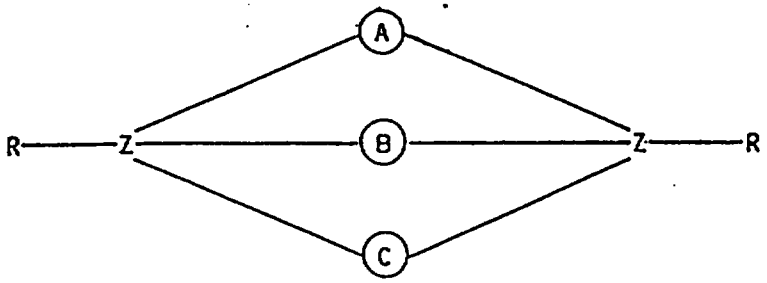
The present invention, which is the result of work carried out in collaboration with Professor J.M. LEHN and his research team at the Université Louis Pasteur in Strasbourg, relates to the field of fluorescence and more particularly to a novel family of macropolycyclic rare earth complexes, or rare earth cryptates, which are suitable as fluorescent tracers, especially in immunoassays.

European patent application EP-A-0 180 492 discloses macropolycyclic rare earth complexes consisting of at least one rare earth salt complexed by a macropolycyclic compound of the general formula:



I

in which Z is a trivalent or tetravalent atom, R is nothing or represents hydrogen, the hydroxyl group, an amino group or a hydrocarbon radical, and the divalent radicals Ⓐ, Ⓑ and Ⓒ independently of one another are hydrocarbon chains which may or may not contain one or more heteroatoms and may or may not be interrupted by a heteromacrocycle, at least one of the radicals Ⓐ, Ⓑ and Ⓒ also containing at least one molecular unit or essentially consisting of a molecular unit, said molecular unit possessing a greater triplet energy than the emission level of the complexed rare earth ion.

These cryptates are particularly suitable as

tracers for biological products in methods of immunological detection and determination by fluorescence.

When these rare earth cryptates are used specifically to label biological molecules by means of a covalent bond, they can be substituted on one or more of their constituent molecular units by one or more sufficiently accessible substituents possessing one or more molecular units which permit covalent coupling with the biological molecule under operating conditions compatible with its biological reactivity.

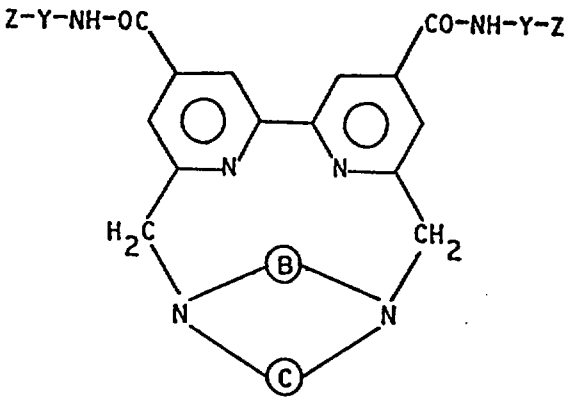
Non-limiting examples which may be mentioned among these molecular units are alkylamino, arylamino, isothiocyano, cyano, isocyano, thiocyano, carboxyl, hydroxyl, mercapto, phenol, imidazole, aldehyde, epoxide, halide, thionyl, sulfonyl, nitrobenzoyl, carbonyl, triazo, succinimido, anhydride, halogenoacetate, hydrazino, dihalogenotriazinyl and other radicals (Biol. Chem. 245, 3059 (1970)). The length of the arm bonding the cryptate to the biological molecule of interest can vary from 1 to 20 atoms, for example, and can contain not only carbon atoms but also heteroatoms such as N, O, S and P.

Two families of functionalized rare earth cryptates have now been found which can be used as fluorescent tracers for biological molecules.

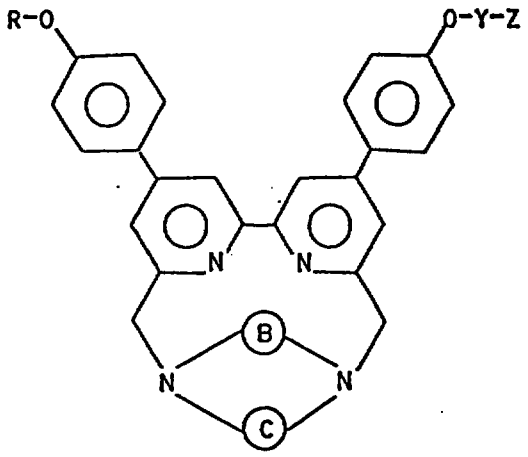
The invention further relates to the methods of obtaining these cryptates and to the synthesis intermediates.

Finally, it further relates to the application of these rare earth cryptates as fluorescent tracers for biological substances.

The functionalized rare earth cryptates according to the present invention consist of at least one rare earth salt complexed by a macropolycyclic compound having one of formulae I or II below:

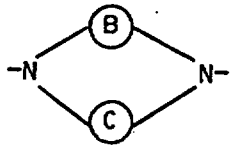


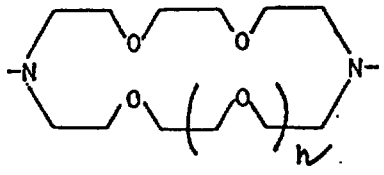
I



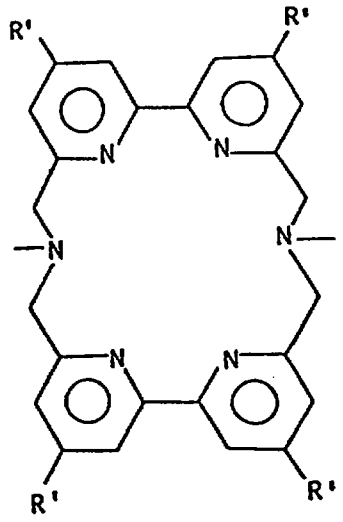
II

in which:

- the ring of the formula  is one of the following rings:

1) 

n = 0 or 1
[N_2O_4] macrocycle or (22) ring
[N_2O_3] macrocycle or (21) ring

2)  bis-bipyridine macrocycle

- Y is a spacer group or arm consisting of a divalent organic radical selected from linear or branched C_1 to C_{20} alkylene groups, which may or may not contain one or more double bonds and/or may or may not be interrupted by one or more heteroatoms such as oxygen, nitrogen, sulfur or phosphorus, from C_5-C_8 cycloalkylene groups or from C_6 to C_{14} arylene groups, said alkylene, cycloalkylene or arylene groups being unsubstituted or substituted by alkyl, aryl or sulfonate groups;
- Z is a functional group capable of bonding covalently with a biological substance;
- R is a methyl group or represents the group -Y-Z; and
- R' is hydrogen or a group -COOR", in which R" is a C_1 to C_{10} alkyl group and preferably represents the methyl, ethyl or tert-butyl group, or R' is a group -CO-NH-Y-Z.

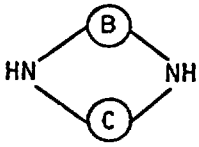
In the present description, the expression "functional group capable of bonding covalently with a biological substance" denotes any functional group capable of bonding covalently, directly or after activation, with at least one of the functional groups naturally present

in or artificially introduced into said biological substance. Such functional groups are, in particular, NH_2, COOH, SH or OH groups. Such groups, and the activation methods, are described in detail by P. TIJSSEN in "Practice and Theory of Enzyme Immunoassays", Elsevier 1985, this document being incorporated in the present description by way of reference.

