* crude **4**; *Lane 2,* crude **10**; *Lane 3,* crude **7**; *Lane 4,* crude **11**; *Lane 5,* crude **9**; *Lane 6,* crude **15**; *Lane 7,* crude **7**; *Lane 8,* crude **12**; *Lane 9,* crude **6**; *Lane 10,* crude **13**; *Lane 11,* crude **5**; *Lane 12,* crude **14**.

with high efficiency as well as the attachment of the Ru complex at the 5′-end. The coupling of the latter caused a relatively strong retardation in gel electrophoresis. The pure compounds were obtained by electroelution from the preparative gel electrophoresis. Alternatively, they could also be purified and isolated easily by reversed-phase HPLC due to the higher retention time caused by the lipophilicity of the Ru complex (*Figs. 4* and *5*).

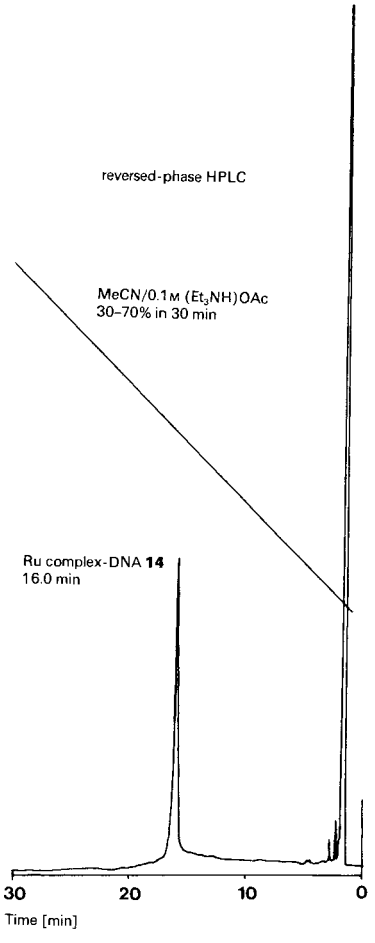



Fig. 4. *Reversed-phase HPLC of crude* **14**

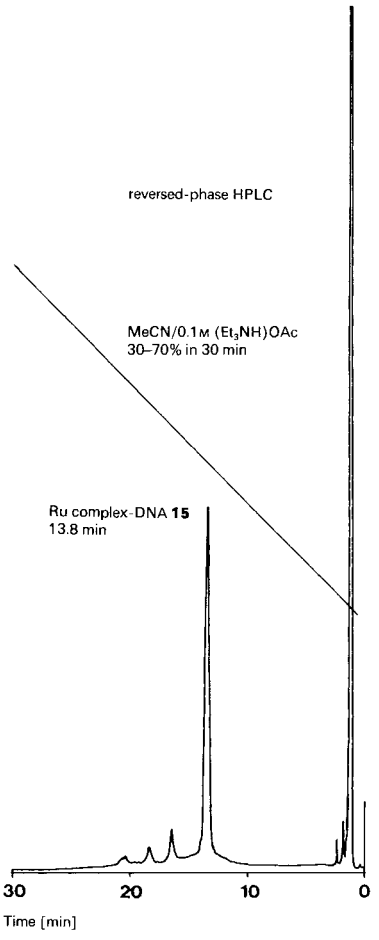



Fig. 5. *Reversed-phase HPLC of crude* **15**

2.2. *Spectroscopic Properties.* The spectroscopic properties of the lumazine deoxyriboside were studied in the free form **1** as well as incorporated into the single-stranded oligodeoxynucleotides. No significant changes in the spectroscopic behavior could be detected. However, the emission spectrum was strongly pH-dependent.

With a peak fluorescence intensity at $\lambda_{EM}$ 466 nm in a phosphate buffer saline solution (PBS; pH 6.9), the overlap of the emission spectrum of the lumazine chromophore **1** with the absorption spectrum of the Ru complex through its MLCT band is very good (see

above, *Fig. 1*). Also, the absorption spectrum of **1** with its highest absorption at 324 nm matches relatively well with the emission wavelength of the $N_2$ laser (337 nm). However, the molecular absorption coefficient at 337 nm is relatively small ($\varepsilon = 6800\ M^{-1}cm^{-1}$), that is, of the same order of magnitude as that of the Ru complex at the same wavelength. Although not quite optimal, this lumazine/Ru complex combination can be used as a model to study this type of energy-transfer systems.

