219. Bathophenanthroline-ruthenium(II) Complexes as Non-Radioactive Labels for Oligonucleotides which Can Be Measured by Time-Resolved Fluorescence Techniques

by **Willi Bannwarth*, Dieter Schmidt, Robert L. Stallard, Claudia Hornung, Reinhard Knorr,** and **Francis Müller**

Central Research Units, *F. Hoffmann-La Roche & Co. Ltd.*, CH–4002 Basel

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The specific attachment of bathophenanthroline-ruthenium(II) complexes as non-radioactive label molecules to synthetically 5′-NH_2-modified oligonucleotides is described. After excitation by light pulses, the fluorescence of these labels can be measured by a time-resolved mode with high sensitivity. No quenching takes place due to coupling of the Ru complexes to the DNA. Ru-complex-labelled oligonucleotides still hybridize specifically to complementary DNA sequences, and no quenching is observed in the course of the hybridization process.

1. Introduction. – With a relatively new technique termed as DNA-probe technology, it is possible to screen directly genetic material for defects or the presence of foreign DNA [1]. The method is based on the specific hybridization of a probe DNA with the corresponding complementary DNA sequence. The main goals with this approach are the diagnosis of genetic and infectious diseases and of cancer as well as the diagnosis of predispositions to certain types of diseases. Our work in this field has concentrated on the replacement of radioactive labels by non-radioactive reporter systems. The most prominent system of this type is the biotin-avidin system [2].

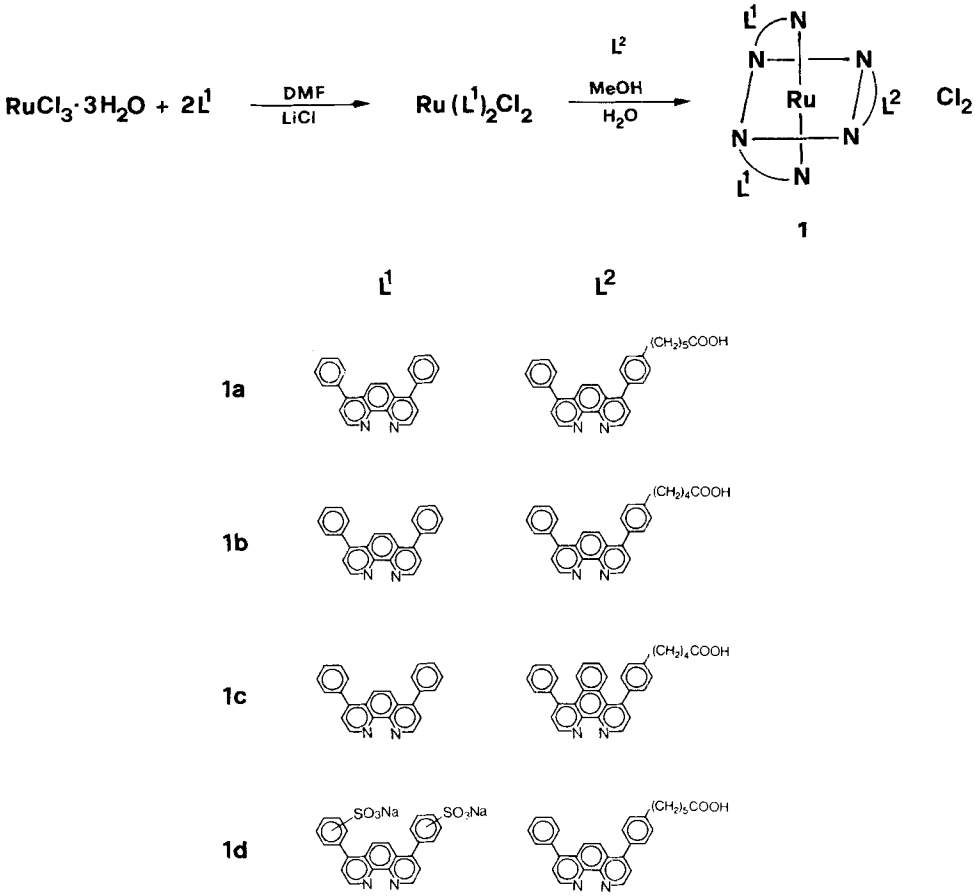
We have focused on organometallic complexes showing a strong fluorescence once they are being excited, usually with a laser. Among these, the lanthanide- and especially Eu complexes are already well established [3]. They show a strong and long-lasting fluorescence after excitation which allows to detect this fluorescence by a time-resolved technique, thereby virtually eliminating the disturbing background fluorescence.

A great disadvantage of the Eu complexes is their relatively low thermodynamic stability resulting in their dissociation at low concentrations. This problem can be overcome by, for example, embedding the Eu ion into cryptates containing bipyridine ligands [4] [5].

Our goal was to use Ru(II) charge-transfer complexes of type **1** for the non-radioactive labelling of biological probes. Bathophenanthroline-ruthenium(II) complexes show a number of properties which make them ideal candidates for being applied in this sense. The complexes are thermodynamically very stable, and, therefore, they do not dissociate at low concentrations, they are chemically very inert and, furthermore, they show a strong and long-lasting fluorescence after excitation by light pulses of short duration which allows their detection by time-resolved measurement techniques.

2. Results and Discussion. – 2.1. *Bathophenanthroline-ruthenium(II) Complexes.* Our intention was to couple Ru(bathophenanthroline) complexes specifically at the 5′-end to synthetic DNA probes *via* a relatively stable amide bond. The end-specific attachment would ensure a minimal interference of the complexes during hybridization processes, especially if a spacer molecule is introduced between the Ru complex and the DNA. In

