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A SIMPLE SPECIFIC LABELLING FOR OLIGONUCLEOTIDES BY BATHOPHENANTHROLINE-Ru^{II} COMPLEXES AS NONRADIOACTIVE LABEL MOLECULES

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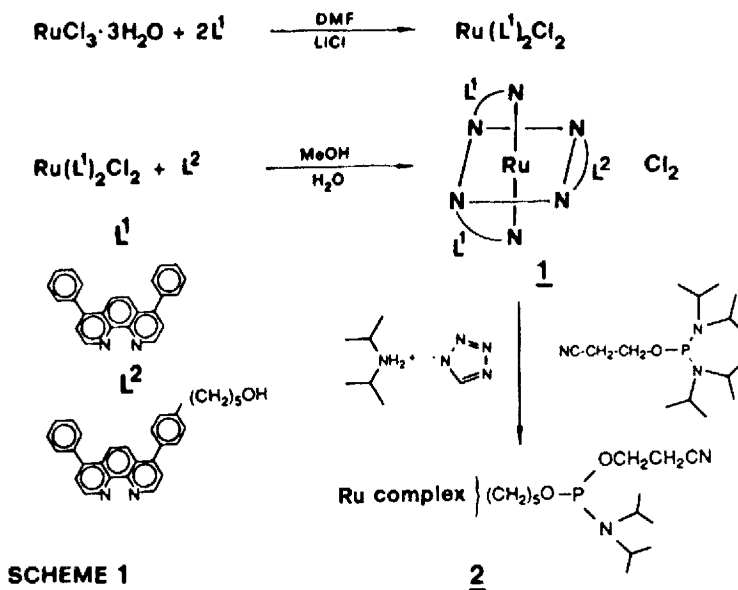
Abstract: A method is described for the specific coupling of bathophenanthroline-Ru^{II} complexes as nonradioactive label molecules to oligonucleotides directly in the course of their synthesis on a solid support by the phosphoramidite procedure.

Currently there is a great interest in the replacement of radioactive labels for biomolecules by nonradioactive systems with the most prominent being the biotin avidin system[1]. Recently we have published the use of Ru^{II} (bathophenanthroline) complexes as an alternative nonradioactive reporter system[2]. This label could be applied in DNA probe technology in order to detect viral or bacterial infections as well as for the diagnosis of genetic diseases[3]. The bathophenanthroline-Ru^{II} complexes are chemically and thermodynamically very stable and show a strong and long lasting fluorescence after excitation by light pulses of short duration which allows the detection of the fluorescence by a time resolved mode thereby eliminating disturbing short lived background fluorescence. This could lead to a high sensitivity comparable with ^{32}P as label. The Ru^{II}-complexes therefore represent an interesting alternative to the already well established Eu^{III}-complexes used for this purpose[4].