Amino, thio, cyano, isocyano, isothiocyano, thiocyano, carboxyl, hydroxyl, maleimido, succinimido, mercapto, phenol, imidazole, aldehyde, epoxide, halide, thionyl, sulfonyl, nitrobenzoyl, carbonyl, triazo, anhydride, halogenoacetate, hydrazino, acridine and other groups may be mentioned in particular as examples of functional groups which are appropriate for the purposes of the invention.

The particularly preferred groups are the amino, thio and carboxyl groups, which have to be activated prior to covalent coupling with the biological substance, and the maleimido, succinimido and isothiocyanate groups, which can bond with the biological substance directly.

In general, the cryptates according to the invention can be obtained either

- by condensation of the ring 

with a 6,6'-dihalogenomethyl-2,2'-bipyridine disubstituted in the 4,4'-positions, subsequent substitution by the group -NH-Y-Z or the group -Y-Z and complexation of the resulting macropolycyclic compound by a rare earth salt, or

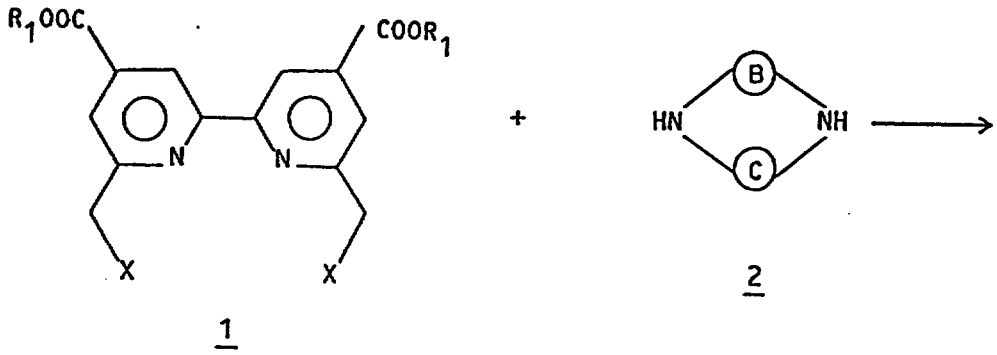
- by condensation of one molecule of 6,6'-diaminomethyl-2,2'-bipyridine disubstituted in the 4,4'-positions with two molecules of a 6,6'-dihalogenomethyl-2,2'-bipyridine disubstituted in the 4,4'-positions, subsequent substitution by the group -NH-Y-Z or the group -Y-Z and complexation of the resulting macropolycyclic

compound by a rare earth salt.

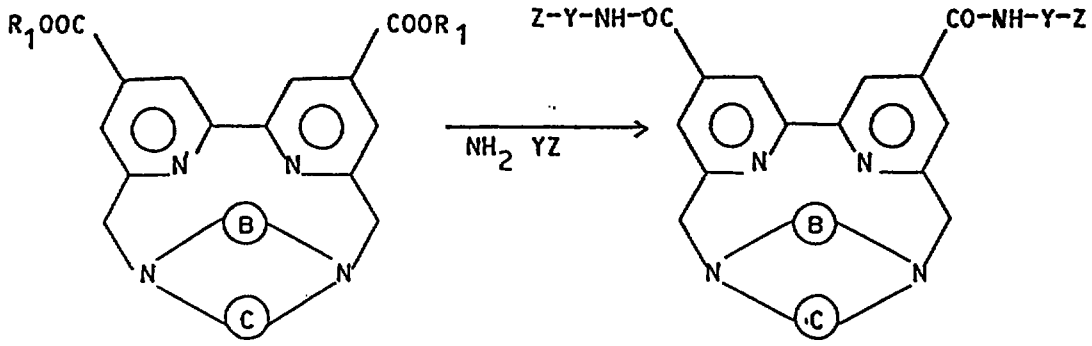
It will be noted that, in the above methods, the complexation by a rare earth salt can take place before the substitution step. This complexation is preferably carried out before the substitution step.

The different methods of obtaining the cryptates according to the invention will now be described in detail.

METHOD A:



1 2



3 4

in which X is a halogeno group and R_1 is an alkyl group containing from 1 to 10 carbon atoms and preferably represents the methyl, ethyl or tert-butyl group.

In this method, the halogenated bipyridine derivative 1 is condensed with the macrocycle 2.

This reaction is advantageously carried out in an anhydrous organic solvent, for example acetonitrile, in the presence of a base such as an alkali metal ion carbonate, for example sodium carbonate or lithium carbonate, at the reflux temperature of the solvent. The macrobicycle 3 obtained is then in the form of a cryptate of the alkali metal ion used, for example in the form of a sodium cryptate.

The macrobicyclic compound 3 is then subjected to aminolysis by reaction with an amine of the formula H_2N-Y-Z, in which the groups Y and Z are as defined above, the functional group Z having been blocked, if necessary, by conventional means. The reaction is advantageously carried out under a nitrogen atmosphere and at room temperature. When the reaction is complete, the excess amine is removed by appropriate means and the cryptate of formula 4 is recovered by conventional means.

The compound obtained in this way is then converted to a rare earth cryptate by ion exchange, a solution of the alkali metal cryptate of the macrobicycle 4 in methanol being refluxed, if appropriate in the presence of chloroform, with a solution of a rare earth halide in methanol.

As indicated earlier, the alkali metal ion cryptate 3 can be converted to a rare earth cryptate, prior to aminolysis, by ion exchange according to the procedure described above.

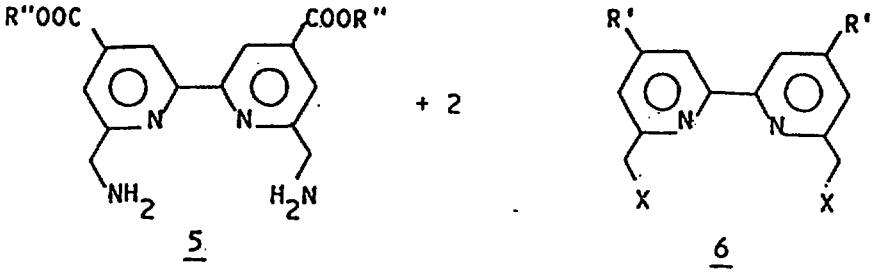
The macrobicyclic compound 3 is a key intermediate for the synthesis of the cryptates of the invention. This intermediate can take the form of an alkali metal ion cryptate or a rare earth cryptate. It forms a further subject of the invention. The particularly preferred intermediates for the purposes of the invention