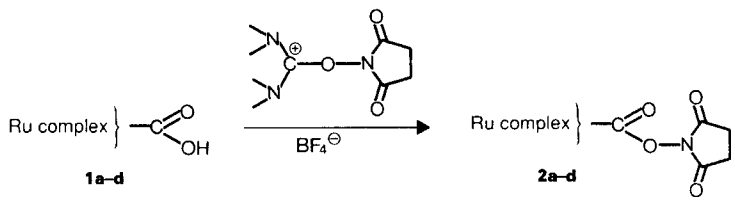
Scheme 1



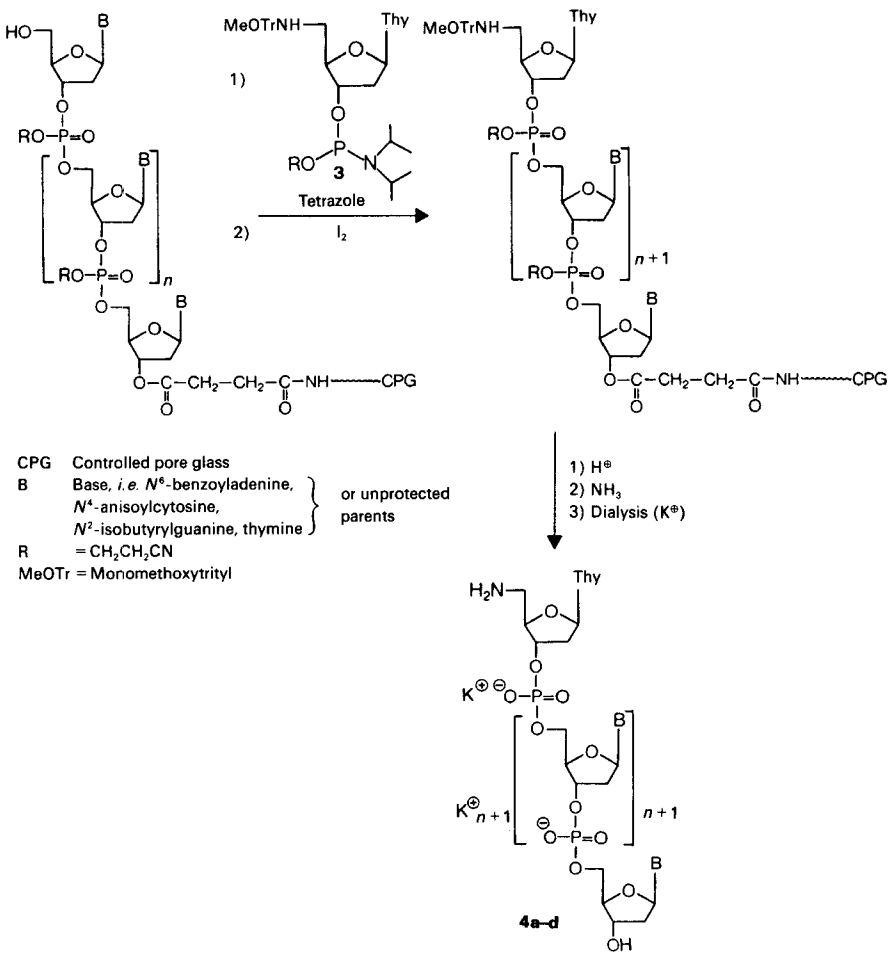
order to reach this goal, one of the phenanthroline ligands must bear a linker arm and a terminal carboxy group attached to it. On the other hand, the DNA fragment has to be modified with an NH_2 group at the 5′-end to which the Ru complexes can be coupled specifically. According to *Scheme 1,* we have prepared a few suitable Ru complexes **1a–d** [6]. They differ either in the length of the spacer arm or in the bathophenanthroline ligands as such.

The complexes **1a–d** were then transformed to the corresponding esters **2a–d** of *N*-hydroxysuccinimide *(Scheme 2)*. This type of activated ester was chosen, since the

Scheme 2



Scheme 3



DNA to be reacted with needs the presence of H_2O to get solubilized, and succinimido esters show a relatively great stability even in the presence of aqueous medium. Usually, the preparation of such esters is performed with dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide. We have found that the esterification can be performed in a much cleaner way with *N,N,N',N'*-tetramethyl(succinimido)uronium tetrafluoroborate (TSU) [7], a reagent which was used for the hardening of gelatine [8]. After a reaction time of *ca.* 2 h and a slight excess of *ca.* 10% of TSU, the Ru complexes were transformed completely into their succinimido esters as checked by TLC. After this activation, the crude reaction mixture could be used directly for the coupling with the amino-modified DNA fragments. But we were also able to isolate and purify the activated complexes **2a–d** by short-column chromatography [9].

2.2. *5'-Amino-modified DNA Fragments.* There exists a number of different methods for the introduction of an NH_2 group at the 5'-end of synthetic DNA fragments [10–17]. In the most recently published methods, this functional group is introduced by a modified building unit, a phosphoramidite or a phosphodiester, at the end of the synthesis of the DNA fragment on a solid support.

Currently, we prefer mainly two strategies to introduce the NH_2 function at the 5'-end of synthetic DNA fragments, and both strategies allow the application of standard phosphoramidite cycles. In the first one, the phosphoramidite **3** of 5'-amino-5'-deoxythymidine was introduced as building block to produce the 5'-NH_2-modified DNA fragments **4a–d** according to *Scheme 3* (see *Table 1*). The attachment of **3** proceeded with the same high yield as with unmodified standard phosphoramidites. After deprotection and a dialysis step, the crude mixture could be used directly for the coupling with the succinimido esters of the Ru complexes. The dialysis step had to be introduced in order to replace the NH_4^+ ions of the phosphodiester functions by K^+ ions since the NH_4^+ ions could interfere during the coupling reaction with the NH_2 group at the 5'-end of the DNA.

Table 1 [a])

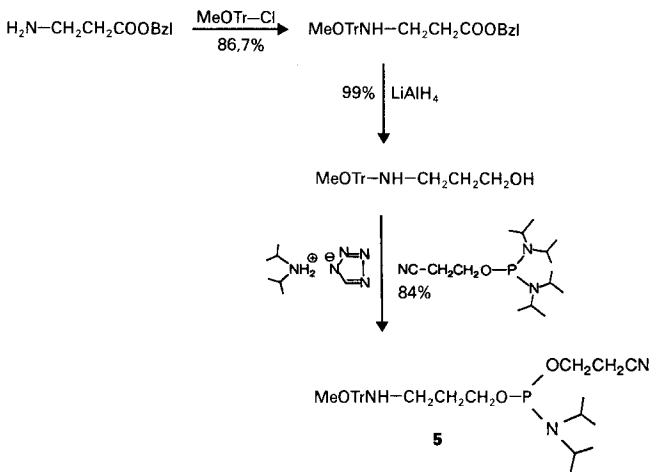
4a	d(nh$_2$T-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T-G)
4b	d(nh$_2$T-T-T-T-C-T-G-G-A-T-C-C-C-T-G-A-G-C-C-T-G-T-T-C)
4c	d(nh$_2$T-G-A-C-G-T-T-G-T-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T-G)
4d	d(nh$_2$T-G-T-T-G-A-C-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C)
4e	d($H_2N(CH_2)_3OPO_3^{\ominus}$T-T-T-T-C-T-G-G-A-T-C-C-C-T-G-A-G-C-C-T-G-T-T-C) [b])

[a]) nh$_2$ means replacement of the 5'-terminal OH by NH_2.

