

640982

COMMONWEALTH OF AUSTRALIA  
 PATENTS ACT 1952  
 APPLICATION FOR A STANDARD PATENT

F.Hoffmann-La Roche AG, of 124 Grenzacherstrasse, CH-4002, Basel, SWITZERLAND,  
 hereby apply for the grant of a standard patent for an invention entitled:

Energy Transfer Systems

which is described in the accompanying complete specification.

Details of basic application(s):-

<u>Basic Applic. No:</u>	<u>Country:</u>	<u>Application Date:</u>
235/90	CH	25 January 1990

The address for service is:-

**Spruson & Ferguson**  
 Patent Attorneys  
 Level 33 St Martins Tower  
 31 Market Street  
 Sydney New South Wales Australia

DATED this EIGHTEENTH day of JANUARY 1991

F.Hoffmann-La Roche AG

By:



Registered Patent Attorney

TO: THE COMMISSIONER OF PATENTS  
 OUR REF: 151333  
 S&F CODE: 55541

§ 019491 210191

COMMONWEALTH OF AUSTRALIA

THE PATENTS ACT 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION FOR A PATENT

In support of the Convention Application made for a patent for an invention entitled:

AUSTRALIA CONVENTION STANDARD & PETTY PATENT DECLARATION

RAN 4090/202

Title of Invention

Energy transfer systems

Full name(s) and address(es) of Declarant(s)

1 Roland Borer of 10 Stockackerstrasse, CH-4153 Reinach / Switzerland

do solemnly and sincerely declare as follows:-

Full name(s) of Applicant(s)

1. I am authorised by F.HOFFMANN-LA ROCHE AG of 124 Grenzacherstrasse, CH-4002 Basle, Switzerland

the applicant(s) for the patent to make this declaration on its/their behalf.

2. The basic application(s) as defined by Section 141 of the Act was/were made

Basic Country(ies)

in Switzerland

Priority Date(s)

on January 25, 1990

Basic Applicant(s)

by  F.HOFFMANN-LA ROCHE AG

the inventor(s) cited in paragraph 3.

Full name(s) and address(es) of inventor(s)

3. 1) Wilhelm Bannwarth 3 Liebenbergstrasse D-7888 Rheinfelden-Beuggen / Germany and

2) Francis Müller 18 Seltisbergerstrasse CH-4059 Basle / Switzerland

(respectively)

is/are the actual inventor(s) of the invention and the facts upon which the applicant(s) is/are entitled to make the application are as follows:

( ) the inventor(s) have assigned the invention to Hoffmann-La Roche Inc., Nutley, USA, who have re-assigned all their rights for Australia to the Applicant.

(X) the Applicant is the assignee of the invention from the inventor(s).

4. The basic application(s) referred to in paragraph 2 of this Declaration was/were the first application(s) made in a Convention country in respect of the invention(s) the subject of the application.

Set out how Applicant(s) derive title from actual inventor(s) e.g. The Applicant(s) is/are the assignee(s) of the invention from the inventor(s)

To:

Declared at Basle this 17th day of December 1990

The Commissioner of Patents, COMMONWEALTH OF AUSTRALIA

Signature of Declarant(s)

Roland Borer

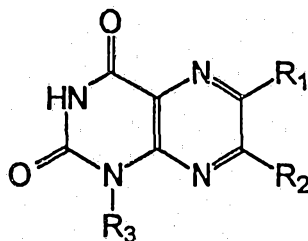


AU9169855

(12) PATENT ABRIDGMENT (11) Document No. AU-B-69855/91  
 (19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 640982

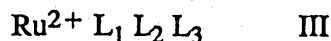
- (54) Title  
**ENERGY TRANSFER SYSTEMS**
- (51)<sup>5</sup> International Patent Classification(s)  
 C12Q 001/68 C07D 475/02 C12N 015/11 C07F 009/6561
- (21) Application No. : 69855/91 (22) Application Date : 21.01.91
- (30) Priority Data
- (31) Number (32) Date (33) Country  
 235/90 25.01.90 CH SWITZERLAND
- (43) Publication Date : 01.08.91
- (44) Publication Date of Accepted Application : 09.09.93
- (71) Applicant(s)  
 F.HOFFMANN-LA ROCHE AG
- (72) Inventor(s)  
 WILHELM BANNWARTH; FRANCIS MULLER
- (74) Attorney or Agent  
 SPRUSON & FERGUSON , GPO Box 3898, SYDNEY NSW 2001
- (57) Claim

1. Energy-transfer systems consisting of two organic compounds, one of which is a chromophore of the lumazine type and the other, with which it interacts, is an Ru complex, characterised in that the chromophore of the lumazine type is a lumazine derivative of the general formula:



II-1

in which  $R_1$  and  $R_2$  represent an optionally substituted  $C_{1-10}$ -alkyl group and  $R_3$  represents 1'-ribose or 1'-(2'-deoxyribose) and the ruthenium complex is a compound of the general formula



where the ligands  $L_1$ ,  $L_2$  and  $L_3$  are identical or different and represent charge-transfer units, and the ligand  $L_3$  is substituted by a group A-X where A represents an alkylene group which can also carry sulphonamide, thioether, ether, carboxyl or carboxamide functionalities, and X represents an aldehyde, carboxyl, hydroxyl, amino or thiocyanate group, halogen or a phosphite or phosphate group or a modified phosphate group, for example a phosphonate or thiophosphate group, or any other suitable functionality.

(11) AU-B-69855/91  
(10) 640982

-2-

2. Energy-transfer systems according to claim 1, characterised in that one or more chromophores of the lumazine type are incorporated at the end of a DNA or RNA sequence or within a DNA or RNA sequence in place of one or more nucleosides in the DNA or RNA sequence, and the DNA or RNA sequence is bound to a ruthenium complex of the general formula III in which the charge-transfer units  $L_1$ ,  $L_2$  and  $L_3$  are identical or different and represent bipyridyl, bathophenanthroline or benzobathophenanthroline groups, each of which can optionally be substituted.

3. Energy-transfer systems according to claim 2 with one of the following formulae



in which the ruthenium complex of the general formula m is linked via a very stable phosphodiester linkage to the DNA or RNA, and the chromophores of the lumazine type are 1-(2'-deoxy- $\alpha$ -D-ribofuranosyl)-6,7-dimethyl-lumazine [Lu].

640982

FORM 10

S & F Ref: 151333

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE:

Class      Int Class

Complete Specification Lodged:  
Accepted:  
Published:

Priority:

Related Art:

---

Name and Address  
of Applicant:

F.Hoffmann-La Roche AG  
124 Grenzacherstrasse  
CH-4002, Basel  
SWITZERLAND

Address for Service:

Spruson & Ferguson, Patent Attorneys  
Level 33 St Martins Tower, 31 Market Street  
Sydney, New South Wales, 2000, Australia

Complete Specification for the invention entitled:

Energy Transfer Systems

The following statement is a full description of this invention, including the best method of performing it known to me/us

Abstract

Energy-transfer systems which can be used, inter alia, for measuring distances within or between different molecules are described.

5500

101

22

5

The detection and the identification of DNA or RNA can be effected by hybridisations with corresponding complementary nucleotide strands. These hybridisations take place with very high specificity and therefore have a high potential for the diagnosis and detection of diseases (Methods Enzymology 68, 373 [1979]).

10

One technique for carrying out such hybridisation experiments is the so-called Southern Blot method (J. Mol. Biol. 98, 503 [1975]), which, however, is rather complicated and has the further disadvantage that, as a rule, radioactive isotopes, e.g.  $^{32}\text{P}$ , are used in this technique. This is why many efforts have been made on the one hand to simplify the technique of the hybridisation methods, and on the other hand to replace the radioactivity by suitable non-radioactive reporter molecules.

20

One possibility for both simplifying the hybridisation technique and, at the same time, replacing the radioactivity is provided by fluorescent systems where an energy transfer takes place from a donor to an acceptor. Such energy-transfer systems were predicted by Förster (Ann. Phys. 2, 55 [1948]). At the same time, Förster (supra) produced a relation between the efficiency of energy transfer and the distance between donor and acceptor (so-called Förster equation). If the emission band of the donor overlaps the absorption band of the acceptor, energy can be transferred from the donor to the acceptor, and the efficiency of this energy transfer decreases with the 6th power of the distance between donor and acceptor. In other words, the intensity of energy transfer increases as the distance between donor and acceptor decreases.