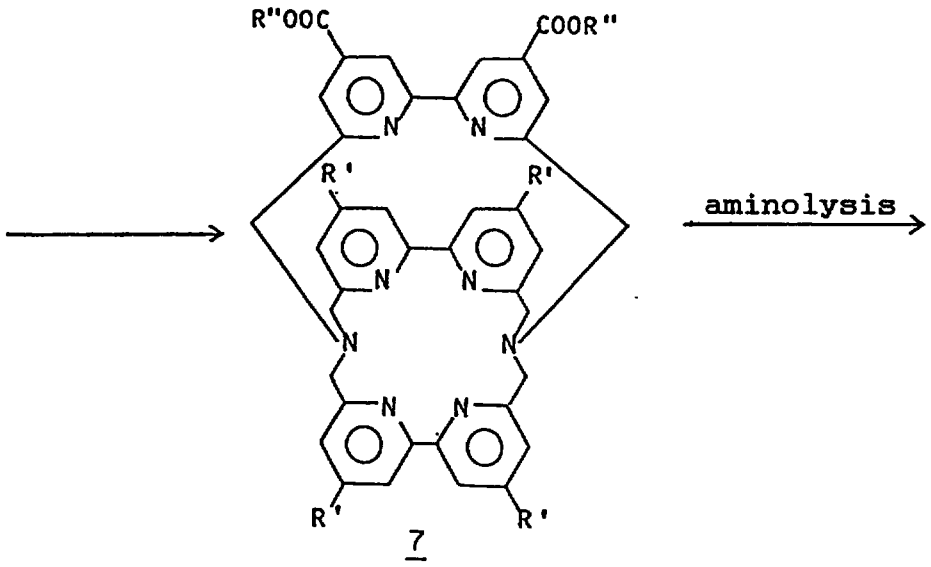
are the compounds of formula 3 in which R_1 is the CH_3, C_2H_5 or t-C_4H_9 group.

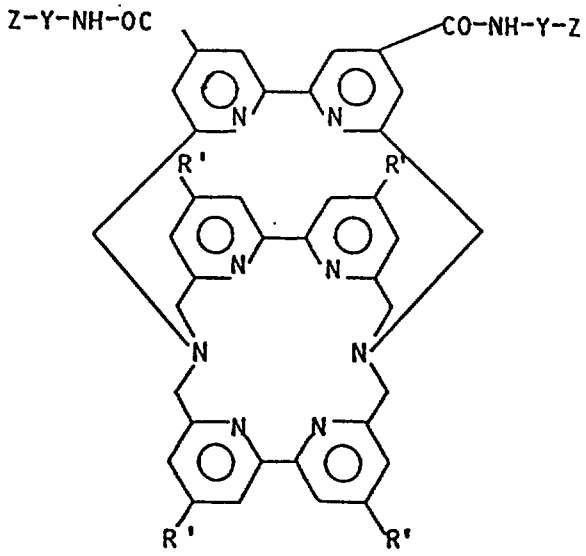
In a modified way of carrying out this method, the alkali metal ion cryptate can be decomplexed first by conversion to the corresponding silver cryptate by reaction with an excess of silver nitrate, and then by treatment of the latter with H_2S [Helvetica Chimica Acta, vol. 67 (1984) 2264-2269]. This gives the macropolycyclic compound 3 in the form of the free cryptand, also a synthesis intermediate, which can then be complexed by conventional means to form a rare earth cryptate.

It will be noted that the cryptates of formula (I) or (II) in which Z is other than an amino group can be obtained from the cryptates of formula (I) or (II) in which Z is the amino group by conventional methods well known to those skilled in the art.

METHOD B:



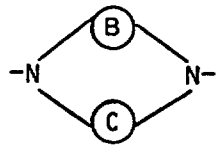




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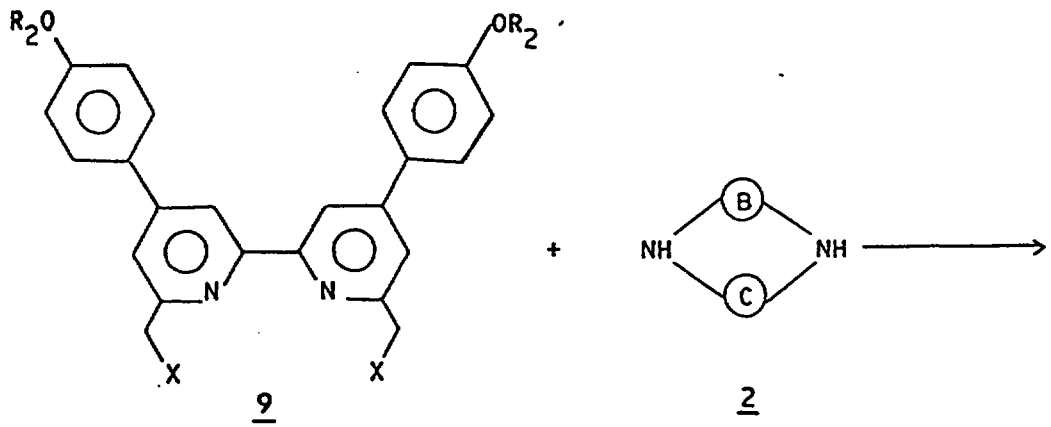
In this method, two molecules of the halogenated derivative 6 are condensed with one molecule of the amino derivative 5. This condensation reaction is advantageously carried out under the same conditions as in the first step of method A, i.e. in an anhydrous organic solvent such as acetonitrile, in the presence of a base such as an alkali metal ion carbonate, for example sodium carbonate or lithium carbonate. The reaction is carried out at the reflux temperature of the solvent. The macropolycyclic compound 7 is obtained, which is a key intermediate for the synthesis of the cryptates according to the invention and also falls within the scope of the invention. This macropolycyclic complex is in the form of an alkali metal ion cryptate, which is advantageously converted to a rare earth cryptate, before aminolysis, according to the procedure defined above. As for method A, the alkali metal ion cryptate 7 can be decomplexed, in a modified way of carrying out the present method, to give the corresponding macropolycyclic compound 7, or free cryptand, which is then recomplexed to form a rare earth

cryptate.

The compound 7 is then subjected to aminolysis according to the procedure defined above to give the macropolycyclic complex 8, i.e. a cryptate according to the invention of formula I in which  is the bis-bipyridine macrocycle and Y, Z and R' are as defined above.

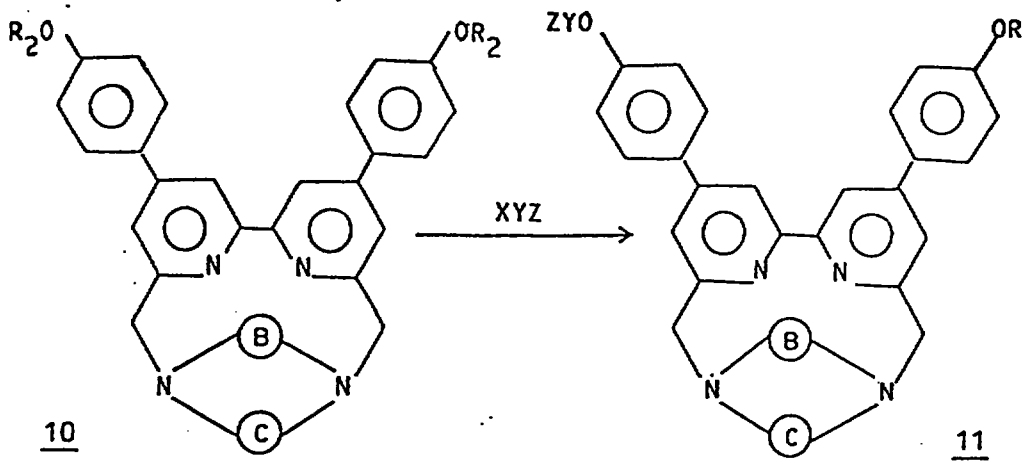
The amino derivative 5 used as a starting material in method B is obtained from the halogenated derivative 1 by conversion to the corresponding azido compound by reaction with NaN_3 under reflux in an organic solvent such as tetrahydrofuran, and then reduction of the resulting azido compound in the presence of a reduction catalyst such as palladium-on-charcoal, under a hydrogen atmosphere, at room temperature.