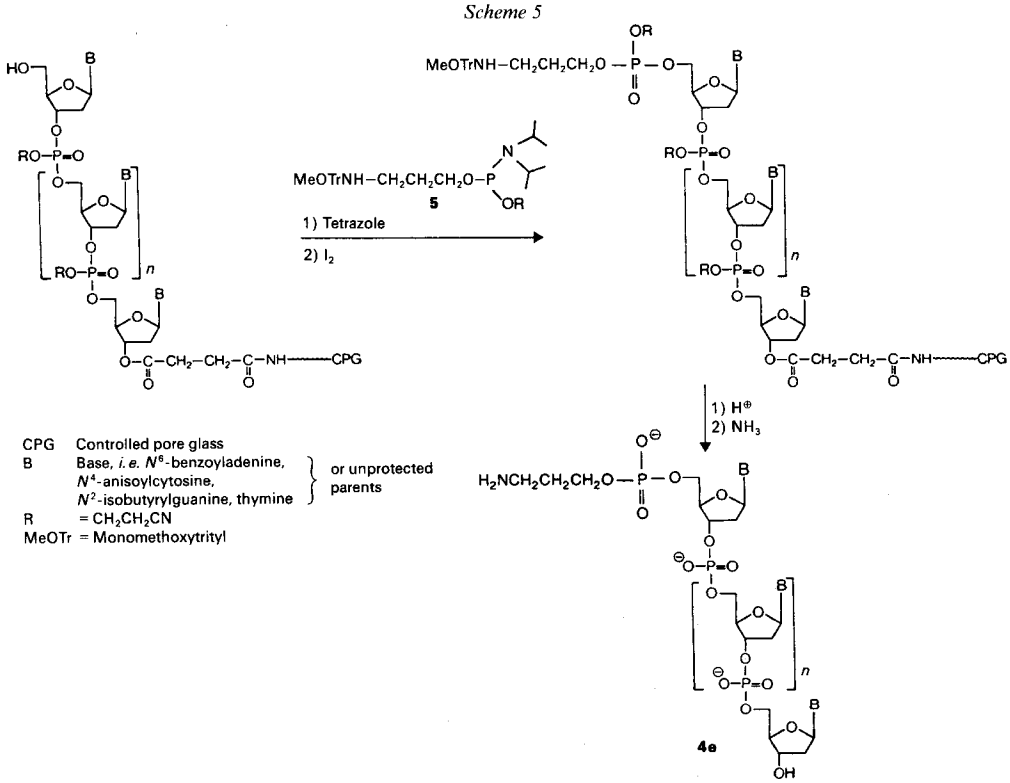
[b]) Replacement of 5'-terminal OH by $H_2N(CH_2)_3OPO_3^-$.

The second approach used the building unit **5** which allows to introduce a spacer arm between the amide bond and the DNA fragment. This building block was synthesized in very good yield starting from *β*-alanine benzyl ester according to *Scheme 4*. Independently, this building block was mentioned in [15] where it was synthesized starting from aminopropanol. The modification with **5** proceeded then according to *Scheme 5* (→**4e**; see *Table 1*).

Scheme 4



Scheme 5



The synthesis of the fragments was performed on a large scale (5–10 μmol) on controlled pore glass as solid support [18] using (2-cyanoethyl) phosphoramidites [19] as building units and our standard technologies [20] [21]. After the deprotection, the crude mixture was dialysed two times against KCl, and this mixture was then used for the coupling with the corresponding activated Ru complexes. As a typical example, *Fig. 1*

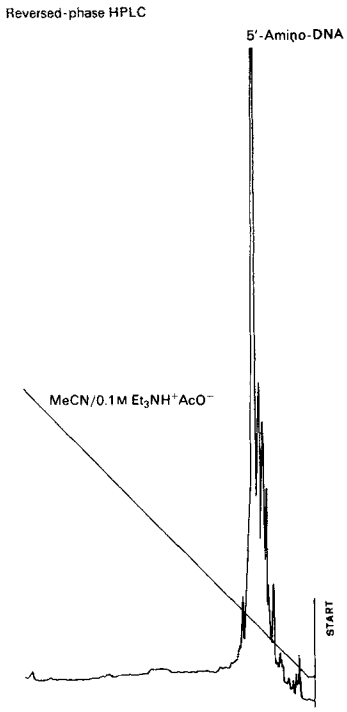


Fig. 1. *Reversed-phase HPLC of crude 5'-NH_2-modified DNA fragment* **4a**

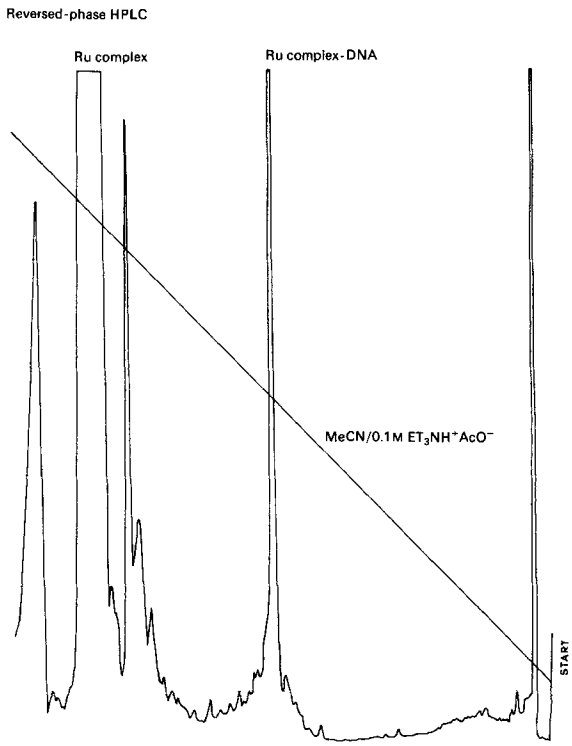
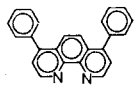
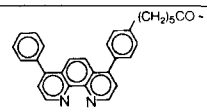
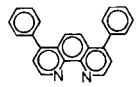
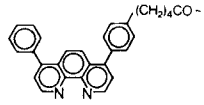
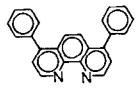
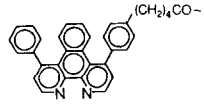
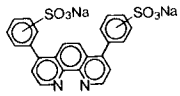
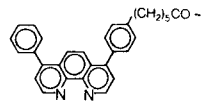
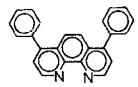
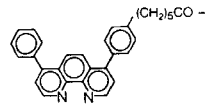
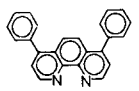
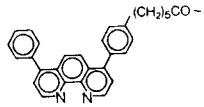
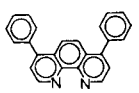
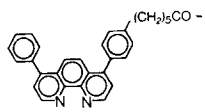
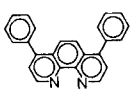
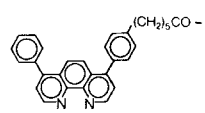


Fig. 2. *Reversed-phase HPLC of the crude reaction mixture of the preparation of* **6a**

shows the reversed-phase HPLC of a crude reaction mixture which indicates that, in this case, at least 62% of the mixture is represented by the desired 5'-NH_2-modified DNA fragment **4a**.

2.3. *Ru Complexes Attached to Amino-Modified DNA.* The coupling between the succinimido esters of the Ru complexes and the NH_2-modified DNA fragments **4a–e** was carried out in dioxane/DMF/H_2O to ensure a complete solubility of the very lipophilic Ru complexes and the hydrophilic DNA fragments. The reactions were carried out in the presence of (i-Pr)$_2$EtN (*Hünig* base) and with a 25-fold excess of the Ru complexes **2a–d** according to *Scheme 6*. The larger excess of the Ru complexes caused problems in the isolation of the thus prepared coupling products **6a–h** (see *Table 2*), since even after a

Table 2 [a])