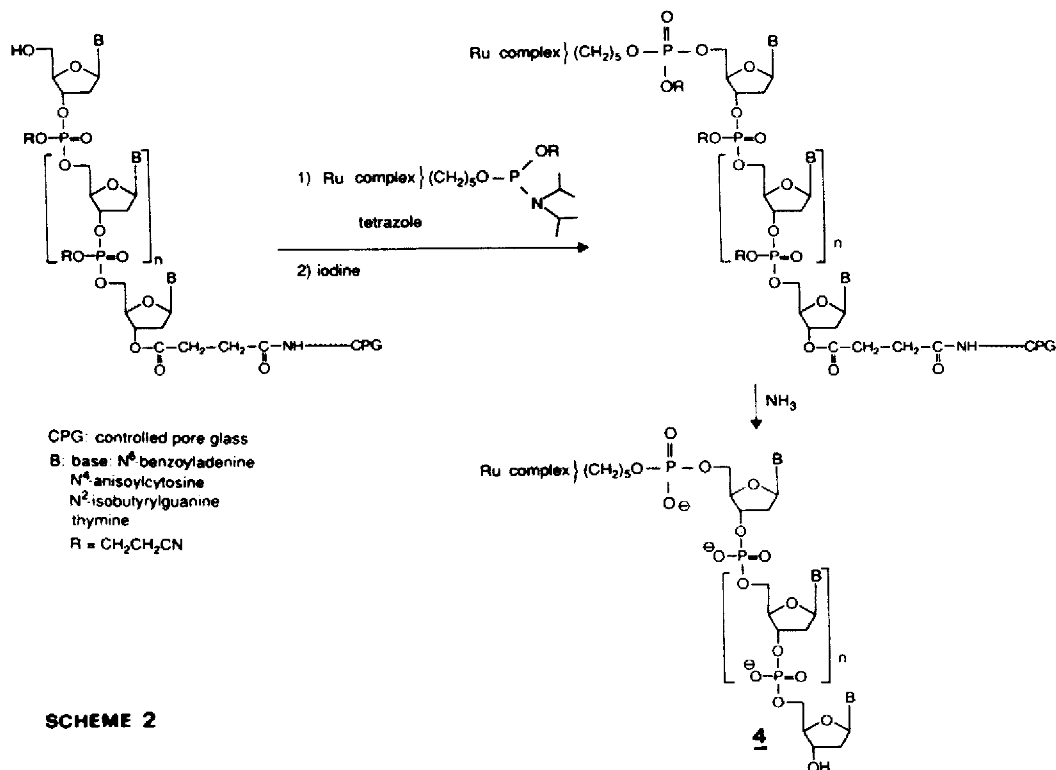


SCHEME 1

In the recently described procedure we have coupled the Ru^{II} complexes to the DNA via a carboxamide bond. For this attachment the DNA had to be modified specifically with a primary amino group at the 5'-end followed by a dialysis step.The coupling had to be performed in solution with a relatively large excess of Ru complex bearing a hydroxysuccinimide activated carboxy group.

Here we would like to report a specific attachment of such Ru^{II} complexes to synthetic DNA fragments directly in the course of their synthesis on a solid support avoiding this cumbersome attachment procedure. The new method is amenable to standard solid phase DNA synthesis by the phosphoramidite approach [5,6].

In order to reach this goal we have synthesized the bathophenanthroline-Ru^{II} complex 1 bearing a hydroxy function which is attached via a spacer arm to one of the bathophenanthroline ligands.This hydroxy group is transferred into the phosphoramidite **2** according to *Scheme 1.* Compound **2** can then be coupled in the course of a standard cycle of the phosphoramidite DNA synthesis approach to synthetic DNA fragments (*Scheme 2).*



SCHEME 2

After the coupling of the phosphoramidite **2** to the DNA which is still attached to the solid support a single deprotection step with ammonia leads directly to Ru^{II} complex-labelled DNA fragments in which the nonradioactive label is coupled via a phosphodiester linkage to the DNA.

Attempts to isolate and purifiy the phosphoramidite **2** resulted only in moderate yields.Therefore we have prepared **2** *in situ* [6] and have used it either directly or after hydrolysis of excess (β-cyanoethoxy) bis (diisopropylamino) phosphine applied for the *in situ* preparation. In this manner a high coupling yield of **2** could be obtained. The deprotection step with conc. ammonia does not influence the Ru^{II} (bathophenanthroline) complex due to its high chemical stability. The complex as such shows a high lipophilicity which leads to a relatively strong retardation in polyacrylamide gel electrophoresis as well as in reversed phase HPLC compared to the unlabelled DNA fragment **3** as shown in *Fig.1* and *2*. (*Fig.1*: PAGE; lane 1: crude 24 mer (**3**): lane 2: crude **4**, *Fig. 2*: HPLC of crude **4**).