METHOD C:



9 + 2

R_2 = H or CH_3



According to this procedure, the appropriately substituted, halogenated bipyridine derivative 9 is first condensed with the macrocycle 2. This reaction is advantageously carried out in an anhydrous organic solvent, for example acetonitrile, in the presence of a base such as an alkali metal ion carbonate, for example sodium carbonate or lithium carbonate. This gives the macropolycyclic compound 10 in the form of an alkali metal ion cryptate. The reaction is preferably carried out at the reflux temperature of the solvent.

The macropolycyclic compound 10 is then substituted by reaction with a halide of the formula XYZ, Z and Y being as defined above and X being a halide ion.

When Z is a cyano group, it can be converted to an amino group by reduction with conventional reducing agents.

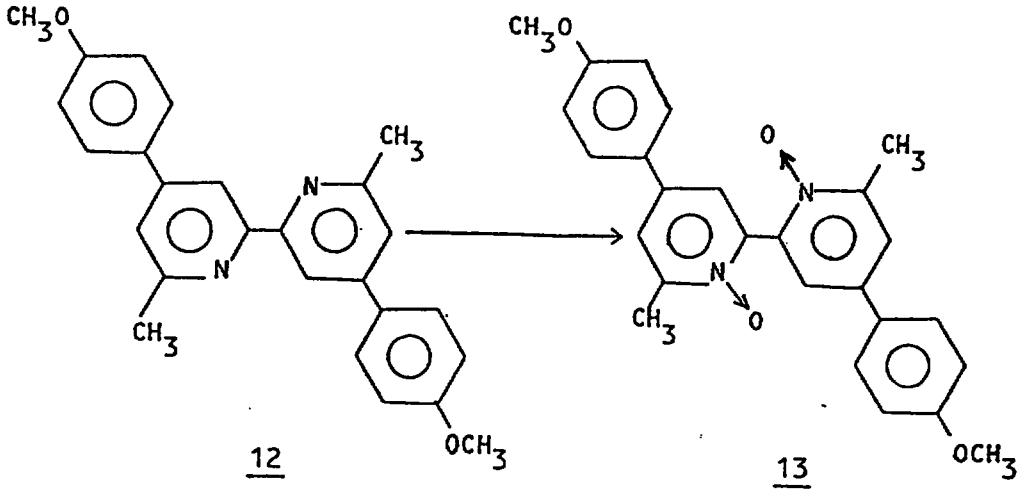
The compound 11 obtained is then converted to a rare earth cryptate by ion exchange, for example according to the procedure defined above. Advantageously, a rare earth halide is dissolved in methanol, and a solution of the alkali metal ion cryptate in methanol, with a small volume of chloroform if necessary, is added. The mixture is refluxed under an inert atmosphere and the disappearance of the alkali metal ion cryptate is followed by

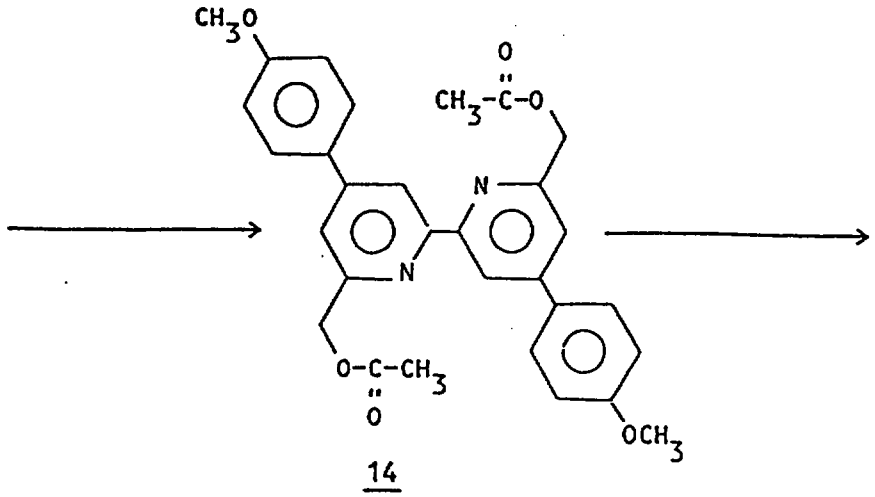
thin layer chromatography.

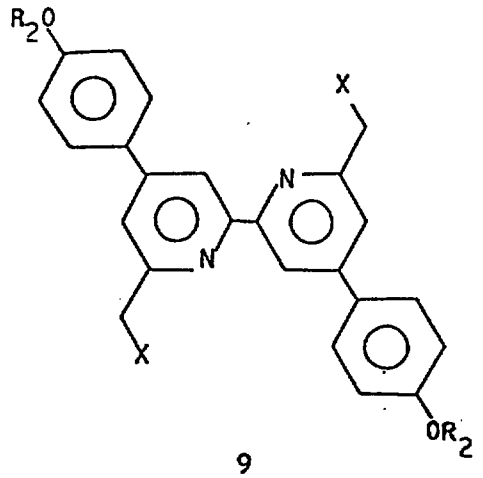
In a modified way of carrying out the method, the alkali metal ion cryptate can be decomplexed according to the procedure defined above (see method A) and converted to the desired rare earth cryptate.

When the reaction is complete, the rare earth cryptate is isolated from the reaction medium by conventional methods. It is in the form of a crystalline solid.

The halogenated bipyridine derivative 9 used in the above procedure can be obtained by the method represented by the reaction scheme below:







R_2 = H or CH_3

9

6,6'-Dimethyl-4,4'-di(p-methoxyphenyl)-2,2'-bipyridine of formula 12 can be obtained from 1,6-di(p-methoxyphenyl)hexa-1,5-diene-3,4-dione by Kröhnke's method described in SYNTHESIS (1976), 1.

The compound 13 is obtained by reacting the compound 12 with m-chloroperbenzoic acid in an organic solvent such as chloroform.

The derivative 14 is then prepared by refluxing a suspension of the compound 13 in acetic anhydride.

Refluxing a solution of the compound 14 with a hydrohalic acid in acetic acid solution gives the compound 9 in which R_2 is H or CH_3, at least one of the radicals R_2 being hydrogen.

The compound of formula 9 is a key intermediate for the preparation of the compounds of the invention and forms a further subject of the invention.

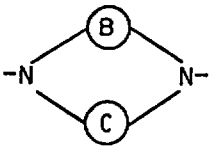
The compounds according to the invention, which are stable rare earth cryptates, are soluble in water and some organic solvents such as methanol or DMSO.