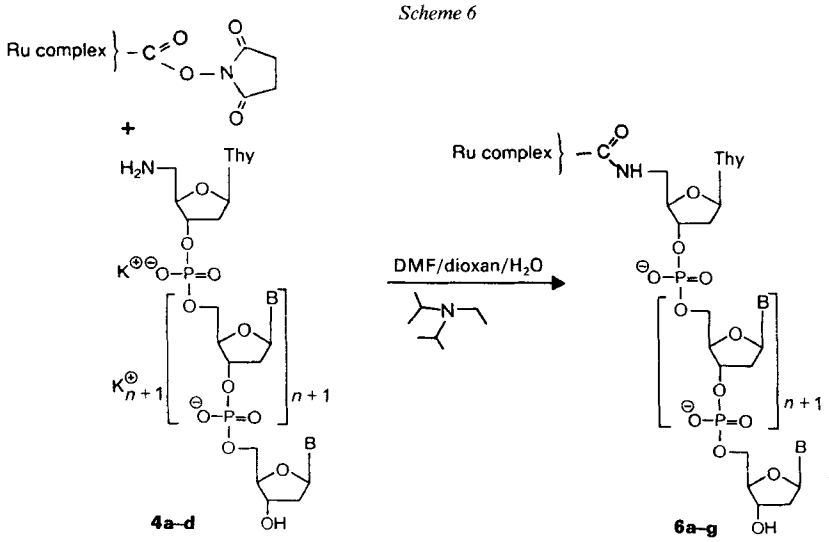
Compound	Ru Complex L^1	Ru Complex L^2	DNA
6a			d(-nhT-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T-G)
6b			d(-nhT-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T-G)
6c			d(-nhT-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T-G)
6d			d(-nhT-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T-G)
6e			d(-nhT-T-T-T-C-T-G-G-A-T-C-C-C-T-G-A-G-C-C-T-G-T-T-C)
6f			d(-nhT-G-A-C-G-T-T-G-T-A-A-A-A-C-G-A-G-G-C-C-A-G-T-G)
6g			d(-nhT-G-T-T-G-A-C-A-A-G-A-A-T-C-C-T-C-A-C-A-A-T-A-C-C)
6h			d(-HN(CH$_2$)$_3$OPO$_3^{\ominus}$T-T-T-T-C-T-G-G-A-T-C-C-C-T-G-A-G-C-C-T-G-T-T-C)[b])

[a])[b]) See *Table 1.*

complete reaction, only a moderate amount of the reaction mixture represented the expected products. Isolation and purification was possible by HPLC on reversed-phase *C18* columns with a very high gradient of MeCN in 0.1M $Et_3NH^+AcO^-$. The unreacted DNA was eluted at a relatively low gradient and the unreacted Ru complex at a very high MeCN content due to its high lipophilicity, the desired product being somewhere in the middle between these two extremes. A typical example is shown in *Fig. 2.*

Since the vast excess of Ru complex limited the HPLC column capacity, we removed the unreacted Ru complex prior to the HPLC separation by extracting it with $CHCl_3$. *Fig. 3* shows an HPLC after one single extraction step with $CHCl_3$ indicating that most of the Ru complex had been removed. A comparison of the HPLC

Scheme 6



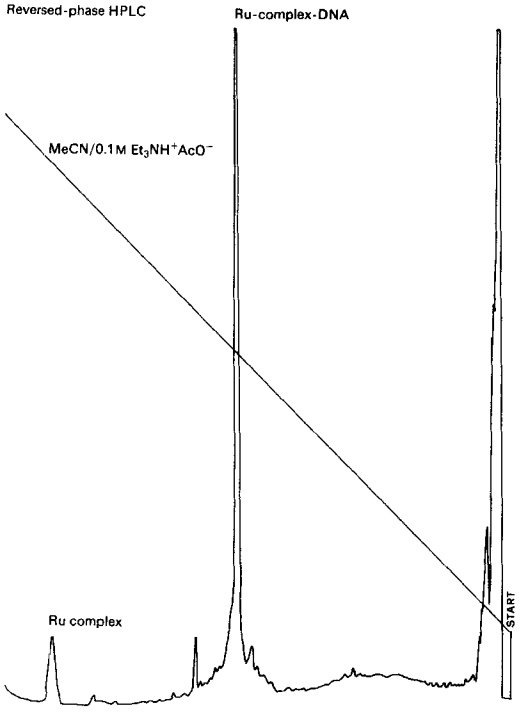


Fig. 3. *Reversed-phase HPLC of the crude reaction mixture of the synthesis of* **6a** *after* $CHCl_3$ *extraction*

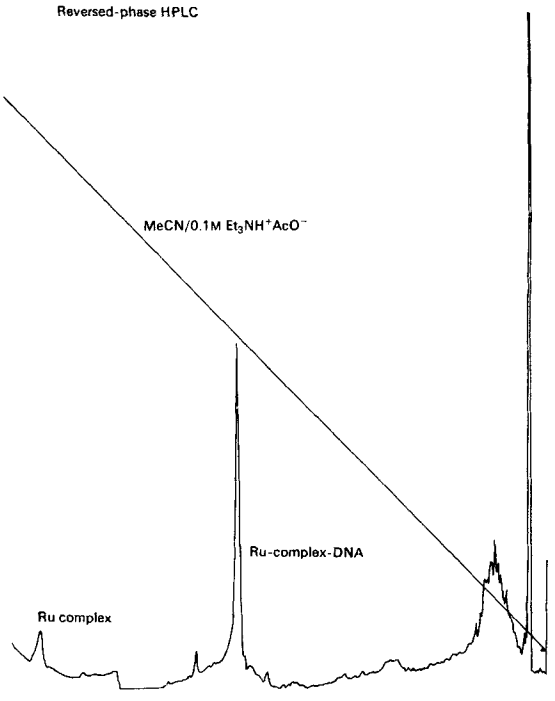


Fig. 4. *Reversed-phase HPLC corresponding to the one shown in* Fig. 3 *but run under the same conditions as the one shown in* Fig. 1

separations in *Figs. 4* and *1* run under the same conditions illustrates that virtually all 5'-NH_2-DNA had been coupled to the Ru complex.

The coupling of the Ru complexes to the DNA fragments led to a relatively strong retardation in the mobility in polyacrylamide gel electrophoresis compared to the NH_2-modified DNA fragments as such *(Fig. 5)*.