25

Since then, energy-transfer measurements have become a useful tool for measuring distances both within and between various molecules (Stryer, Ann. Rev. Biochem. 47, 819 [1978]). Such measurements of distance via the efficiency of energy transfer between donor and acceptor molecules are suitable for immunoassays and for DNA Hybridisation assays (J. Biol. Chem. 251, 4172 [1976]; Anal. Biochem. 108, 176 [1980]; Clin. Chem. 29, 1582 [1983];

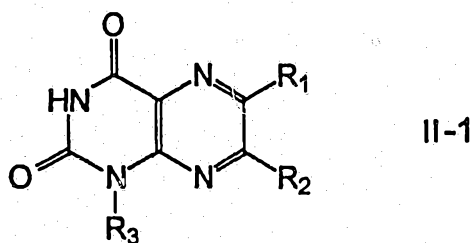
Chemiluminescent and Fluorescent Probes for DNA-Hybridisation Systems [1985]; Kingsburg, D.T. and Falkow S., Eds., 345-356, Academic Press, New York).

The present invention relates to novel energy-transfer systems which are suitable for measuring distances both within and between various molecules and which consist of two organic compounds, one of which is a chromophore of the lumazine type and the other, with which it interacts, is a ruthenium complex.

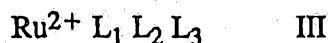
These energy-transfer systems can also, in the wider sense, be defined as donor-acceptor energy-transfer systems. The donor component is to be understood to mean, in general, those compounds which are able to absorb light from an energy source and then release it to an acceptor. By acceptor is generally meant those compounds which are able to absorb this energy released by the donor.

Such acceptors are, according to the present invention, Ru complexes. The energy absorption takes place via the long wavelength metal to ligand charge transfer (MLCT) band of these Ru complexes. The term "metal to ligand charge transfer" (MLCT) band designates the transition from a d electron of the Ru (II) ion to a  $7\pi^*$  electron of the ligand system of the ruthenium complex. In this connection, see also Crosby, J. Chem. Education 60, 791-796 [1983].

In accordance with a first embodiment of this invention, there is provided energy-transfer systems consisting of two organic compounds, one of which is a chromophore of the lumazine type and the other, with which it interacts, is an Ru complex, characterised in that the chromophore of the lumazine type is a lumazine derivative of the general formula



in which  $R_1$  and  $R_2$  represent an optionally substituted  $C_{1-10}$ -alkyl group and  $R_3$  represents 1'-ribose or 1'-(2'-deoxyribose) and the ruthenium complex is a compound of the general formula



where the ligands  $L_1$ ,  $L_2$  and  $L_3$  are identical or different and represent charge-transfer units, and the ligand  $L_3$  is substituted by a group A-X where A represents an alkylene





group which can also carry sulphonamide, thioether, ether, carboxyl or carboxamide functionalities, and X represents an aldehyde, carboxyl, hydroxyl, amino or thiocyanate group, halogen or a phosphite or phosphate group or a modified phosphate group, for example a phosphonate or thiophosphate group, or any other suitable functionality.

- 5 By analogous hydroxyl compounds are meant compounds with one or more hydroxyl groups via which covalent coupling to other molecules can be brought about. By lumazine derivatives are meant both the  $\alpha$  and the  $\beta$  anomers. The determination of configuration of the  $\alpha$ - and  $\beta$ -anomers is based upon  $^1\text{H-NMR}$  as published in the relevant scientific literature (Chem. Ber. 106,1401-1417 [1973], Liebigs Ann. Chem. 1217-1234 [1977]).
- 10 New investigations of ours using X-ray analysis however showed that the formerly designated  $\alpha$ -anomere is in reality the  $\beta$ -anomer and vice versa.

As used herein, the term " $\text{C}_{1-10}$ -alkyl" represents a straight-chain or branched, optionally substituted alkyl chain which contains 1-10 C atoms, such as, for example, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec.butyl, tert.butyl, n-pentyl,

- 15 n-hexyl, etc. Preferred alkyl groups are methyl groups.

By substituted alkyl groups are meant those which do not have an adverse effect on the energy-transfer ability.



Examples of charge-transfer units L<sub>1</sub>, L<sub>2</sub> and L<sub>3</sub> are bipyridyl, bathophenanthroline or benzobathophenanthroline, each of which can optionally be substituted.

The alkylene group A can be straight-chain or branched. A is very particularly preferably a -(CH<sub>2</sub>)<sub>4</sub>- or -(CH<sub>2</sub>)<sub>5</sub>-group and X is an -OH group.

Preferred energy-transfer systems according to the invention are DNA or RNA sequences which contain covalently bonded chromophores of the lumazine type and which are covalently linked either in modified form, preferably in amino-modified form, or in unmodified form directly or via a spacer group by reaction with a ruthenium complex of the formula III. Covalent bonding at the 5' end of the DNA or RNA sequences, at the 3' end or within the DNA or RNA sequences, which are appropriately modified for this purpose, is preferred.

According to the present invention, the chromophores of the lumazine type can be incorporated at the end of the DNA or RNA sequence or within the DNA or RNA sequence in place of a nucleoside, it being possible for the incorporation to take place in a desired manner. However, it is also possible for several, preferably 2-8, consecutive chromophores of the lumazine type to be incorporated at the end of the DNA or RNA sequence, or within the DNA or RNA sequence, in place of suitable nucleosides. It would also be conceivable to modify the chromophores of the lumazine type in such a way that they can also be bonded to appropriately modified bases or to the sugar-phosphate backbone.

Particularly preferred energy-transfer systems according to the invention are:



where the ruthenium complex (Ru complex) of the general formula III is linked via a very stable phosphodiester linkage to the DNA, and the chromophores of the lumazine type are 1-(2'-deoxy- $\alpha$ -D-ribofuranosyl)-6,7-dimethyl-lumazine (Lu) (see Figure 1).

The chromophores of the lumazine type and ruthenium complexes of the formula III can, however, also be used incorporated in different DNA or RNA sequences as energy-transfer systems. However, they can also be used incorporated in peptides or polypeptides as energy-transfer systems. Equally conceivable is use in other types of molecules.

The term "DNA or RNA sequences" represents natural or synthetically prepared, unmodified or modified DNA or RNA sequences.

The ruthenium complexes of the general formula III can be prepared as described in European Patent Application, Publication No. 178450.

The coupling of the ruthenium complexes of the general formula III to the DNA or RNA sequences which contain one or more chromophores of the lumazine type is carried out in a manner known per se. A possible way of coupling to the modified DNA or RNA comprises treating the ruthenium complex of the formula III and the modified DNA or RNA sequence with a water-soluble carbodiimide derivative, for example with N-cyclohexyl-N'-(2-morpholinoethyl)carbodiimide-methyl-p-toluenesulphonate. A particularly preferred coupling agent is 1,1,3,3-tetramethyl-2-succinyluronium tetrafluoroborate (1:1), called "TSU" hereinafter (the aforementioned "TSU" can be prepared as described in published Japanese Patent Application No. 166730/86). The coupling is preferably carried out in a solvent mixture, for example composed of DMF, dioxane or water. It has been found, surprisingly, that activation of carboxyl functionalities with TSU takes place even in the presence of water.

However, the coupling can also be carried out directly, for example via a phosphodiester linkage which is formed in a solvent, such as acetonitrile or absolute pyridine. For a direct coupling, the ruthenium complexes are converted into a form suitable for the coupling, preferably into phosphoramidite, H-phosphonate or activated phosphate functionalities.

The phosphoramidite derivatives of the ruthenium complexes can be prepared in situ for the coupling in the oligonucleotide synthesis (Bannwarth and Schmidt, Tetrahedron Letters 30, 1513 (1989)).