Upon excitation at 337 nm, the fluorescence intensity $I_F$ at 618 nm (emission wavelength of the Ru complex) of the oligonucleotides **11**–**15**, labelled at the 5′-end with the Ru complex and containing one or more lumazine-chromophore moieties will be composed of 3 different components: *a*) fluorescence of the lumazine moiety at 618 nm ($I_{F1}$), *b*) fluorescence of the Ru-complex moiety due to direct excitation ($I_{F2}$), and *c*) fluorescence of the Ru-complex moiety due to energy transfer ($I_{F3}$). Thus the fluorescence due to energy transfer is determined by *Eqn. 1*.

$$I_{F3} = I_F - I_{F1} - I_{F2} \tag{1}$$

$I_{F1}$ and $I_{F2}$ were obtained by measurements on compounds **8** and **10**, respectively, in the same buffer system. Actually, the fluorescence intensity $I_{F1}$ of the lumazine moiety at 618 nm was very weak (*ca.* 0.5% of that of $I_{F2}$) and can be neglected. The fluorescence intensities were measured in two different ways; by conventional and by time-resolved fluorometry. This latter method used the 0.7-ns light pulses at 337 nm from a $N_2$ laser as excitation. The fluorescence light was detected by a photomultiplier using the photon-counting technique. In order to assess the efficiency $E$ of the energy transfer, we define the expression given in *Eqn. 2*.

$$E = \frac{I_F - I_{F2}}{I_{F2}}\ 100\ [\%] \tag{2}$$

The efficiencies obtained from intensity measurements using *Eqn. 2* are summarized in the *Table*. Distance sensing from the Ru complex appears through the incorporation of the lumazine moiety at different positions in the DNA strand, the Ru complex being always located at the 5′-end. An interpretation according to the *Förster* formula which predicts a

Table. *Efficiencies* E *of Energy Transfer for* **11**–**15**

| | **11** | **12** | **13** | **14** | **15** |
|---|---|---|---|---|---|
| $E$[%] | 13.0 | 11.2 | 5.0 | 3.9 | 43.5 |

6th power dependence of the energy transfer upon distance was not possible here because of the unknown structure of the oligonucleotide. The energy-transfer efficiency $E$ was *ca.* 13% between donor and acceptor in the nearest vicinity (compound **11**) and decreased with increasing distance between donor and acceptor. In compound **15** which has 5 consecutive lumazine moieties between the oligonucleotide and the Ru complex, the efficiency for the energy transfer was *ca.* 45%. These data were also confirmed by fluorescence lifetime measurements.

For lifetime measurements, the energy-transfer efficiency $E$ is given by *Eqn. 3*

$$E = 1 - \frac{\tau}{\tau_o} \tag{3}$$

where $\tau$ and $\tau_0$ are the fluorescence lifetimes of the energy donor in presence or in absence of the energy acceptor, respectively. Lifetimes $\tau = 12.85$ ns and $\tau_0 = 14.79$ ns were measured for **11** and **8**, giving, therefore, a transfer efficiency of 13%.

The incorporation of lumazine moieties into Ru-complex-labelled DNA allows the excitation of the Ru complex at the wavelength of the $N_2$ laser with about the same efficiency as through the excitation at 453 nm with the help of the dye laser so that the latter can be omitted.

**3. Conclusion.** – We showed that 1-(2′-deoxy-β-D-ribofuranosyl)-6,7-dimethyllumazine (**1**) can be incorporated as its phosphoramidite **3** with high efficiency into synthetic oligodeoxynucleotides during their syntheses on a solid support. The corresponding oligonucleotides are then labelled at the 5′-end with a bathophenanthroline-ruthenium(II) complex, also directly on the support material. We were able to show that the lumazine chromophore incorporated into the oligonucleotide can transfer light from a $N_2$ laser onto the attached Ru complex. In this respect, the combination represents an energy-transfer system with the lumazine moiety as a donor and the Ru complex as acceptor. The introduction of the lumazine-Ru-complex system at the 5′-end of oligonucleotides allows the direct application of a $N_2$ laser in order to perform time-resolved fluorometry with the same high efficiency as that obtained with Ru complexes excited by a combination of a $N_2$ and a dye laser.

Since the efficiency $E$ in energy-transfer systems is highly dependent on the distance between donor and acceptor, the determination of $E$ allows to measure distances so that the system can be applied as a spectroscopic ruler. The combination of the lumazine chromophore and the Ru complex is especially suited for distance measurements within synthetic DNA since the lumazine moiety can be positioned at will into the DNA. More generally, the system should be useful for the study of molecule interactions.

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## Experimental Part

1. *General.* All solvents were of highest purity available. The phosphoramidite of the [$Ru^{II}$(bathophenanthroline)] complex was prepared *in situ* as described earlier [6]. DNA syntheses were performed on controlled pore glass (CPG) as solid support [7] applying phosphoramidite chemistry and using our standard technology [8] [9]. Time-resolved fluorescence measurements were carried out in a volume of 100 µl on a home-made instrument [10]. Short column chromatography (CC) [11]: silica gel *60* (0.063–0.040 mm, *Merck*). TLC: HPTLC silica-gel plates (*Merck*). $^1$H-NMR (250 MHz): chemical shifts in $\delta$ (ppm) rel. to TMS. 1-(2′-Deoxy-β-D-ribofuranosyl)-6,7-dimethyllumazine (**1**) was prepared according to [12]; and the β-D-configuration assigned according to X-ray analysis and NMR investigations (manuscript in preparation; the configurations given in [12] have to be reversed).