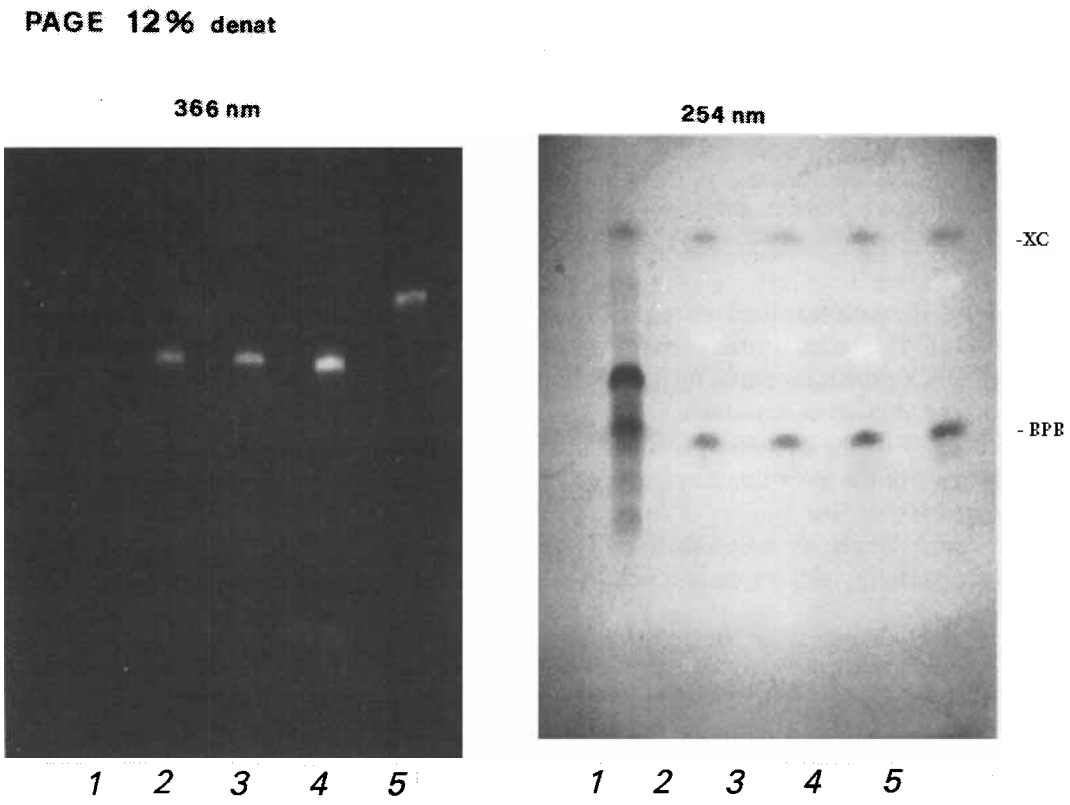


Fig. 5. *UV-Shadowing polyacrylamide gel of Ru-complex-labelled DNA sequence* **6a-d**. Lane *1*: crude **4a**; lane *2*: HPLC-purified **6a**; lane *3*: HPLC-purified **6b**; lane *4*: HPLC-purified **6c**; lane *5*: HPLC-purified **6d**.

2.4. *Properties of Compounds* **6a–h**. a) *Time-Resolved Fluorescence*. The coupling products **6a–h** show the characteristic metal to ligand absorption band of the Ru(bathophenanthroline) complexes at 440 and 464 nm and the fluorescence at 616 nm when measured in phosphate buffer saline (PBS; 150 mM NaCl, 10 mM p_i). The detection limit is below 10^{-14} mol/l when measured by time-resolved fluorescence techniques after excitation by a pulsed dye laser. At this sensitivity, the signal/noise ratio is 2. This value is the same as for free Ru(bathophenanthroline) complexes indicating that no quenching takes place due to the coupling of these complexes to DNA molecules. The same was true when Ru-complex-labelled DNA fragments were hybridized to a complementary single-stranded DNA sequence. Since the measurements are routinely carried out in a volume of

Table 3. *Fluorescence Life Times of* **6a**

Medium [a])	Concentration [% w/w]	Fluorescence life time [μsec]		
			+0.1M/l Na_2SO_3	+0.1M/l NH_4PF_6 +0.1M/l Na_2SO_3
Thioglycerol/phthalic acid		7.2		
PBS, H_2O		2.0	4.2	
PBS + 12-DAPS	0.7	1.3	1.9	7.5
PBS + CHAPS	2.5	3.0	1.9	5.2
PBS + DDPC	1.9	1.3	4.0	6.8
PBS + β-OG	3	1.9	1.7	4.7
PBS + *Triton X-100*	2	1.9	2.2	5.4
PBS + SDS	2.9	1.2	6.0	7.4

[a]) PBS = phosphate buffer saline (150 mM NaCl, 10 mM *pi*); DAPS = 3-(dimethyl-ammonio)-1-propane-sulfonate, zwitterionic detergent; CHAPS = 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, zwitterionic detergent; DDPC = dodecylphosphorylcholine, nonionic detergent; SDS = sodium dodecyl sulfate, anionic detergent; β-OG = β-ocytl glucoside.

100 μl, the detection limit corresponds to 10^{-18} mol or $6 \cdot 10^5$ molecules, which is almost the level of ^{32}P-labelled probes prepared by nick translation and more sensitive then ^{32}P-labelled DNA probes prepared by polynucleotide kinase and (γ-^{32}P)ATP.

The number of detectable molecules is mainly determined by the test volume and the efficiency of the excitation of the Ru complexes by the pulsed dye laser (10–15%). A decrease of the test volume and a more efficient excitation process should, therefore, lead to an even smaller number of detectable molecules.

Surprisingly, we were able to influence the decay time τ of the fluorescence as well as the quantum yields by certain additives like detergents, salts, and reducing agents in a

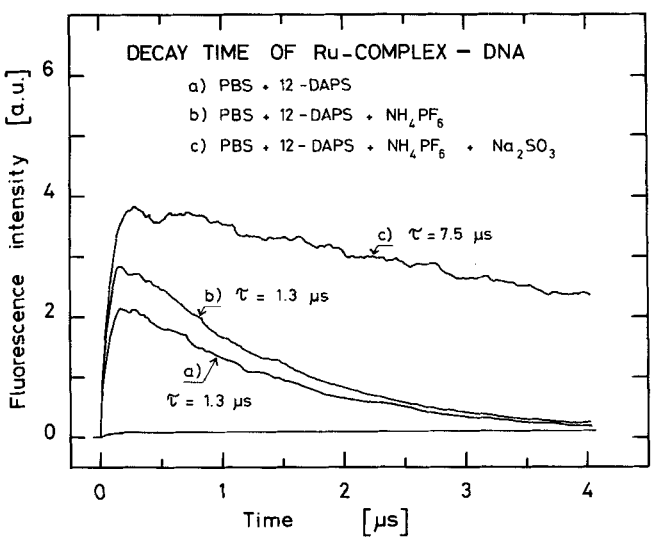


Fig. 6. *Typical curves obtained for the fluorescence decay of* **6a**

positive manner. Thus, the decay time could be extended up to 7.5 μsec compared to the 2 μsec when measuring in PBS without additive *(Table 3)*. *Fig. 6* shows a few typical curves for the fluorescence decay of compound **6a**.

b) *Specific Hybridization.* An essential requirement for the Ru-complex-labelled DNA fragments in order to be used in DNA-probe technology is the specific hybridization to complementary single-stranded DNA molecules. In order to demonstrate that the Ru complex has no negative effect on this hybridization, compounds **6a** and **6d** of which the DNA fragments represents the M13 universal sequencing primer [22] were tested in several experiments. Firstly, it was demonstrated that **6d** hybridized specifically to its