The ruthenium complexes in the form of the H-phosphonates can be obtained by reaction between the ruthenium complex which has been derivatised with a hydroxyalkyl group and trisimidazolylphosphine (Fröhler et

al., Nucleic Acid Research 14, 5399 (1986)) or salicylchlorophosphine as reagent (Marugg et al., Tetrahedron Letters, 2271 (1986)) and subsequent hydrolysis. The preparation can be carried out as shown in Figure 6. The H-phosphonates obtained in this way can be used in a known manner in the synthesis of oligonucleotides (Garreg et al., *Chemica Scripta* 25, 280 (1985)).

The preparation of the DNA or RNA sequences which have one or more chromophores of the lumazine type incorporated in place of one or more nucleosides or additionally contained at one end of the DNA or RNA sequence is carried out in a manner known per se. The chromophores of the lumazine type are converted into a form suitable for the coupling, preferably into phosphoramidite, H-phosphonate or activated phosphate functionalities. The coupling is preferably carried out on the growing DNA or RNA fragment during the synthesis. The synthesis can be carried out both in liquid phase and on a solid phase, as described, for example, in *Science* 230, 281 (1985), *Chimia* 41, 302 or in "Oligonucleotide Synthesis: A practical Approach", IRL Press, Oxford, UK, M.J. Gait, Ed. (1984).

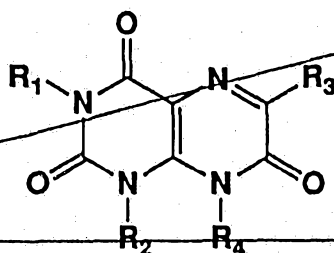
Particularly preferred DNA sequences in which one or more chromophores of the lumazine type are incorporated in place of one or more nucleosides or additionally at the 5' end are:

d(GTLuGACAAGAATCCTCACAATACC) 3',  
d(GTTGALuAAGAATCCTCACAATACC) 3',  
d(GTTGACAALuAATCCTCACAATACC) 3',  
d(LuGTTGACAAGAATCCTCACAATACC) 3' and  
d(LuLuLuLuLuGTTGACAAGAATCCTCACAATACC) 3'

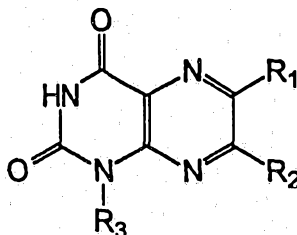
in which Lu represents 1-(2'-deoxy- $\alpha$ -D-ribofuranosyl)-6,7-dimethyl-lumazine.

The present invention also relates to these DNA or RNA sequences.

~~The present invention likewise relates to the lumazine derivatives and similar  $\pi$  systems of the general formula~~



In accordance with a second embodiment of this invention there is provided a lumazine derivative of the general formula



II-1

in which  $R_1$  and  $R_2$  represent an optionally substituted  $C_{1-10}$  alkyl group, and  $R_3$  represents 1'-ribose or 1'-(2'-deoxyribose).

$R_1$  and  $R_2$  is preferably a methyl group.

A particularly preferred lumazine derivative is 1-(2'-deoxy- $\alpha$ -D-ribofuranosyl)-6,7-dimethyl-lumazine (see compound 1 in Figure 2).

The abovementioned lumazine derivatives can be prepared as described by Ritzmann and Pfeleiderer in Chem. Ber. 106, 1401 (1973) or in a manner analogous thereto.

The present invention furthermore relates to phosphoramidites and H-phosphonates of the abovementioned lumazine derivatives, which are suitable for the solid-phase or solution syntheses of oligo- or polynucleotides.



A particularly preferred phosphoramidite of the present invention is 1-(5'-O-4,4'-dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl-3'-O-((2-cyanoethyl)-N,N-diisopropylphosphoramidite)-6,7-dimethyl-lumazine (see compound 3 in Figure 2).

5           The abovementioned phosphoramidites or H-phosphonates of the lumazine derivatives can be prepared by reacting, by known methods, above-mentioned lumazine derivatives which are protected at the 5' end. It is preferable for the 5' end of the lumazine derivative first to be protected by a 4,4'-dimethoxytrityl group, and for the resulting compound then to be reacted  
10           with 2-cyanoethoxy-bis-diisopropylaminophosphine in the presence of diisopropylammonium tetrazolide to give the corresponding phosphoramidite of the lumazine derivative. The particularly preferred phosphoramidite, which has already been mentioned herein, 1-(5'-O-4,4'-dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl-3'-O-((2-cyanoethyl)-N,N-diisopropylphosphoramidite)-6,7-dimethyl-lumazine 3 can be prepared as shown in  
15           Figure 2.

The present invention furthermore relates to lumazine derivatives which allow lumazines to be incorporated at the 3' end of oligonucleotides in solid-phase synthesis.

20           A particularly preferred lumazine derivative of the present invention is 1-(5'-O-4,4'-dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl-3'-O-succinyl)-6,7-dimethyl-lumazine (see compound 16 in Figure 5).

          The abovementioned compound can be prepared by reacting, by known methods, the lumazine derivative 2 protected at the 5' end with a 4,4'-  
25           dimethoxytrityl group. The protected lumazine derivative is preferably reacted with succinic anhydride with activation, and the product is isolated as salt of the acid. 1-(5'-O-4,4'-dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl-3'-O-succinyl)-6,7-dimethyl-lumazine can be prepared as shown in Figure 5.

30           The present invention furthermore relates to controlled pore glass (CPG), which is normally used as support material in solid-phase synthesis, which has been modified with lumazine derivatives (see compound 17 in Figure 5). Other support materials which can be used for the modification, such as, for example, silica gel, can likewise be utilised (F. Chow; T. Kempe; G. Palm; Nucleic Acids Res. 9, 2807 [1981]).

A wide variety of radicals can be incorporated at the 3' end of an oligonucleotide by the linkage of the CPG material to nucleosides and derivatives thereof. In the preferred embodiment of the present invention, the support material is linked to a lumazine derivative which is suitable for the further synthesis of an oligonucleotide.

The functionally modified CPG is prepared in a manner known per se by coupling the 1-(5'-O-4,4'-dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl-3'-O-succinyl)-6,7-dimethyl-lumazine 16 to CPG material suitable for the linkage. In the preferred embodiment, the functionalised support material is prepared as shown in Figure 5 by activation of 16 with mesitylene-2-sulphonyl chloride (MsCl) and 1-methyl-imidazole (MeIm).

The support obtained in this way can be used to introduce lumazine derivatives at the 3' end of oligo- or polynucleotides. This has the advantage that, for example, energy-transfer systems are possible between oligonucleotides which are equipped with donor or acceptor at different ends of the oligonucleotides.

Particularly preferred energy-transfer systems according to the invention having these properties are:



Surprisingly, it has emerged that the chromophores of the lumazine type according to the present invention are suitable for transferring light from a nitrogen laser to ruthenium complexes of the formula III.

The combinations of chromophore of the lumazine type/ruthenium complex of the formula III thus represent energy-transfer systems (with the chromophore of the lumazine type as donor and the ruthenium complex of the formula III as acceptor) which are extremely suitable for measurements of distances within one or between different molecules because, as already stated in the introduction, the Förster equation means that there is strict correlation between energy transfer and the distance between donor and acceptor.

Such measurements of distance can be used to determine molecular associations between various molecules, for example between DNA or RNA sequences and proteins/peptides when one type of molecule is equipped with

the donor and the other with the acceptor. This can be used to detect interactions of these molecules and for detecting the presence or absence of molecules. These detections are particularly suitable for diagnostic assays, such as, for example, immunoassays, receptor screening assays and DNA probe assays.

5

A coupling of the Ru complexes of the formula III to proteins/peptides has been described in European Patent Application, Publication No. 178 450. The modification of lumazine 2'-deoxyribosides at the 5' end with an amino functionality can be carried out in analogy to that of thymidine (Helv. Chim. Acta 71, 1517 [1988]). The coupling of chromophores of the lumazine type to proteins/peptides via carboxamide linkages is to be brought about in this way. This means that the energy-transfer system according to the invention between a chromophore of the lumazine type and an Ru complex of the formula III can be used in those assays in which distances between proteins/peptides play a part, such as, for example, in immunoassays.

10

15

Receptor screening assays can also be designed on this basis.