The compounds of the invention are useful as fluorescent tracers for biological products and are therefore suitable as assay reagents in methods of immunological detection and determination by fluorescence, as well as in so-called assays by competition and so-called

assays by excess.

The invention will now be described in greater detail in the illustrative Examples below.

EXAMPLE 1 - Rare earth cryptate of [(22)(p-OCH_3 p-$OCH_2CH_2NH_2$ diPhbpy)]

Formula II: R = CH_3; Y = CH_2CH_2; Z = NH_2;  is

the (22) macrocycle (method C).

A - 6,6'-Dimethyl-4,4'-di(p-methoxyphenyl)-2,2'-bipyridine (compound 12)

A mixture of 1,6-di(p-methoxyphenyl)hexa-1,5-diene-3,4-dione (3.07 g, 9.5 mmol), prepared by Kröhnke's method, Synthesis (1976), 1, 1-pyridiniopropan-2-one chloride (3.27 g, 19.1 mmol), obtained by mixing equimolar amounts of pyridine and 1-chloroacetone, and ammonium acetate (19 g) in 95.5 ml of CH_3OH is refluxed for 38 h under nitrogen. After the reaction mixture has returned to room temperature, the cream-colored precipitate formed is filtered off and washed with CH_3OH. The crude product is used without further purification. It can be crystallized from CH_2Cl_2/hexane.

Yield: 70%

TLC: R_f = 0.5 (silica, CH_2Cl_2/CH_3OH, 95/5)

MS: 396 (M^+), 381 (M^+-CH_3), 365 (M^+-2CH_3), 353 (M^+-CH_3-CO), 338 (M^+-2CH_3-CO), 198 ($M^+/2$).

B - 1,1'-Di(N-oxide)-6,6'-dimethyl-4,4'-di(p-methoxyphenyl)-2,2'-bipyridine (compound 13)

A solution of m-chloroperbenzoic acid (6 g, 34.8 mmol) in 360 ml of $CHCl_3$ is added dropwise, at 0°C, to a solution of 12 (3.45 g, 8.7 mmol) in 800 ml of $CHCl_3$. The mixture is stirred overnight at room temperature. It is then washed with a saturated solution of $NaHCO_3$ until the pH is alkaline. The organic phase is separated off and concentrated. The addition of hexane causes the

compound to precipitate. The precipitate is filtered off and then redissolved in a CH_2Cl_2/CH_3OH mixture (90/10) and washed with a 2 N solution of sodium hydroxide. The organic phase, which contains the compound 13, is separated off and the solvent is evaporated off. In a separate operation, the filtrate originating from the precipitation is also washed with a 2 N solution of sodium hydroxide and then concentrated and chromatographed on an alumina column using CH_2Cl_2 as the eluent.

Yield: 80% M.p. > 250°C

TLC: R_f = 0.5 (alumina, CH_2Cl_2/CH_3OH, 95/5)

MS: 429 (MH^+), 411 (M^+-H_2O).

C - 6,6'-Diacetoxymethyl-4,4'-di(p-methoxyphenyl)-2,2'-bipyridine (compound 14)

A suspension of 13 (1.21 g, 2.82 mmol) in 18 ml of acetic anhydride is refluxed for 1 h 30 min. The solution obtained is concentrated. 5 ml of H_2O and 20 ml of CH_2Cl_2 are added to the pasty residue and the medium is rendered basic with a saturated aqueous solution of $NaHCO_3$. The organic phase is separated off and the aqueous phase is extracted twice with 30 ml of CH_2Cl_2. The resulting organic phase is dried over Na_2SO_4 and the solvent is evaporated off. The crude product is chromatographed on an alumina column using CH_2Cl_2 as the eluent.

Yield: 90% M.p. = 140-141°C

TLC: R_f = 0.9 (alumina, CH_2Cl_2/CH_3OH, 95/5)

MS: 513 (MH^+), 469 ($M^+-C(O)CH_3$), 453 (M^+-CH_3COOH), 256 ($M^+/2$).

D - 6,6'-Dibromomethyl-4,4'-di(p-methoxyphenyl)-2,2'-bipyridine (compound 9a: R_2 = CH_3)
6,6'-Dibromomethyl-4-p-methoxyphenyl-4'-p-hydroxyphenyl-2,2'-bipyridine (compound 9b: one of the radicals R_2 is H, the other is CH_3)

A solution of 14 (0.31 g, 0.60 mmol) in 5 ml of

33% HBr/AcOH is refluxed for 12 to 13 h. 20 ml of H_2O and 60 ml of $CHCl_3$ are added to the solution at room temperature. The mixture is washed with a saturated solution of $NaHCO_3$ until the pH is neutral. The organic phase is separated off and the aqueous phase is extracted twice with 20 ml of CH_2Cl_2. The solvent is evaporated off from the resulting organic phase and the residue is chromatographed on an alumina column using CH_2Cl_2 and then CH_2Cl_2/CH_3OH (95/5) as the eluents.

Compound 9a: $R_2 = CH_3$

Yield: 16%

Decomposition: 210-215°C

TLC: $R_f > 0.9$ (alumina, CH_2Cl_2/CH_3OH, 95/5)

MS: 556,555,553 (MH^+), 556,554,552 (M^+), 475,473 (M^+-Br), 394 (M^+-2Br).

Compound 9b: one of the radicals R_2 is H

Yield: 35%

TLC: $R_f = 0.4$ (alumina, CH_2Cl_2/CH_3OH, 95/5)

MS: 542,540,538 (M^+), 461,459 (M^+-Br), 380 (M^+-2Br).

E - <u>Sodium cryptate of [(22)(p-OCH$_3$ p-OH diPhbpy)]</u> (compound 10)

A mixture of the (22) macrocycle (0.262 g, 1 mmol) and Na_2CO_3 (1.05 g, 10 mmol) in 600 ml of anhydrous CH_3CN is refluxed for 30 min under nitrogen. A suspension of the compound 9b (0.54 g, 1 mmol) in 450 ml of anhydrous CH_3CN is then added. The mixture is refluxed for 20 h under nitrogen. The solution obtained is filtered hot and the solvent is evaporated off. The crude product is chromatographed on alumina using CH_2Cl_2/CH_3OH (95/5) as the eluent.

Yield: 60-65%

TLC: $R_f = 0.6$ (alumina, CH_2Cl_2/CH_3OH, 90/10)

MS: 663 (M^+), 640 (M^+-Na), 625 ($M^+-Na-CH_3$), 609 ($M^+-Na-OCH_3$), 595 ($M^+-Na-OCH_3-OH$).

F - Sodium cryptate of [(22)(p-OCH_3 p-OCH_2CN diPhbpy)] (compound 11a): Z = CN; Y = CH_2; R = CH_3

An excess of NaH (in powder form) is added under nitrogen to a solution of the sodium cryptate of the compound 10 (0.042 g, 0.066 mmol) in the minimum volume of CH_3CN. The mixture is refluxed under nitrogen for 1 h. The solution is cooled to room temperature and bromoacetonitrile (1.2 equivalents) is then added. The mixture is stirred overnight at room temperature, under nitrogen. 20 ml of H_2O and 10 ml of CH_2Cl_2 are added. The medium is neutralized with a saturated solution of $NaHCO_3$. The organic phase is separated off and the aqueous phase is extracted twice with 20 ml of CH_2Cl_2. The resulting organic phase is dried over Na_2SO_4 and the solvents are then evaporated off. The crude product is chromatographed on an alumina column using CH_2Cl_2/CH_3OH (96/4) as the eluent.