2. *1-[2′-Deoxy-5′-O-(4,4′-dimethoxytrityl)-β-D-ribofuranosyl]-6,7-dimethylpteridine-2,4(1H,3H)-dione* (**2**). Compound **1** (0.15 mmol, 45 mg) was twice taken up in anh. pyridine and evaporated. Then, it was again dissolved in anh. pyridine (5 ml), and 4,4′-dimethoxytrityl chloride (0.25 mmol, 85 mg) was added at r.t. and with stirring. After 1 h, 1 ml of MeOH was added, and after additional 15 min, the mixture was poured into sat. $NaHCO_3$ soln. and extracted with $CH_2Cl_2$ (3 × 30 ml). The combined org. layer was dried ($Na_2SO_4$) and evaporated. The residue was separated by CC (10 g of silica gel, 100 ml of $CH_2Cl_2/Et_3N$ 99:1 and 100 ml of $CH_2Cl_2$/MeOH/$Et_3N$ 97:2:1). The residue of the pure fractions was dissolved in 5 ml of $CH_2Cl_2$ and precipitated into 150 ml of pentane. The

precipitate was dried: 65 mg (71%) of **2**. $^1$H-NMR ($CDCl_3$): 2.24–2.44 (*m*, 1 H–C(2′)); 2.46 (*s*, Me); 2.62 (*s*, Me); 2.86–3.03 (*m*, 1 H–C(2′)); 3.30 (*dd*, 1 H–C(5′)); 3.37 (*dd*, 1 H–C(5′)); 3.78 (*s*, 2 MeO); 3.95–4.05 (*m*, H–C(4′)); 4.73–4.85 (*m*, H–C(3′)); 6.67–6.84 (*m*, H–C(1′), 4 arom. H ($C_6H_4$)); 7.12–7.46 (*m*, 9 arom. H). Anal. calc. for $C_{34}H_{34}N_4O_7 \cdot 0.5\ H_2O$ (619.67): C 65.90, H 5.69, N 9.04; found: C 65.83, H 5.94, N 8.69.

3. *1-[2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-β-*D*-ribofuranosyl]-6,7-dimethylpteridine-2,4(1*H*,3*H*)-dione 3′-*O*-[(2-Cyanoethyl) N,N-Diisopropylphosphoramidite]* (**3**). In 15 ml of MeCN, 0.3 mmol (183 mg) of **2** were dissolved and evaporated. The residue was taken up again in 15 ml of MeCN and (2-cyanoethoxy)bis(diisopropylamino)phosphine (0.6 mmol, 180 mg) and diisopropylammonium tetrazolide (0.3 mmol, 51 mg) were added with stirring. Stirring was continued for 2 h (TLC monitoring), then the mixture was poured into 100 ml of sat. $NaHCO_3$ soln. and extracted 3 times with 30 ml of $CH_2Cl_2$ each. The combined org. layers were dried ($Na_2SO_4$) and evaporated. The residue was separated by CC (10 g of silica gel 100 ml of $CH_2Cl_2/Et_3N$ 99:1 and 100 ml of $CH_2Cl_2/Et_3N$ 98:2): 170 mg (70%) of **3**. Anal. calc. for $C_{43}H_{51}N_6PO_8 \cdot H_2O$: C 62.30, H 6.45, N 10.14; found: C 61.96, H 6.74, N 10.63.

4. *Syntheses of* **4–9**. The synthesis was started with 180 mg of controlled pore glass functionalized with C (4.87 μmol) in a glass-frit system. Elongations were performed with 40 mg of the corresponding 2-cyanoethyl phosphoramidite up to the insertion of **3**. Whenever **3** had to be introduced, an aliquot of 30 mg of solid support carrying the corresponding sequence was removed and the synthesis continued according to *Scheme 2*. For each elongation with **3**, 20 mg of **3** were applied. Of each protected sequence still attached to the solid support, 8 mg were removed, deprotected with ammonia, and checked on a 20% polyacrylamide gel under denaturing conditions (*Fig. 3*) for estimation of the performance of the synthesis of **4–9**.

5. *Syntheses of* **10–15**. From the residual material of the sequences which yielded compounds **4–9** after deprotection, the dimethoxytrityl protecting group was removed. Then the bathophenanthroline-ruthenium(II) complex was coupled with high efficiency applying its *in situ* prepared phosphoramidite as we described recently [6]. Treatment with conc. ammonia yielded directly the crude target compounds **10–15**. Polyacrylamide gel electrophoresis (*Fig. 3*) of the crude compounds as well as of the parent compounds before the coupling with the Ru complex confirmed especially the good yield of the coupling steps with **3** as well as with the Ru complex.

Pure **10–15** were either obtained by HPLC purification on reversed-phase material (see, *e.g.*, *Figs. 4* and *5*) or by prep. gel electrophoresis followed by electroelution.

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