The incorporation of the energy-transfer system in the same molecule allows measurements of distance to be carried out within one molecule. The incorporation of the components of the energy-transfer system in different molecules also allows, however, measurements of distance between different molecules to be carried out. Systems of this type represent the preferred energy-transfer systems according to the invention.

20

25

In addition, the said donors, especially the lumazine derivatives according to the invention, are suitable for replacing the dye laser in the combination of nitrogen and dye laser used for excitation of the ruthenium complexes.

The methodology of the time-resolved fluorescence technique is described, for example, in German Offenlegungsschrift 2628158 or the above-mentioned European Patent Application No. 85.1113777.9 (Publication No. 178450).

30

The examples and figures which follow illustrate the present invention without restricting it.

Figure 1 shows the structure of the 5' end of compound 11 (Example 4).



Figure 2 shows diagrammatically the preparation of 1-(5'-O-4,4'-dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl-3'-O-((2-cyanoethyl)-N,N-diisopropyl-phosphoramidite)-6,7-dimethyl-lumazine 3.

5 Figure 3 shows diagrammatically the synthesis of various DNA sequences which have one or more chromophores of the lumazine type incorporated in place of one or more nucleosides or additionally contained at the 5' end of the DNA sequence.

Figure 4 shows the analysis by gel electrophoresis of compounds 4-9 (Example 3) and 10-15 (Example 4).

10 Figure 5 shows diagrammatically the synthesis of 1-(5'-O-4,4'-dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl-3'-O-succinyl)-6,7-dimethyl-lumazine and of the support modified with lumazine.

Figure 6 shows the synthesis of the Ru complex H-phosphonate.

15 Figure 7 shows the oligonucleotide sequences 20-25 and their use in energy transfer systems.

Figure 8 shows the oligonucleotide sequences 20, 21, 23, 24 and 25 and their use in energy transfer systems.

### Examples

20 All the solvents were of extra high purity. The phosphoramidite of the ruthenium (bathophenanthroline) complex was prepared in situ as described by W. Bannwarth and D. Schmidt (Tetrahedron Lett. 30, 1513, 1989). DNA syntheses were carried out on solid supports (Adams et al., J. Am. Chem. Soc. 105, 661 [1983]) by means of phosphoramidite chemistry using published methods, for example that of Sinha et al., Nucleic Acids Res. 12, 4539 (1984) or Bannwarth, Chimia 41, 302 (1987). Time-resolved fluorescence measurements were carried out in a volume of 100  $\mu$ l using a published apparatus (European Patent Application, Publication No. 178450). Short column chromatography (CC) was carried out as described by Hunt and Rigby (Chem. Ind., London, 1868 [1967]) with silica gel 60 (0.063-0.040 mm, Merck). 1-(2'-Deoxy- $\alpha$ -D-ribofuranosyl)-6,7-dimethyl-lumazine 1 was prepared as described by Ritzmann and Pfleiderer in Chem. Ber. 106, 1401 (1973).

25

30

Example 1

1-(5'-O-4,4'-Dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl)-6,7-dimethyl-lumazine 2

---

5           0.15 mmol (45 mg) of compound 1 was taken up in abs. pyridine and evaporated twice. It was then again dissolved in abs. pyridine (5 ml), 0.25 mmol (85 mg) of 4,4'-dimethoxytrityl chloride was added, and the mixture was stirred at room temperature (RT). After 1 h, 1 ml of methanol was added and, after a further 15 min, the mixture was poured into saturated NaHCO<sub>3</sub>  
10 solution and extracted three times with 30 ml of methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>) each time. The combined organic phases were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered to remove desiccant and concentrated. The residue was fractionated on 10 g of silica gel by short column chromatography using 100 ml of CH<sub>2</sub>Cl<sub>2</sub>/Et<sub>3</sub>N (99/1) and 100 ml of CH<sub>2</sub>Cl<sub>2</sub>/MeOH/Et<sub>3</sub>N (97/2/1). The pure product fractions were collected and concentrated. The residue was dissolved in 5 ml of chloroform and precipitated by dropwise addition to 150 ml of n-pentane. The precipitate was collected and dried and provided 65 mg (48.3 %) of pure product.

Example 2

1-(5'-O-4,4'-Dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl 3'-O-((2-cyanoethyl)-N,N-diisopropyl-phosphoramidite)-6,7-dimethyl-lumazine 3

---

20           0.3 mmol (183 mg) of compound 2 (Example 1) was taken up in 15 ml of MeCN (abs.) and evaporated. The residue was then taken up again in 15 ml of MeCN, 0.6 mmol (180 mg) of 2-cyanoethoxy-bis-diisopropylaminophosphine and 0.3 mmol (51 mg) of diisopropylammonium tetrazolide were added and the mixture was stirred for 2 h. The mixture was then poured into 100 ml of saturated NaHCO<sub>3</sub> solution and extracted three times with 30 ml of CH<sub>2</sub>Cl<sub>2</sub>. The combined organic phases were dried over Na<sub>2</sub>SO<sub>4</sub>, filtered to remove desiccant and concentrated. The residue was fractionated on 10 g of silica gel by short column chromatography using 100 ml of CH<sub>2</sub>Cl<sub>2</sub>/Et<sub>3</sub>N (99/1) and 100 ml of CH<sub>2</sub>Cl<sub>2</sub>/Et<sub>3</sub>N (98/2). The pure product fractions provided 170 mg (70%) of pure product.

Example 3

Synthesis of d(GTTGACAAGAATCCTCACAATACC) 3' 4,  
d(GTLuGACAAGAATCCTCACAATACC) 3' 7,  
d(GTTGALuAAGAATCCTCACAATACC) 3' 6,  
5 d(GTTGACAALuAATCCTCACAATACC) 3' 5,  
d(LuGTTGACAAGAATCCTCACAATACC) 3' 8 and  
d(LuLuLuLuLuGTTGACAAGAATCCTCACAATACC) 3' 9.

---

10 The synthesis was started with controlled pore glass as solid support  
which was functionalised with 180 mg of C (4.87  $\mu$ mol) (Fig. 3). The chain  
extensions were carried out using 40 mg in each case of the appropriate  
 $\beta$ -cyanoethylphosphoramidite until the lumazine deoxyriboside 3 (Example 2)  
was incorporated. Each time a lumazine deoxyriboside phosphoramidite 3 was  
15 incorporated, 30 mg of solid support material with the corresponding sequence  
were separated off and the synthesis was continued as shown in Figure 3. 20  
mg of 3 were used for each chain extension with this amidite. 8 mg of each  
protected sequence still coupled to the solid support material were separated  
off and, after elimination of protective groups with ammonia, fractionated on  
a 20% polyacrylamide gel under denaturing conditions (Figure 4).

Example 4

20 Synthesis of  
Ru complex-d(GTTGACAAGAATCCTCACAATACC) 3' 10,  
Ru complex-d(GTLuGACAAGAATCCTCACAATACC) 3' 12,  
Ru complex-d(GTTGALuAAGAATCCTCACAATACC) 3' 13,  
25 Ru complex-d(GTTGACAALuAATCCTCACAATACC) 3' 14,  
Ru complex-d(LuGTTGACAAGAATCCTCACAATACC) 3' 11 and  
Ru complex-d(LuLuLuLuLuGTTGACAAGAATCCTCACAATACC) 3' 15

---

30 After elimination of the dimethoxytrityl protective group, the batho-  
phenanthroline-ruthenium(II) complex was coupled in the form of its in situ  
prepared phosphoramidite to the compounds 4-9 (Example 3) (Bannwarth and  
Schmidt, Tetrahedron Letters 30, 1513 (1989)). The compounds 10-15 were then  
obtained in impure form by treatment with conc. ammonia. Figure 4 shows  
the analysis of these compounds by 20% polyacrylamide gel electrophoresis.

Compounds 10-15 were obtained in pure form either by reversed-phase HPLC or by preparative gel electrophoresis and subsequent electroelution.