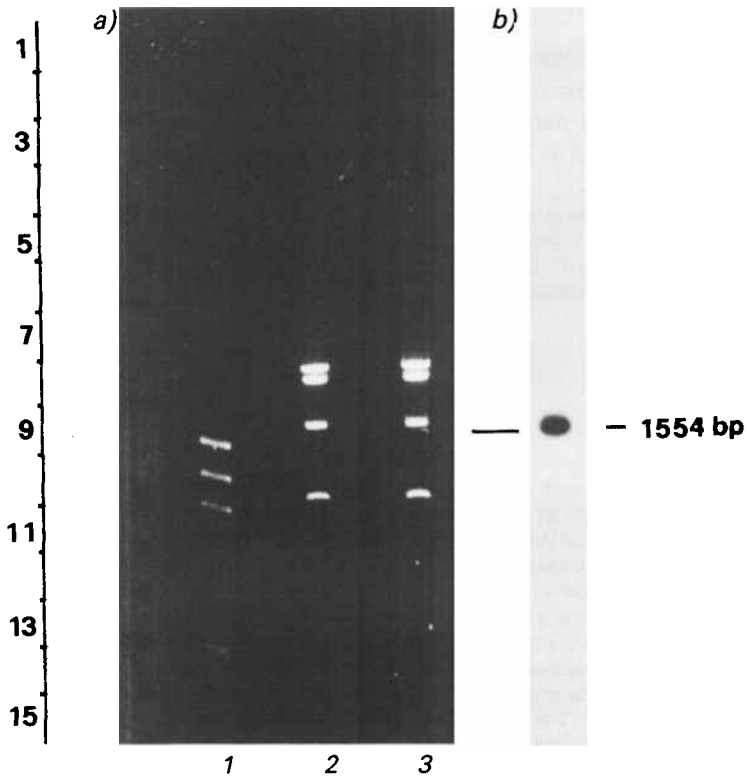


Fig. 7. Southern *hybridization. a*) Lane *1*: HaeIII digest of $\phi \times 174$ Rf-DNA as standard; lane *2* and *3*: SspI digest of M13mp18; *b*) Specific hybridization of **6d** to the 1554 bp fragment of the M13 mp18 SspI digest.

counterpart in a *Southern* DNA hybridization [23] experiment *(Fig. 7)*. Secondly, it was demonstrated that **6a**, when hybridized to a synthetically prepared target, can be elongated specifically by T7 DNA polymerase (data not shown), and thirdly **6a** was shown to produce specific patterns when used in M13 sequencing reactions by the method of *Sanger* [24] (will be published elsewhere).

3. Conclusion. – We have shown that carboxyalkyl-substituted Ru(bathophenanthroline) complexes can be specifically coupled, after their activation by TSU, to synthetically prepared 5′-NH_2-modified DNA fragments. The coupling products can be isolated by reversed-phase HPLC prior to which the large excess of activated and unreacted Ru(bathophenanthroline) complex is removed by extraction. Thus, a number of combinations between different Ru(bathophenanthroline) complexes and different amino-modified DNA fragments have been synthesized. Due to the coupling to the DNA, the relatively long fluorescence decay time of the complexes which is the basis for being applied in time-resolved measurements is not decreased. The same is true when 5′-Ru-complex-labelled DNA fragments are hybridized to a complementary single-stranded DNA sequence. With different additives, the fluorescence decay time is increased up to 7.5 μs. At present, we are able to detect with this method *ca.* $6 \cdot 10^5$ molecules in a test volume of 100 μl.

Furthermore, it is shown that Ru(bathophenanthroline) complex labelled DNA fragments still hybridize specifically to single stranded complementary sequences.

Thus, Ru(bathophenanthroline) complexes seem to be ideal nonisotopic reporter molecules to be applied in DNA probe technology.

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Experimental Part

1. *General.* All solvents were of the highest purity available. The Ru(bathophenanthroline) complexes **1a–d** were synthesized according to [6] [25]. Complex **1d** was a mixture due to the substitution pattern obtained on sulfonylation of bathophenanthroline. The synthesis of building block **3** has been reported earlier [26]. The activation agent *N,N,N′,N′*-tetramethyl(succinimido)uronium tetrafluoroborate (TSU) was prepared according to [8]. DNA synthesis was performed on controlled pore glass as solid support as described earlier [20] [21] [27] [28]. The syntheses were started from 5 μmol of functionalized support, and at the end of the synthesis, either building block **3** or **5** were introduced in a standard cycle in order to modify the DNA fragment with a NH_2 function at the 5′-end. The deprotection procedure of the amino-modified DNA fragments was like in unmodified DNA fragments. After deprotection, the crude mixture was evaporated and then dialysed 2 times against 2 l of H_2O with a 20-fold molar excess of KCl. The restricition enzyme SspI was from *New England Biolabs,* terminal nucleotidyl transferase from *BRL*, M13mp18 Rf from *Pharmacia,* the nylon membranes from *Pall Biosupport,* East Hills, N. Y., and (α^{32}P)ddATP from *Amersham.* Short-column chromatography (CC) [9]: silica gel *60* (0.063–0.040 mm, *Merck*). TLC: HPTLC silica plates *(Merck)*. ^{1}H-NMR (400 MHz): chemical shifts in δ (ppm) rel. to TMS.

2. *(2-Cyanoethoxy)[(diisopropyl)amino]{3-[(4-methoxytrityl)amino]propoxy}phosphine* (**5**). β-Alanine benzyl ester · TsOH (15 mmol, 5.27 g) was taken up in 50 ml of anh. MeCN and evaporated. This was repeated, and the residue was dissolved in 80 ml of anh. pyridine. Then, 30 mmol (9.264 g) of (4-methoxyphenyl)diphenylmethyl chloride and 37.5 mmol (39 ml) of *N*-methylmorpholine were added with stirring. After 4 h (reaction not complete (TLC)), another batch of (4-methoxyphenyl)diphenylmethyl chloride (3 mmol, 926 mg) and of *N*-methylmorpholine (3.75 mmol, 0.38 ml) was added. After 1 h (reaction complete (TLC)), MeOH (100 ml) was added and the mixture stirred for 30 min, then concentrated to *ca.* 100 ml, poured into 400 ml of sat. $NaHCO_3$ soln., and extracted 3 times with 150 ml of CH_2Cl_2 each. The combined org. layers were dried (Na_2SO_4) and evaporated after addition of toluene. The residue was purified by CC (90 g of silica gel; hexane (200 ml), hexane/Et_2O 9:1 (200 ml), hexane/Et_2O 8:2 (200 ml), and hexane/Et_2O 7:3 (200 ml)). The pure fractions (TLC) were crystallized from

Et_2O/hexane: 5.87 g (86.7%) of N-*[(4-methoxyphenyl)diphenylmethyl]-β-alanine benzyl ester*. M.p. 86–87°. ^{1}H-NMR ($CDCl_3$): 1.97 (br. *s* NH); 2.43 (*t*, CH_2NH); 2.53 (*t*, CH_2COOCH_3); 3.77 (*s*, CH_3O); 5.14 (*s*, CH_2O); 6.76–7.45 (*m*, 2 C_6H_5, C_6H_4).

To a soln. of *N*-[(4-methoxyphenyl)diphenylmethyl]-*β*-alanine benzyl ester (3 mmol, 1.36 g) in 50 ml of anh. Et_2O at 0°, 6 mmol (228 mg) of $LiAlH_4$ were added and stirred for 1 h at r.t. Then, 10 ml of MeOH were added dropwise at 0°. The mixture was poured into sat. $NaHCO_3$ soln. (50 ml), Et_2O (200 ml) was added, the org. layer washed 3 times with $NaHCO_3$ soln., dried (Na_2SO_4), and evaporated, and the crude material (1.4 g) purified by CC (15 g of silica gel; Et_2O/pentane 1:1 (200 ml), Et_2O/pentane 2:1 (200 ml), and Et_2O (200 ml)): 1.03 g (99%) of *3-{[(4-methoxyphenyl)diphenylmethyl]amino}propanol*. ^{1}H-NMR ($CDCl_3$): 1.70 (*m*, $CH_2CH_2CH_2$); 2.39 (*t*, CH_2NH); 2.75 (br. *s*, NH, OH); 3.78 (*s*, CH_3O); 3.87 (*t*, CH_2OH); 6.60–7.45 (*m*, 2 C_6H_5, C_6H_4). Anal. calc. for $C_{23}H_{25}NO_2 \cdot H_2O$ (365.47): C 75.59, H 7.45, N 3.83; found: C 75.24, H 7.43, N 3.70.