Yield: 75%

TLC: R_f = 0.5 (bluish violet spot), (alumina, CH_2Cl_2/CH_3OH, 92.5/7.5)

MS: 702 (M^+), 679 (M^+-Na), 663 (M^+-Na-H-CH_3), 654 (MH^+-Na-CN), 640 (MH^+-Na-CH_2CN).

G - Sodium cryptate of [(22)(p-OCH_3 p-$OCH_2CH_2NH_2$ diPhbpy)] (compound 11b: Z = NH_2; Y = -CH_2-CH_2) R = CH_3

2 ml of B_2H_6 (1 M in THF) and a further 5 ml of THF are added to a suspension of 11a (0.078 g, 0.1 mmol) in 8 ml of anhydrous THF. The mixture is stirred overnight at room temperature. 10 ml of CH_3OH are added and the mixture is stirred for 20 to 30 min. The solvents are evaporated off. 20 ml of an H_2O/concentrated HCl mixture (1/1) are added to the residue. A yellow precipitate and the evolution of a gas are observed. Dissolution takes place gradually. The solvents are evaporated off and the residue obtained is treated with

an aqueous solution of NaOH (6 N). The amine 11b is extracted with 25 ml of CH_2Cl_2. The yield is quantitative. The formation of the compound is demonstrated by ^{1}H-NMR.

H - Complexation reaction: Exchange of sodium for an Ln^{3+} ion (Eu, Tb)

$LnCl_3.xH_2O$ (0.1 mmol) is dissolved in 25 ml of CH_3OH, and a solution of the previously obtained sodium cryptate (0.06 mmol) in 4-8 ml of $CHCl_3$ (minimum volume) is added. The mixture is refluxed under a nitrogen atmosphere (for 6 hours to 4 days, depending on the sodium cryptate used). The disappearance of the sodium cryptate is followed by TLC (alumina, CH_2Cl_2/CH_3OH, 90/10). When the reaction is complete, the reaction mixture is filtered, if necessary, and the solvents are evaporated off. The residue is redissolved in 15-20 ml of CH_3OH, and ether is added until a turbidity persists (5 ml to 25-30 ml). The rare earth cryptate formed crystallizes or precipitates very rapidly or after several hours, depending on the nature of the cryptate. The formation of the cryptate is demonstrated by ^{1}H-NMR and UV/visible spectroscopy.

This procedure gave the europium cryptate and the terbium cryptate of formula 11, respectively, which have the following spectroscopic characteristics:

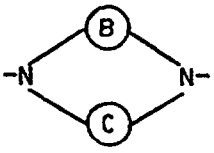
Europium cryptate: UV/visible in CH_3OH

280 nm maximum; 292 nm shoulder; 320 nm maximum.

Terbium cryptate: UV/visible in H_2O

282 nm; 310 nm shoulder.

EXAMPLE 2 - Rare earth cryptate of [bpy.bpy.(p-OCH_3 p-$OCH_2CH_2NH_2$ diPhbpy)]

R = CH_3; Y = $-CH_2-CH_2-$; Z = NH_2; R' = H; 

is the bis-bipyridine macrocycle (method C).

A - Sodium cryptate of [bpy.bpy.(p-OCH_3 p-OH diPhbpy)]
(compound 10)

A mixture of the bis-bipyridine macrocycle (0.48 g, 1.21 mmol) and Na_2CO_3 (1.16 g, 11 mmol) in 480 ml of anhydrous CH_3CN is refluxed for 30 min under nitrogen. A suspension of previously prepared 6,6'-dibromomethyl-4-p-methoxyphenyl-4'-p-hydroxyphenyl-2,2'-bipyridine (see Example 1 section D) (0.65 g, 1.20 mmol) in 340 ml of CH_3CN is added. The resulting solution is refluxed for 24 h under nitrogen. After it has cooled to room temperature, the reaction mixture is filtered and the solvent is evaporated off. The crude product is then chromatographed on an alumina column using CH_2Cl_2/CH_3OH (98/2) as the eluent.

Yield: 45%

TLC: R_f = 0.4 (alumina, CH_2Cl_2/CH_3OH, 90/10)
violet spot (254 nm), green spot (366 nm)

MS: a) CI (NH_3): 795 (M^+), 773 (MH^+-Na)
b) FAB$^+$ (thioglycerol): 875 (MBr^+), 795 (M^+).

The sodium cryptate obtained was converted to the terbium cryptate by the procedure described in Example 1H. This cryptate has the following spectroscopic characteristics:

- UV/visible in H_2O:
  244 nm (maximum); 303 nm (maximum)
- UV/visible in CH_3OH:
  244 nm (maximum); 301 nm (maximum); 318 nm (shoulder).

B - Sodium cryptate of [bpy.bpy.(p-OCH_3 p-OCH_2CN diPhbpy)]

A solution of NaOH in CH_3OH (1.5 equivalents in 2 ml) is added to a solution of the sodium cryptate of 10 (0.21 g, 0.24 mmol) in 12 ml of CH_2Cl_2 and 12 ml of CH_3OH. The mixture is refluxed for 1 h 30 min under nitrogen. After cooling to room temperature, the solvent is evaporated off; the water formed during the reaction

is removed by azeotropic distillation with toluene. The product obtained in this way is dried overnight with a vane pump. The bright orange compound obtained is dissolved in 35 ml of THF (the solution is turbid). As soon as bromoacetonitrile is added (30 µl, 1.8 equivalents), the solution becomes clear. The reaction is allowed to proceed overnight at room temperature under nitrogen, with stirring. 20 ml of H_2O and about 10 ml of CH_2Cl_2 are added. The medium is neutralized with a saturated solution of $NaHCO_3$. The organic phase is separated off and the aqueous phase is extracted twice with 20 ml of CH_2Cl_2. The resulting organic phase is dried over Na_2SO_4 and the solvents are then evaporated off. The crude product is chromatographed on an alumina column using CH_2Cl_2/CH_3OH (95/5) as the eluent.

Yield: 92%

MS: FAB^+: 834 (M^+).

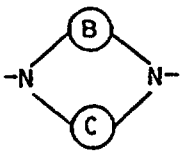
The sodium cryptate obtained was converted to the corresponding terbium cryptate by the procedure described in Example 1. This cryptate has the following spectroscopic characteristics:

- UV/visible in CH_3OH: 300 nm (maximum); 314 nm (shoulder).

C - <u>Rare earth cryptate of [bpy.bpy.(p-OCH_3 p-$OCH_2CH_2NH_2$ diPhbpy)]</u>

The rare earth cryptate of the above compound is obtained by the procedure described in section G of Example 1.

<u>EXAMPLE 3</u> - <u>Preparation of the europium cryptate of [(bis-bpy) bpy-di(amidoethyleneamine)]</u> (method A)

Compound of formula I: Z = NH_2; Y = $-CH_2-CH_2-$; 

is the bipyridine macrocycle and R' is hydrogen.