Example 5

5 Fluorescence measurements with compounds 11-15 to determine the efficiency of energy transfer

---

The fluorescence intensities of compounds 11-15 were measured using the time-resolved fluorescence technique. Compounds 11-15 were excited by pulses of light from a nitrogen laser (0.7 ns at 337 nm), and the fluorescent light was measured using a photomultiplier.

10 The measured fluorescence intensity  $I_F$  at 618 nm (emission wavelength of the ruthenium complex) can be described by the sum of 3 components:

$$I_F = I_{F1} + I_{F2} + I_{F3}.$$

15  $I_{F1}$  is the lumazine fluorescence intensity at 618 nm.  $I_{F2}$  represents the emission of the Ru complex caused by direct excitation, and  $I_{F3}$  designates the contribution to the fluorescence intensity from the energy transfer. Since  $I_{F1}$  is virtually zero, the measured fluorescence intensity can be written as follows:

$$I_F \sim I_{F2} + I_{F3}.$$

The table which follows lists the efficiencies (E) of energy transfer for compounds 11-15. These were calculated using the formula

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

20

25

30

Compound	E (%)
11	15.0
12	12.6
13	5.4
14	4.1
15	77.0

The table reveals a correlation between the efficiency of energy transfer and the distance between donor (chromophore of the lumazine type) and acceptor (ruthenium complex). In addition, the transfer efficiency can be increased by incorporating several donors, as shown by compound 15.

5

Example 6

Synthesis of 5' d(TGGGATAGGTGGATTAT-LuLuLuLu) 3' 20

---

A. Synthesis of 1-(5'-O-4,4'-dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl-3'-O-succinyl)-6,7-dimethyl-lumazine 16.

10

0.29 mmol (175 mg) of compound 2 (Example 1) was taken up in dry pyridine and evaporated several times. The residue was taken up in 5 ml of dry CH<sub>2</sub>Cl<sub>2</sub>, and 0.91 mmol (91 mg) of succinic anhydride, 0.33 mmol (41 mg) of DMAP and 0.91 mmol (126  $\mu$ l) of Et<sub>3</sub>N were added, and the mixture was stirred at room temperature for five hours. The mixture was poured into 10 ml of 1% strength acetic acid and extracted three times with 50 ml of CH<sub>2</sub>Cl<sub>2</sub> each time. The combined organic phases were dried and concentrated. The residue was purified by column chromatography on 20 g of silica gel using CH<sub>2</sub>Cl<sub>2</sub>/Et<sub>3</sub>N (99/1) and an increasing ethanol gradient (1, 3 and 5%). Pure fractions were collected and concentrated. The product (TLC; R<sub>f</sub>: 0.32; CH<sub>2</sub>Cl<sub>2</sub>/MeOH 9/1) was dissolved in 5 ml of CH<sub>2</sub>Cl<sub>2</sub>/1% NEt<sub>3</sub> and precipitated in 350 ml of n-pentane. The precipitate was collected and dried. Yield: 165 mg (79%) of 16 as triethylammonium salt.

15

20

B. Functionalisation of the CPG support with 1-(5'-O-4,4'-dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl-3'-O-succinyl)-6,7-dimethyl-lumazine 16 to give compound 17.

25

2.5 g of the CPG support and 0.18 mmol (130 mg) of compound 16 were evaporated in 10 ml of dry pyridine several times. The residue was taken up in 10 ml of dry pyridine, and 4.6 mmol (1.0 g) of mesitylene-2-sulphonyl chloride and 4 mmol (0.4 ml) of 1-methylimidazole were added. The mixture was shaken occasionally during the reaction time (18 hours). The mixture was filtered and washed with pyridine and ether. Unreacted amino groups were blocked by addition of 15 ml of a solution of 1 g of DMAP, 2 ml of Ac<sub>2</sub>O and 2 ml of lutidine. After 30 minutes the solution was filtered and the functionalised support was washed with pyridine and ether and dried. UV

30

measurement of the eliminated DMTr group (499 nm) revealed that the loading on the support 17 was 27  $\mu\text{mol/g}$ .

C. Synthesis of the oligonucleotide 3'-derivatised with lumazine 5' d(TGGGATAGGTGGATTATLuLuLuLu) 3' 20.

5 The oligonucleotide was synthesized using the support 17 prepared in part B. and using the lumazine phosphoramidite 3 from Example 2. For this, 1.08  $\mu\text{mol}$  (40 mg) of the support were coupled with 24  $\mu\text{mol}$  (20 mg) of the compound 3 and 240  $\mu\text{mol}$  (17 mg) of tetrazole in 0.5 ml of dry MeCN in a standard reaction cycle. After the incorporation of the lumazine chromophores  
10 on the 3' end, the synthesis was continued with normal phosphoramidites. After deprotection with  $\text{NH}_3$ , the oligonucleotide 20 was isolated by electroelution after preparative gel electrophoresis.

Example 7

Synthesis of Ru complex-d(ATAATCCACCTATCCCAGTAGGAGAAAT) 3' 21

---

A. Synthesis of the H-phosphonate of the bathophenanthroline-Ru(III) complex 18.

Process A:

0.8 mmol (17  $\mu\text{l}$ ) of  $\text{PCl}_3$  was added by syringe to a solution of 2.7 mmol (180 mg) of imidazole and 2.8 mmol (380  $\mu\text{l}$ ) of  $\text{Et}_3\text{N}$  in 10 ml of dry MeCN within 5 minutes under argon, and the mixture was stirred at room temperature (RT) for 30 minutes. Separately, 0.1 mmol (126 mg) of the Ru complex was evaporated with dry MeCN and taken up in 10 ml of MeCN and then added to the mixture containing the trisimidazolylphosphine. The mixture was stirred at RT for 2 h and then poured into 100 ml of 0.1 M TEAA pH 7.0 and extracted with  $\text{CH}_2\text{Cl}_2$  (3x50 ml). The combined organic phases were dried over  $\text{Na}_2\text{SO}_4$ . The residue from evaporation was digested with ether and then washed. 120 mg (86%) of the H-phosphonate 18 were obtained after drying.

Process B:

0.1 mmol (126 mg) of the Ru complex was evaporated with MeCN and dissolved in 5 ml of dry MeCN and 1 ml of dry pyridine, and a solution of 0.5 mmol (101 mg) of salicylchlorophosphine in 2 ml of dry MeCN was added. This reaction mixture was stirred at RT for 1.5 h, poured into 50 ml of 0.1 M TEAA and worked up as described in process A. Yield: 120 mg (86%) of the H-phosphonate 18.

B. Coupling of Ru complex H-phosphonates to oligonucleotides.

The oligonucleotide with the sequence d(ATAATCCACCTATCCCAGT-AGGAGAAAT) 3' was prepared in a known manner and left on the support with the 5' end deprotected. 26  $\mu$ mol (36 mg) of compound 18 were evaporated with dry MeCN and dissolved in 1 ml of dry pyridine. 0.5 ml of this solution was added at the same time as 7.3  $\mu$ l of pivaloyl chloride dissolved in 0.5 ml of dry MeCN to 0.4  $\mu$ mol of the oligonucleotide d(ATAATCCACCTAT-CCCAGTAGGAGAAAT) 3' bound to the support. After a coupling time of 4 minutes, the support was washed and again reacted with the same amount of reagents. This was followed by oxidation with 1 ml of 0.2 M I<sub>2</sub>/THF and 1 ml of Et<sub>3</sub>N/H<sub>2</sub>O/THF (1:8:1, v/v) and washing steps with MeCN and ether. 10 mg of the support were treated with 700  $\mu$ l of conc. ammonia at 67°C for two hours for the deprotection. Polyacrylamide gel electrophoresis showed complete conversion of the initial oligonucleotide into the oligonucleotide 21 labelled with the Ru complex.

Example 8

Fluorescence measurements with compounds 20-25 to determine the efficiency of energy transfer

In order to determine the energy transfer between lumazine and Ru complex which are each linked to different molecules, the oligonucleotides 20 and 23 and the oligonucleotide 21 labelled with Ru complex were hybridised on a synthetic template 22 and the energy transfer was determined.

The oligonucleotides 24 and 25 were employed as negative controls (no energy transfer) (see Fig. 7). To characterise the energy transfer, the following table lists the ratios of the measured fluorescence intensities I<sub>F</sub> and I<sub>F2</sub> of the

tested oligonucleotides. The definitions used for  $I_F$  and  $I_{F2}$  in this connection are those of Example 5.