The protected amino alcohol (2.6 mmol, 904 mg) was evaporated 3 times with anh. MeCN (50 ml each). The residue was taken up in 50 ml of anh. CH_2Cl_2, and 1.5 mmol (258 mg) of diisopropylammonium tetrazolide followed by 3 mmol (905 mg) of (2-cyanoethoxy)bis(diisopropylamino)phoshine were added. After stirring for 90 min the mixture was poured into 100 ml of sat. $NaHCO_3$ soln. and extracted 3 times with CH_2Cl_2, the combined org. phase dried (Na_2SO_4) and evaporated, and the oily residue purified by CC (15 g silica gel; pentane/Et_2O/Et_3N 75:24:1 (200 ml) and pentane/Et_2O/Et_3N 66:33:1 (200 ml)): 1.19 g (84%) of pure **5**. ^{1}H-NMR ($CDCl_3$): 1.10, 1.16 (2*d*, CH_3CH); 1.64 (*t*, NH); 1.80 (*m*, $CH_2CH_2CH_2$); 2.21 (*dd*, CH_2NH); 2.52 (CH_2CN); 3.45–3.82 (*m*, CH_2O, $(CH_3)_2CH$); 3.78 (*s*, CH_3O); 6.78–7.47 (*m*, 2 C_6H_5, C_6H_4). Anal. calc. for $C_{32}H_{42}N_3O_3P$ (347.46): C 70.18, H 7.73, N 7.67; found: C 70.06, H 7.87, N 7.80.

3. *Activated Complex* **2a**. Under exclusion of moisture, **1a** (100 μmol, 128.4 mg) was dissolved in anh. DMF (2 ml; distilled from Ph_3SiCl). TSU (110 μmol, 36 mg) and (i-Pr)$_2$ EtN (110 μmol, 14.2 mg, 18.7 μl) were added and stirred for 2 h at r.t. Then, the DMF was removed under high vacuum and the residue suspended in Et_2O. After filtration, it was washed several times with Et_2O and dried: 114 mg (82.6%) of **2a** as a pale red powder, which can be used directly for the coupling with amino-modified DNA. For characterization, it was purified by CC (4 g of silica gel; $CHCl_3$ and increasing parts of EtOH (2% steps)): 90 mg (65.2%) of pure **2a**.

IR (KBr): 1808, 1778 (5-ring imid); 1737 (CO, ester); 1205 (ester). ^{1}H-NMR ($CDCl_3$): 1.45–1.86 (3*m*, $CH_2(CH_2)_3CH_2$); 2.62 (*t*, CH_2COO); 2.73 (*t*, $C_6H_4CH_2$); 2.82 (*s*, $OCCH_2CH_2CO$); 7.35, 7.37 (2*m*, 2 H of C_6H_4); 7.48–7.66 (*m*, 25 H of C_6H_5, 2 H of C_6H_4); 7.83 (*m*, 3 H–C(3), 3 H–C(8)); 8.18–8.28 (*m*, 3 H–C(5), 3 H–C(6)); 8.46–8.53 (*m*, 3 H–C(2), 3 H–C(9)).

4. *Activated Complex* **2b** was prepared in the same way and with the same molar amounts as **2a**: 105 mg (76.8%). Part of this material (58 mg) was purified by CC as described for **2a**: 34 mg of pure **2b**. IR (KBr): 1808, 1778, (5-ring imide); 1737 (CO, ester); 1204 (ester). ^{1}H-NMR ($CDCl_3$): 1.83 (*m*, $CH_2CH_2CH_2CH_2$); 2.66 (*t*, CH_2CO); 2.76 (*t*, $C_6H_4CH_2$); 2.83 (*s*, $OCCH_2CH_2CO$); 7.37, 7.39 (2 *m*, 2 H of C_6H_4); 7.50–7.66 (*m*, 25 H of 5 C_6H_5, 2 H of C_6H_4); 7.83 (*m*, 3 H–C(3), 3 H–C(8)); 8.18–8.28 (*m*, 3 H–C(5), 3 H–C(6)); 8.49 (*m*, 3 H–C(2), 3 H–C(9)).

5. *Activated Complex* **2c** was prepared in the same way and with the same molar amounts as **2a**: quant. yield. After CC as described for **2a** over 5.2 g of silica gel, 91 mg (64.2%) of pure **2c** were obtained. IR (KBr): 1808, 1784 (5-ring imide); 1737 (CO, ester); 1204 (ester). ^{1}H-NMR ($CDCl_3$): 1.82 (*m*, $CH_2CH_2CH_2CH_2$); 2.66 (*t*, CH_2COO); 2.76 (*t*, $C_6H_4CH_2$); 2.83 (*s*, $OCCH_2CH_2CO$); 7.15 (2 H of CH=CH); 7.31, 7.33 (2*m*, 2 H of C_6H_4); 7.40–7.79 (*m*, 25 H of 5 C_6H_5, 2 H of C_6H_4, 2 H of benzo); 7.79–7.90 (*m*, 2 H–C(3), 2 H–C(8)); 8.20 (*s*, 2 H–C(5), 2 H–C(6)); 8.39, 8.46, 8.54 (3*m*, 3 H–C(2), 3 H–C(9)).

6. *Activated Complex* **2d**. In DMF (2 ml) and H_2O (0.2 ml), **1d** (30 μmol, 51 mg) was reacted with TSU (70 μmol, 23 mg) in the presence of (i-Pr)$_2$EtN (70 μmol, 9.0 mg, 11.9 μl) at r.t. After 1 h stirring (TLC control), the DMF was evaporated and the residue suspended in Et_2O. After filtration, it was washed several times with Et_2O and dried: 57 mg (100%) of **2d** which was used without further purification for the coupling reaction with NH_2-modified DNA. IR (KBr): 1808, 1780 (5-ring imide); 1736 (CO, ester); 1188 (SO_3).

7. *General Coupling Procedure* (*Synthesis of* **6a**). In a 1.5-ml *Eppendorf* tube, 40 *OD* (260 nm) units of crude NH_2-modified DNA fragment **4a** were taken up in DMF/dioxane/H_2O 1:1:1 (600 μl). After the addition of 6 mg (4.35 μmol) of **2a** and 6.8 μl (40 μmol) of (i-Pr)$_2$EtN (clear soln.), the coupling was performed for 24 h in the dark at r.t. with slow shaking. An aliquot of the mixture was purified directly by reversed-phase HPLC with a gradient of 5–90% MeCN in 0.1M $Et_3NH^+AcO^-$ (pH 7.0; *Fig. 2*): **6a** at *ca.* 50% MeCN. The residual reaction mixture was evaporated in the speed vacuum concentrator. The pellet was suspended in H_2O/$CHCl_3$, and part of the aq. layer was again purified by reversed-phase HPLC under the same conditions as the crude reaction mixture (*Fig. 3*). Pure

6a was collected and the solvent evaporated at the speed vacuum concentrator. This was repeated several times after the addition of H_2O/EtOH giving **6a** as an orange pellet.