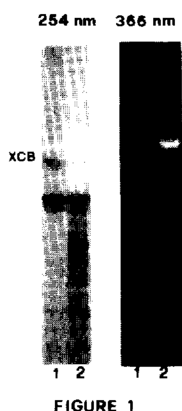


FIGURE 1

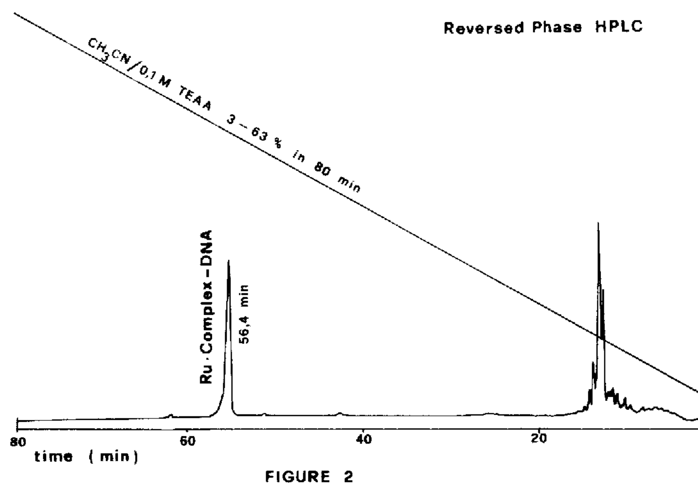


FIGURE 2

Compared to our previous coupling procedure for Ru^{II} (bathophenanthroline) complexes to synthetic DNA fragments this new approach offers several advantages. It is much easier to perform and the excess of **2** can be removed after the coupling reaction by simple washing steps. Furthermore there is no need for a functionalization of the DNA with a primary amino group and no dialysis step prior to the coupling has to be performed. With the new procedure the Ru^{II} complex is bound to the DNA via a very stable phosphodiester linkage.The Ru^{II} complex-labelled DNA **4** can be worked up and isolated by standard procedures in which the complex serves as a purification handle.

Since the Ru^{II} complex-labelled primers can also be used for DNA sequencing by the dideoxy method [8] the described procedure garanties also a rapid, simple and efficient synthesis of such sequencing primers [9].

General procedure.

In situ preparation of **2**.

After the addition of anh. acetonitrile to 20 μmol (25 mg) of **1** it was evaporated three times and taken up in 200μl of acetonitrile. After the addition of 10 μmol (1.7 mg) of diisopropyl ammonium tetrazolide and 100 μl of an acetonitrile solution containing 20 μmol (6mg) of (β-cyanoethoxy) bis

(diisopropylamino) phosphine it was reacted for 3 h leading to **2** which was used directly as such for the coupling reaction.

Alternatively the reaction mixture was poured after 3 h into 50 ml of sat. $NaHCO_3$ solution. It was extracted three times with CH_2Cl_2 and the combined organic layers were dried with Na_2SO_4 and evaporated which was repeated twice after addition of anh. acetonitrile. The residue containing 2 was taken up in 300 µl of anh. acetonitrile and this solution was applied for the coupling reaction.

Coupling of **2** to DNA sequences:

Compound **2** prepared in one of the ways mentioned above was added to 0.18 µmol (corresponding to 5 mg of support) of the sequence d(GTTGACAAGAATCCTCACAATACC) (3) which was still attached in the protected form (but lacking the 5'-DMT-group) to the solid support. This was followed by the addition of 0.7 ml of a 0.48 M solution of tetrazole in anh. acetonitrile. After 10 min. the excess of **2** was removed and after oxidation with 1 ml of a 0.2 M iodine solution in THF/lutidine/water (8/2/0,2) for 30 sec it was washed with acetonitrile and ether and dried.

For the deprotection the material was treated with 500 µl of conc. ammonia for 90 min at 66 °C. After removal from the support it was taken up in 100 µl of water, 100 µl of dioxane and precipitated with 600 µl of THF. The pellet was dissolved and purified by gel electrophoresis or by reversed phase HPLC.

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