	Hybrid	$I_F/I_{F2}$
5	<u>22/21/25</u>	1.0
	<u>22/21/24</u>	1.1
	<u>22/21/23</u>	1.9
	<u>22/21/20</u>	2.2

In the cases where oligonucleotides 24 and 25 were used (as negative controls), no energy transfer was observed ( $I_F/I_{F2} \sim 1$ ). Where there was appropriate hybridisation (hybrid 22/21/20 and 22/21/23), energy transfer was possible and was indicated by doubling of the signalling intensity for the fluorescent light from the energy transfer ( $I_F/I_{F2} \sim 2$ ).

Thus measurement of the energy transfer made it possible to state clearly whether hybridisation of the two donor/acceptor oligonucleotides took place on a target which was sought, in this case a DNA sequence.

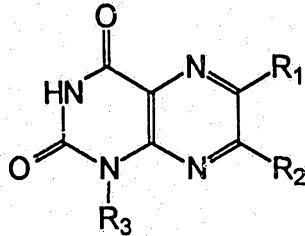
The same sequences 21-25 were used to determine the energy transfer between two separate molecules without the necessity for the latter, as described above, to be attached by a third molecule (Fig. 8). This made use only of the specificity of recognition between the two compounds of the energy-transfer system. In the present case, the oligonucleotides 21/20 and 21/23 hybridised with one another because of their base composition, and formed with their chromophores an energy-transfer system. By contrast, the oligonucleotide 24 labelled with a chromophore was unable to hybridise with 21. Although the oligonucleotide 25 hybridised with 21, it did not carry a chromophore which was required for an energy-transfer system. As the table below shows, the fluorescence measurements revealed the expected energy transfers only with the pairs 21/20 and 21/23, while the other two pairs 21/24 and 21/25 showed no transfers (controls).

	Hybrid	$I_F/I_{F2}$
30	<u>21/20</u>	1.9
	<u>21/23</u>	1.8
	<u>21/24</u>	1.0
35	<u>21/25</u>	1.0



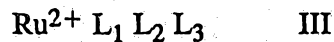
The claims defining the invention are as follows:

1. Energy-transfer systems consisting of two organic compounds, one of which is a chromophore of the lumazine type and the other, with which it interacts, is an Ru complex, characterised in that the chromophore of the lumazine type is a lumazine  
5 derivative of the general formula



II-1

in which R<sub>1</sub> and R<sub>2</sub> represent an optionally substituted C<sub>1-10</sub>-alkyl group and R<sub>3</sub> represents 1'-ribose or 1'-(2'-deoxyribose) and the ruthenium complex is a compound of the general  
10 formula



10

where the ligands L<sub>1</sub>, L<sub>2</sub> and L<sub>3</sub> are identical or different and represent charge-transfer units, and the ligand L<sub>3</sub> is substituted by a group A-X where A represents an alkylene group which can also carry sulphonamide, thioether, ether, carboxyl or carboxamide functionalities, and X represents an aldehyde, carboxyl, hydroxyl, amino or thiocyanate  
15 group, halogen or a phosphite or phosphate group or a modified phosphate group, for example a phosphonate or thiophosphate group, or any other suitable functionality.

2. Energy-transfer systems according to claim 1, characterised in that one or more chromophores of the lumazine type are incorporated at the end of a DNA or RNA sequence or within a DNA or RNA sequence in place of one or more nucleosides in the  
20 DNA or RNA sequence, and the DNA or RNA sequence is bound to a ruthenium complex of the general formula III in which the charge-transfer units L<sub>1</sub>, L<sub>2</sub> and L<sub>3</sub> are identical or different and represent bipyridyl, bathophenanthroline or benzobathophenanthroline groups, each of which can optionally be substituted.

3. Energy-transfer systems according to claim 2 with one of the following  
25 formulae



30



in which the ruthenium complex of the general formula m is linked via a very stable phosphodiester linkage to the DNA or RNA, and the chromophores of the lumazine type are 1-(2'-deoxy- $\alpha$ -D-ribofuranosyl)-6,7-dimethyl-lumazine [Lu].

4. Energy-transfer systems according to claim 1, characterised in that the chromophores of the lumazine type and the ruthenium complexes are incorporated in different modified or unmodified DNA or RNA sequences.

5. Use of the energy-transfer systems according to any one of claims 1-4 for measuring distances within a molecule or between different molecules, in particular for measuring distances within a DNA or RNA sequence or between different DNA or RNA sequences.

6. Use according to claim 5 in diagnostic assays.

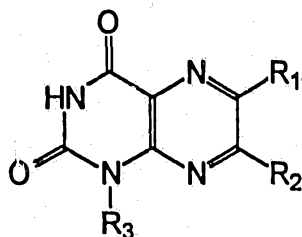
7. DNA or RNA sequences which contain one or more chromophores of the lumazine type as defined in claim 1.

8. DNA sequences according to claim 7 with the following formulae

15 d(LuGTTGACAAGAATCCTCACAATACC) 3',  
 d(GTLuGACAAGAATCCTCACAATACC) 3',  
 d(GTTGALuAAGAATCCTCACAATACC) 3',  
 d(GTTGACAALuAATCCTCACAATACC) 3',  
 d(LuLuLuLuLuGTTGACAAGAATCCTCACAATACC) 3',  
 20 d[TGGGATAGGTGGATTAT-LuLuLuLu] 3'  
 d[CTACTGGGATAGGTGGA-LuLuLuLu] 3' and  
 d[TCAACGTATGTTACCGC-LuLuLuLu] 3'.

9. DNA sequences according to claim 8, characterised in that the chromophores of the lumazine type (Lu) are 1-(2'-deoxy- $\alpha$ -D-ribofuranosyl)-6,7-dimethyl-lumazine.

25 10. Lumazine derivatives of the general formula



II-1

in which  $R_1$  and  $R_2$  represent an optionally substituted  $C_{1-10}$ -alkyl group, and  $R_3$  represents 1'-ribosyl or 1'-(2'-deoxyribosyl).



11. Phosphoramidites or H-phosphonates of the lumazine derivatives according to claim 10, in particular 1-(5'-O-4,4'-dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl 3'-O-((2-cyanoethyl)-N,N-diisopropyl-phosphoramidite)-6,7-dimethyl-lumazine.

12. Support material modified with lumazine derivatives according to claim 11, in particular 1-(5'-O-4,4'-dimethoxytrityl-2'-deoxy- $\alpha$ -D-ribofuranosyl-3'-O-succinyl)-6,7-dimethyl-lumazine.

13. Use of the support material according to claim 12 in solid-phase synthesis.

14. Energy-transfer systems consisting of two organic compounds substantially as hereinbefore described with reference to any one of the Examples.

15. DNA or RNA sequences which contain one or more chromophores of the lumazine type substantially as hereinbefore described with reference to any one of the Examples.

16. A lumazine derivative substantially as hereinbefore described with reference to any one of the Examples.

17. Process for preparing energy-transfer systems consisting of two organic compounds substantially as hereinbefore described with reference to any one of the Examples.

18. Process for preparing DNA or RNA sequences which contain one or more chromophores of the lumazine type substantially as hereinbefore described with reference to any one of the Examples.

19. Process for preparing support material linked to lumazine derivatives substantially as hereinbefore described with reference to any one of the Examples.