All the other combinations **6b–h** *(Table 2)* were prepared and purified in the same way as **6a**.

8. *Time-Resolved Fluorescence Measurements.* They were carried out in 100-µl cuvettes on our home-made instrument composed of a N_2 and a dye laser for the excitation and a photomultiplier for the detection [25]. The detection limit is defined as the concentration of Ru(bathophenanthroline) complex or Ru(bathophenanthroline) complex attached covalently to DNA giving a response which is twice as high as the one for the background. The compound to be investigated was dissolved and measured in PBS (150 mM NaCl, 10 mM p_i) alone and with different additives (see *Table 3* for **6a**). With **6a** the detection limit was $3 \cdot 10^{-14}$ mol/l in a mixture of phthalic acid/thioglycerol 3:1 (*v:v*) where the decay time τ for the fluorescence was 7.2 µsec. Since the measurements were carried out in a volume of 100 µl, this corresponds to $3 \cdot 10^{-18}$ mol.

When **6a** was hybridized in 10 mM p_i/0.5M NaCl to a synthetically prepared complementary sequence d(C-G-C-T-T-C-A-C-T-G-G-C-C-G-T-C-G-T-T-T-T-A-C-A-A-C-G-T-C), no changes neither in the fluorescence intensity nor in the decay time could be observed compared to **6a** alone without this complementary sequence.

9. Southern *Hybridization.* In a restriction digest, 5 µg of M13mp18 Rf-DNA were digested with 30 U of SspI in 33 mM *Tris*-acetate (pH 7.9), 66 mM KOAc, 10 mM $Mg(OAc)_2$, and 0.5 mM DTT for 2 h at 37°. The resulting restriction fragments were separated on a 0.7% agarose gel containing 300 ng/ml of ethidium bromide. When the 1554 bp fragment which contains the complementary region to **6d** had moved for 8.5 cm *(Fig. 7a)*, the gel was soaked twice for 15 min in 0.24M HCl, rinsed with H_2O, soaked twice for 15 min in 0.5N NaOH/1M NaCl, and finally twice in 0.5M *Tris*-HCl (pH 8.9)/3 M NaCl. The gel was then blotted overnight by diffusion in 20 × SSC (3M NaCl, 0.28M sodium citrate) as described in [29]. After transfer, the membrane was dried, and 1-cm-wide strips were cut from the membrane corresponding to the lanes of the SspI digest of M13mp18. Two such strips (corresponding to 1-µg digest) were used after prehybridization with sonicated calf thymus DNA for the hybridization with 20 pmol of **6d** which had been elongated and thereby labelled with (α-^{32}P)ddATP by terminal nucleotidyl transferase according to [29] and **6d** itself.

Both hybridization steps were performed in a roller tube turned in an incubator set at 37° for 3 h. The nonspecifically bound DNA probe was washed off with 2 × SSC. An autoradiograph of the hybridization strip with the radioactively labelled probe showed a specific hybridization with the 1554 bp fragment of the SspI digest *(Fig. 7b)*. The hybridization strip with **6d** (only labelled with the Ru complex) was divided into squares according to the numbers in *Fig. 7*, and each square was individually washed three times at 65° for 10–20 min in 300 µl of 0.1 × SSC. The washings were evaporated separately in the speed vacuum concentrator, taken up in 75 µl of H_2O and measured by time-resolved fluorescence technique, whereupon fluorescence could be detected in square *9*.

REFERENCES

[1] L.S. Lerman, Ed., 'DNA Probes', Cold Spring Harbor Laboratory, New York, 1986.
[2] J.J. Leary, D.J. Brigati, D.C. Ward, *Proc. Natl. Acad. Sci. USA* **1983,** *80,* 4045.
[3] W.D. Horrocks, Jr., M. Albin, *Prog. Inorg. Chem.* **1984,** *31,* 1.
[4] B. Alpha, J.M. Lehn, G. Mathis, *Angew. Chem.* **1987,** *99,* 259.
[5] B. Alpha, V. Balzani, J.M. Lehn, S. Perathoner, N. Sabbatini, *Angew. Chem* **1987,** *99,* 1310.
[6] P. Belser, A. v. Zelewski, *Helv. Chim. Acta* **1980,** *63,* 1675.
[7] R. Knorr, A. Trzeciak, W. Bannwarth, D. Gillessen, submitted to *Tetrahedron Lett.*
[8] Japanese Pat. Appl. 223457,1984; *Fuji Photo Film Co., Ltd.*
[9] B.J. Hunt, W. Rigby, *Chem. Ind. (London)* **1967,** 1868.
[10] A. Chollet, E.H. Kawashima, *Nucleic Acids Res.* **1985,** *13,* 1529.
[11] L. Wachter, J.-A. Jablonski, K.L. Ramachandran, *Nucleic Acids Res.* **1986,** *14,* 7985.
[12] L.M. Smith, S. Fung, M.W. Hunkapiller, T.J. Hunkapiller, L.E. Hood, *Nucleic Acids Res.* **1985,** *13,* 2399.
[13] S. Agrawal, C. Christodoulou, M.J. Gait, *Nucleic Acids Res.* **1986,** *14,* 6227.
[14] J.M. Coull, H.L. Weith, R. Bischoff, *Tetrahedron Lett.* **1986,** *27,* 3991.
[15] B.A. Connolly, *Nucleic Acids Res.* **1987,** *15,* 3131.
[16] B.S. Sproat, B. Beijer, P. Rider, *Nucleic Acids Res.* **1987,** *15,* 6181.
[17] T. Tanaka, T. Sakata, K. Fujimoto, M. Ikehara, *Nucleic Acids Res.* **1987,** *15,* 6209.
[18] S.P. Adams, K.S. Kavka, E.J. Wykes, S.B. Holder, G.R. Galluppi, *J. Am. Chem. Soc.* **1983,** *105,* 661.

[19] N. D. Sinha, J. Biernat, J. McManus, H. Köster, *Nucleic Acids Res.* **1984,** *12,* 4539.
[20] H. R. Kiefer, W. Bannwarth, 'Immunol. Methods 3', Eds. I. Lefkovits and B. Pernis, Academic Press, New York, 1985, p. 69.
[21] W. Bannwarth, *Chimia* **1987,** *41,* 302.
[22] J. Messing, *Meth. Enzymol.* **1983,** *101,* 20.
[23] E. M. Southern, *J. Mol. Biol.* **1975,** *98,* 503.
[24] F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.* **1977,** *74,* 5463.
[25] F. Müller, D. Schmidt, Eur. Patent Application 85 111 377.9.
[26] W. Bannwarth, *Helv. Chim. Acta* **1988,** *71,* 1517.
[27] W. Bannwarth, P. Iaiza, *DNA* **1986,** *5,* 413.
[28] W. Bannwarth, A. Trzeciak, *Helv. Chim. Acta* **1987,** *70,* 175.
[29] T. Maniatis, in 'Molecular Cloning', Cold Spring Harbor Laboratory, New York, 1982.