A - Preparation of the sodium cryptate of [(bis-bpy) bpy diester]

Compound of formula 1 in which $R_1 = CH_3$

A mixture of 0.300 g ($7.61.10^{-4}$ mol) of the bis-bipyridine macrocycle and 1.10 g ($10.4.10^{-3}$ mol) of Na_2CO_3 is refluxed for 30 min in 450 ml of CH_3CN. A solution of 0.350 g ($7.64.10^{-4}$ mol) of 6,6'-dibromomethyl-(4,4'-p-dimethoxycarbonyl)-2,2'-bipyridine (compound 1; X = Br) in 375 ml of CH_3CN is then added over 60 min, with vigorous stirring. The reaction mixture is stirred for a further 18 hours under reflux and then cooled to room temperature and filtered. The solvent is removed under vacuum on a rotary evaporator. The solid obtained is dissolved in $CHCl_3$ and chromatographed on an alumina column (with a small amount of silica on the top) using $CHCl_3/CH_3OH$ (98/2) as the eluent. The solvents are removed under vacuum and 0.230 g (38%) of the expected complex is obtained. This has the following physicochemical characteristics:

Melting point > 240°C

^1H-NMR (CD_3OD): 3.89 (S, 8H, CH_2-bpy); 3.98 (S, 4H, CH_2-bpy diE); 4.03 (S, 6H, $COOCH_3$); 7.42 (d, J = 7.4 Hz, 4H-bpy); 7.92 (t-J = 7.4 Hz, 4H-bpy); 7.96 (d, J = 1.2 Hz, 2H-bpy diE); 8.10 (d, J = 7.4 Hz, 4H-bpy); 8.60 (d, J = 1.2 Hz, 2H-bpy diE) (diE = diester)

^{13}C-NMR ($CDCl_3$): 52.9, 59.5, 119.7, 120.4, 123.4, 124.1, 138.2, 139.7, 155.2, 155.7, 158.3, 160.4, 164.9

$C_{40}H_{34}N_8O_4NaBr.4H_2O$ (865.71):

Calculated (%): C 55.49; H 4.89; N 12.94;

Found (%): C 55.44; H 4.33; N 13.10.

B - Aminolysis of the above cryptate

The cryptate obtained above (0.100 g, $1.26.10^{-4}$ mol) is subjected to aminolysis by being added in portions to 4 ml of ethylenediamine which has been distilled over KOH and heated to 90°C beforehand. The reaction

mixture obtained is stirred for 1 hour and then cooled to room temperature. The excess ethylenediamine is then removed under vacuum to give an oil. This is taken up in 2 ml of a $CHCl_3/CH_3OH$ mixture (2/1), which is in turn removed under vacuum. Purification is repeated 5 times to give the expected complex with a yield of 89%.

C - Conversion of the above cryptate to the corresponding europium cryptate

The europium cryptate of [(bis-bpy) (bpy-di-(amidoethyleneamine))] was obtained by the procedure described in section H of Example 1; it has the following spectroscopic characteristics:

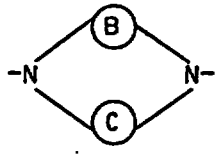
UV/visible in H_2O

240 nm; 303 nm (maxima)

EXAMPLE 4 - Preparation of the europium cryptate of [(bis-bpy) (bpy-di(amidoethlyleneamine))]

(method A)

Compound of formula I: Z = NH_2; Y = $-CH_2-CH_2-$; R' = H;



is the bipyridine macrocycle.

A - Preparation of the europium cryptate of [(bis-bpy) (bpy diester)]

The sodium cryptate obtained in section A of the previous Example (0.10 mmol) is dissolved in 8 ml of $CHCl_3$ and added to a solution of $EuCl_3.6H_2O$ (0.15 mmol) in 40 ml of CH_3OH. The solution is stirred at the reflux temperature, under nitrogen, until the sodium cryptate has disappeared (TLC, aluminum oxide, eluent: CH_2Cl_2/ CH_3OH (90/10)). The reaction time is of the order of 48 hours. The reaction mixture is cooled to room temperature and filtered if turbid. The solvents are removed on a rotary evaporator and the residue is redissolved in 20-25 ml of CH_3OH. Ethyl ether is added dropwise until a very slight turbidity persists. The europium cryptate

crystallizes in this way at room temperature. The solvents are removed by pipette and the cryptate is dried. Yield: about 40% (first crystallization). The filtrate is concentrated to dryness and the operation is repeated.

The product obtained has the following physicochemical characteristics:

^{1}H-NMR (D_2O, ref = t-BuOH): 10.51 (2H, S, CH(bpy diE)), 8.66 (2H, S, CH-bpy diE), 8.06 (4H, t, CH-bpy), 7.08 (4H, d, CH-bpy), 6.74 (4H, d, CH-bpy), 4.66 (6H, S, OCH_3), 0.9 (8H, S, CH_2-bpy), -0.78 (4H, S, CH_2-bpy diE)

$C_{40}H_{34}N_8O_4EuCl_3NaCl.9/2H_2O$ (1088.58)

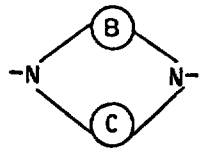
% calculated:	C 44.13;	H 3.98;	N 10.29
% found:	C 43.93;	H 3.79;	N 9.88
	C 44.06;	H 3.70;	N 10.03

B - <u>Aminolysis of the europium cryptate</u>

The europium cryptate obtained above (40 mg) is added in portions to 40 ml of ethylenediamine which has been distilled over KOH beforehand. The clear solution is stirred for 3 hours under nitrogen at room temperature. The ethylenediamine is removed under vacuum on a rotary evaporator. The residue is taken up in a few milliliters of CH_2Cl_2/MeOH (2/1) and the solvents are removed under vacuum. The operation is repeated 5 times to give a perfectly solid compound. The cryptate obtained is dried for 24 hours under a vane pump vacuum. Melting point = 168-170°C.

This europium cryptate has the same characteristics as the europium cryptate obtained in the previous Example. These two Examples therefore show that the complexation of the alkali metal ion cryptate by a rare earth ion can be carried out before or after substitution.

EXAMPLE 5 - Preparation of the europium or terbium cryptate of [(22) bpy di(amidoethyleneamine)]

Compound of formula I =  is the (22) macrocycle; Z = NH_2; Y = $-CH_2-CH_2-$

A - Preparation of the sodium cryptate of [(22) bpy diester]

A mixture of 0.114 g ($4.37.10^{-4}$ mol) of the (22) macrocycle and 0.460 g ($4.34.10^{-3}$ mol) of Na_2CO_3 is refluxed for 30 min in 130 ml of CH_3CN. A solution of 0.200 g ($4.37.10^{-4}$ mol) of the compound 1 in 220 ml of CH_3CN is then added over 60 min, with vigorous stirring. The reaction mixture is stirred for a further 15 hours under reflux and then cooled to room temperature and filtered. The solvent is removed under vacuum on a rotary evaporator. The solid obtained is dissolved in CH_2Cl_2 and purified by chromatography on an alumina column (with a small amount of silica on the top). An impurity is eluted first with CH_2Cl_2. The macrobicycle formed is then eluted with CH_2Cl_2/CH_3OH (98/2). The solvents are removed under vacuum to give 0.135 g (61%) of the macrobicycle.