**Dated 28 June, 1993**  
**F. Hoffmann-La Roche AG**

**Patent Attorneys for the Applicant/Nominated Person**  
**SPRUSON & FERGUSON**



FIG 1

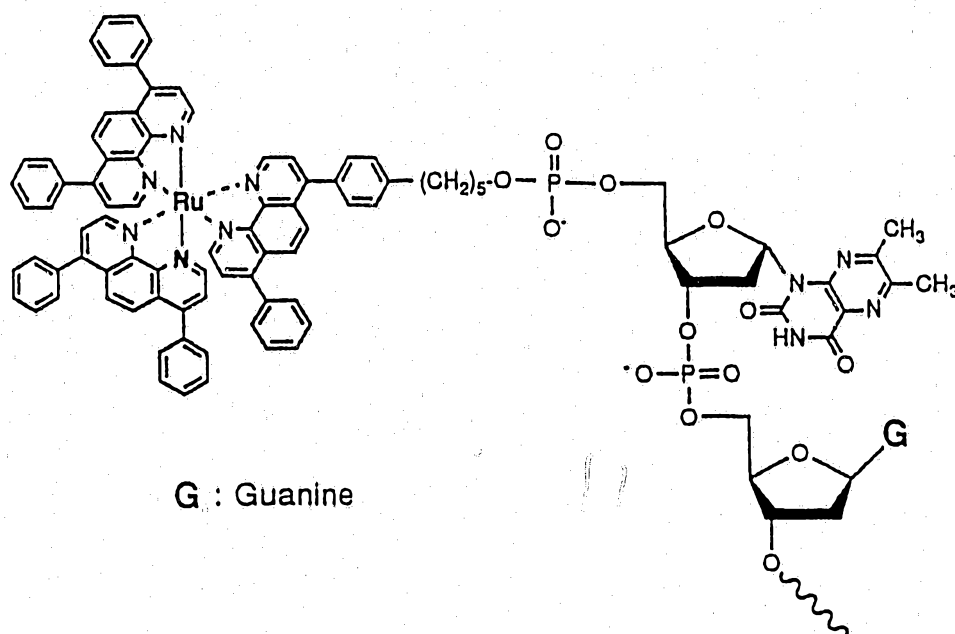


FIG 2

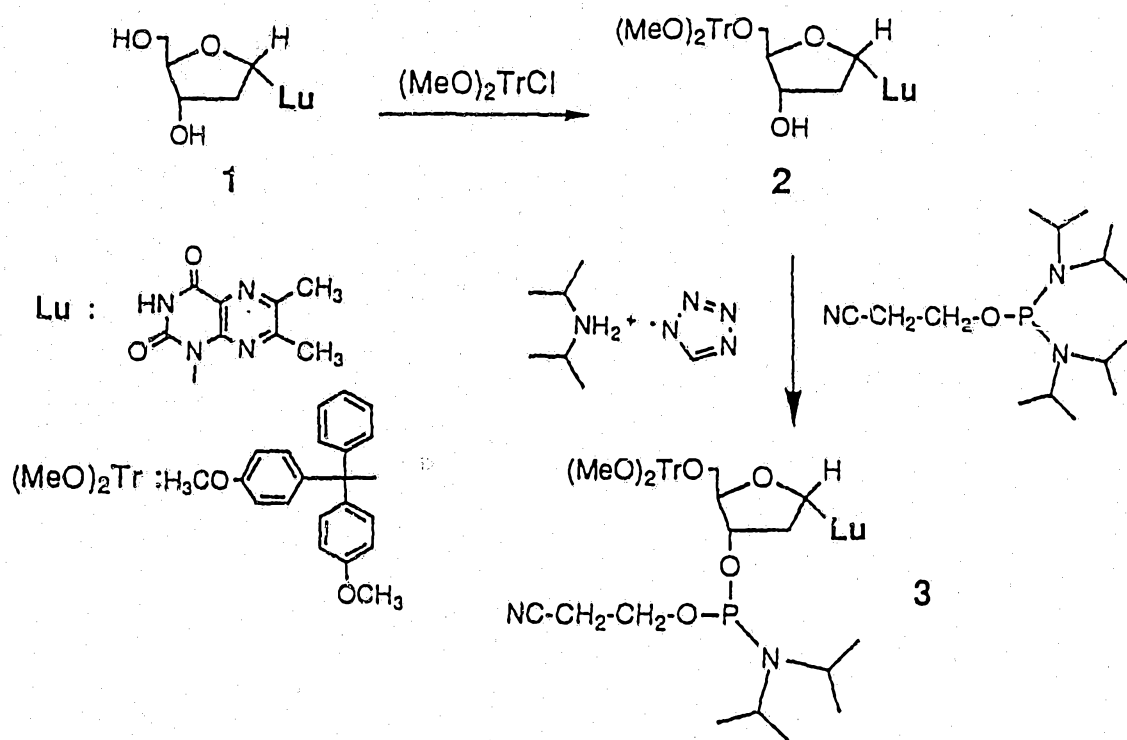
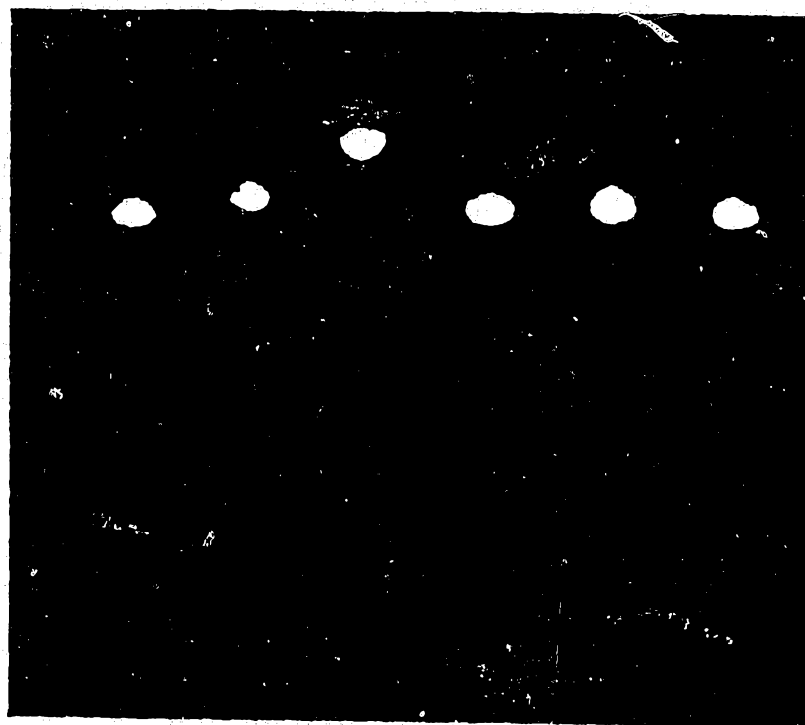


FIG 3

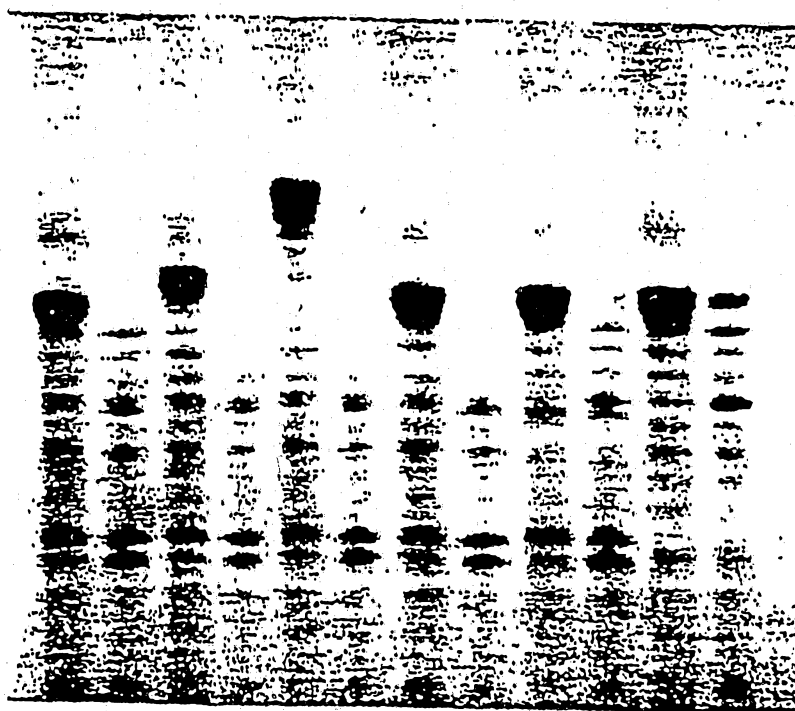


FIG 4



366 nm

10 11 15 12 13 14



254 nm

4 8 9 7 6 5

FIG 5

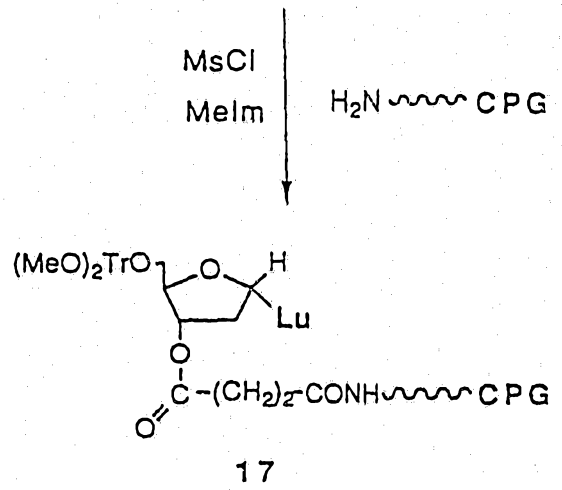
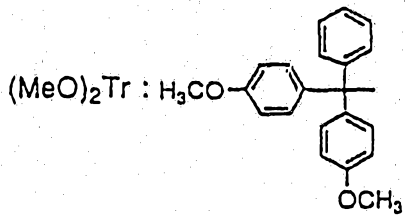
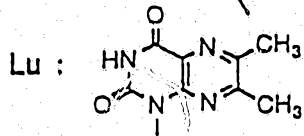
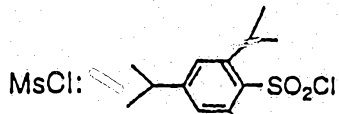
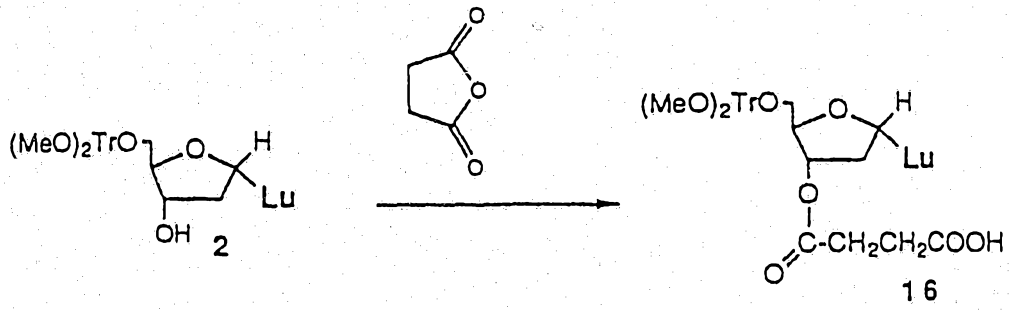




FIG 6

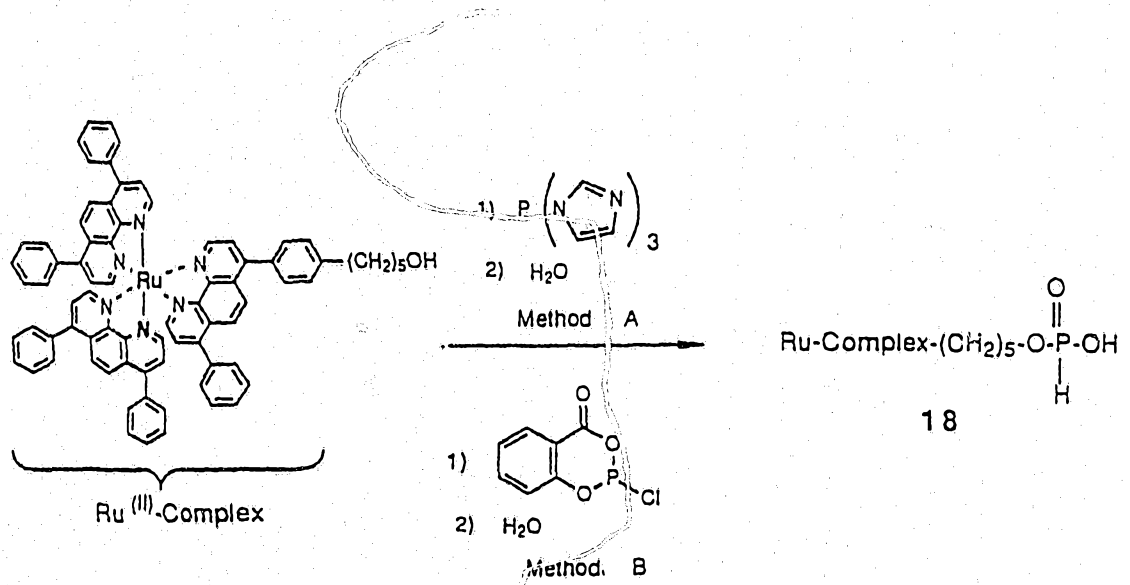
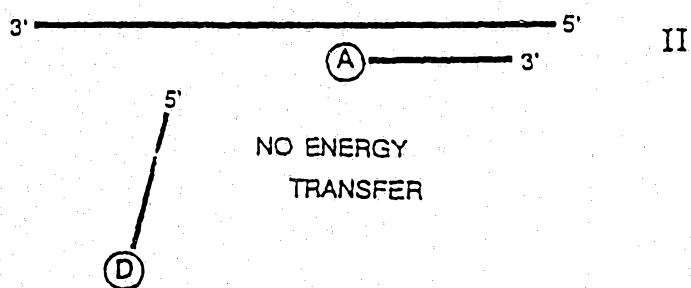
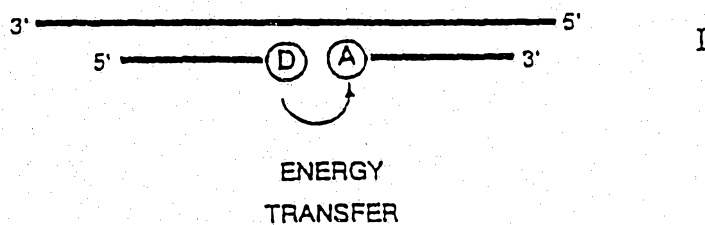


FIG 7



d(ACCCTATCCACCTAATAAAAAATATTAGGTGGATAGGGTCATCCTCTTTA)5'

22

d(TGGGATAGGTGGATTAT-Lu<sub>4</sub>) Ru-d(ATAATCCACCTATCCCAGTAGGAGAAAT)

20

21

ENERGY  
TRANSFER

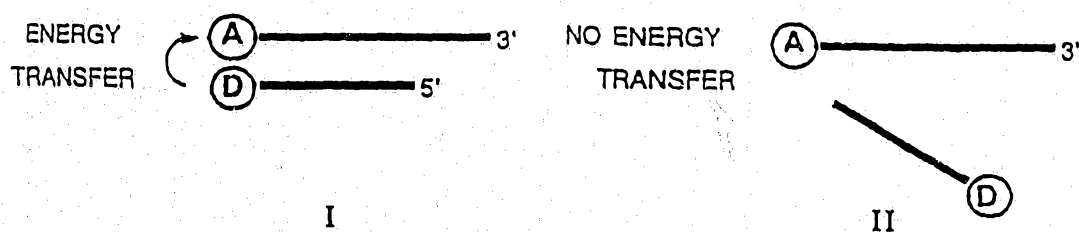
d(CTACTGGGATAGGTGGA-Lu<sub>4</sub>) 23

d(TCAACGTATGTTCCACC-Lu<sub>4</sub>) 24

d(TGGGATAGGTGGATTAT) 25

} Controls

FIG 8



Ru-d(ATAATCCACCTATCCCAGTAGGAGAAAT)<sub>3</sub> 21

d(Lu<sub>4</sub>-TATTAGGTGGATAGGGT)<sup>5'</sup> 20

d(Lu<sub>4</sub>-AGGTGGATAGGGTCATC)<sup>5'</sup> 23

d(Lu<sub>4</sub>-GCCACTTGTATGCAACT)<sup>5'</sup> 24

d(TATTAGGTGGATAGGGT)<sup>5'</sup> 25

} Controls