B - Preparation of the europium or terbium cryptate of [(22) bpy diester]

The europium and terbium cryptates of [(22) bpy diester] were obtained by the procedure described in Example 1H; they have the following spectroscopic characteristics:

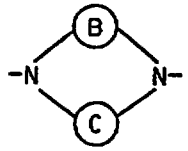
- europium cryptate: UV/visible in CH_3OH
  242 nm and 324 nm (maxima)
- terbium cryptate: UV/visible in CH_3OH
  242 nm and 325 nm (maxima)

C - Preparation of the europium cryptate of [(22) bpy di(amidoethyleneamine)]

The europium cryptate obtained above (0.100 g, $1.43.10^{-4}$ mol) is added in portions to 5 ml of ethylenediamine which has been distilled over KOH and heated to 90°C beforehand. The reaction mixture is stirred for 1 hour and then cooled to room temperature. The excess ethylenediamine is removed under vacuum to give an oil. This is taken up in 2 ml of $CHCl_3$, which is in turn removed under vacuum. The purification is repeated 3 times to give 93 mg (90%) of the europium cryptate. This cryptate has the following spectroscopic characteristics:
UV/visible in H_2O:
240 and 315 nm (maxima)

The terbium cryptate obtained in section B can be subjected to aminolysis in the same way.

EXAMPLE 6 - Preparation of the sodium cryptate of [(bpy dicarbomethoxy)a] (method B)

Formula I: R' = R" = OCH_3; 

is the bis-bipyridine macrocycle.

A - Synthesis of 4,4'-dicarbomethoxy-6,6'-diazidomethyl-2,2'-bipyridine

A mixture of 4,4'-dicarbomethoxy-6,6'-dibromomethyl-2,2'-bipyridine (0.50 g, $1.10.10^{-3}$ mol) and NaN_3 (1.0 g, $15.4.10^{-3}$ mol) is refluxed for 36 hours in 15 ml of THF. The reaction mixture is cooled to room temperature, filtered on celite and concentrated to dryness. The residue is dissolved in $CHCl_3$ and chromatographed on a silica column using $CHCl_3$ as the eluent.

Evaporation of the solvent gives 0.40 g (95%) of the expected compound, which has the following physicochemical characteristics:
M.p. = 168-170°C

^{1}H-NMR ($CDCl_3$): 4.02 (S, 6H, $COOCH_3$); 4.63 (S, 4H, CH_2-H_3); 7.94 (d, J = 1.3 Hz, 2H); 8.96 (d, J = 1.3 Hz, 2H);

^{13}C-NMR ($CDCl_3$): 52.7, 55.1; 120.0; 121.5; 139.7; 156.0, 156.8; 165.3.

IR (KBr): 1715 cm^{-1} (ester), 2090 cm^{-1} (azide)

MS: 383 $((MH)^+)$

$C_8H_{14}N_8O_4$ (382.34)

% calculated: C 50.27; H 3.69; N 29.31

% found: C 50.37; H 3.51; N 27.69.

B - Synthesis of 4,4'-dicarbomethoxy-6,6'-diaminomethyl-2,2'-bipyridine (compound 5)

A mixture of the compound obtained according to A (0.126 g, $3.30.10^{-4}$ mol) and 12.6 mg of 10% Pd/C in 38 ml of CH_2Cl_2/CH_3OH (2/1) is stirred at room temperature under a hydrogen atmosphere for 12 hours.

The reaction mixture is filtered on célite and evaporation of the solvents gives 0.100 g (92%) of the expected compound, which is used without purification in the following step.

C - Synthesis of the sodium cryptate of [bpy-dicarbomethoxy]a (compound 7)

A mixture of 6 (R' = OCH_3; X = Br) (0.280 g, $6.11.10^{-4}$ mol) and Na_2CO_3 (0.65 g, $6.13.10^{-3}$ mol) is heated to the reflux temperature in 500 ml of CH_3CN. 0.100 g ($3.03.10^{-4}$ mol) of 5 is then added and the reaction mixture is stirred under reflux for a further 48 hours. The reaction mixture is cooled to room temperature and filtered. The solvent is removed under vacuum. The residue is taken up in $CHCl_3$ and chromatographed on an alumina column (with a small amount of silica on the top) using $CHCl_3$/MeOH (98/2) as the eluent. Evaporation of the solvents gives 0.132 g (40%) of the macrobicycle 7 (R" = R' = OCH_3).

M.p. > 250°C

^{1}H-NMR ($CDCl_3$): 4.02 (S, 18H, $COOCH_3$); 4.08 (S, 12H, CH_2); 7.96 (d, J = 1.2 Hz, 6H); 8.49 (d, J = 1.2 Hz, 6H); ^{13}C-NMR ($CDCl_3$): 53.2; 59.2; 120.0; 123.8; 139.9; 155.6; 159.9; 164.8.

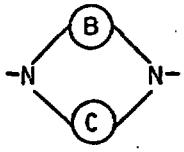
$C_{48}H_{42}N_8O_{12}.NaBr.3H_2O$ (1079.84)

% calculated: C 53.39; H 4.48; N 10.38

% found: C 53.40; H 4.40; N 9.75.

The cryptate obtained above can be subjected to aminolysis with ethylenediamine by the procedure described in the previous Examples. This generally gives a mixture of cryptates monosubstituted and disubstituted to hexasubstituted by the group $CO-NH-CH_2-CH_2-NH_2$. These compounds can be separated by conventional means.

EXAMPLE 7 - Preparation of the europium cryptate of [bis-(bpy dicarboxybutoxy), bpy-diamidoethylene-amine]

Formula I = Y = $-CH_2-CH_2-$; Z = NH_2;  is the bis-bipyridine macrocycle; R' = t-Bu (tert-butyl or Bu^t)

A - Preparation of the sodium cryptate of [bis(bpy dicarboxybutoxy), bpy dicarboxymethoxy]

The sodium cryptate of formula 7 (R' = COO(t-C_4H_9); R" = $COOCH_3$) was obtained by the procedure described in Example 6, using the compound 5 obtained in section B of said Example and compound 6 (R' = COO(t-C_4H_9); X = Br); it has the following physicochemical characteristics:

M.p. > 220°C

^{1}H-NMR ($CDCl_3$): 1.60 (S, 36H, $COOBu^t$); 3.99; 4.02; 4.06 (3S, 18H, CH_2-bpy diCOOMe + CH_2-bpy diCOOBu^t + $-COOCH_3$); 7.81 (S, 4H, bpy diCOOBu^t); 7.95 (S, 2H, bpy diCOOMe); 8.36 (S, 4H, bpy diCOOBu^t); 8.45 (S, 2H, bpy diCOOMe)

^{13}C-NMR ($CDCl_3$): 28.0; 53.1; 59.1; 83.4; 119.8; 119.9; 123.4; 123.7; 139.8; 141.7.

$C_{60}H_{66}N_8O_{12}.NaBr.3H_2O$ (1248.18)

% calculated: C 57.73; H 5.81; N 8.98

% found: C 57.99; H 5.31; N 9.03

This sodium cryptate was then converted to the corresponding europium cryptate by the procedure described above.

This was then subjected to aminolysis with ethylenediamine.

In a variant, it is possible first to subject the above compound 7 to aminolysis and then to convert the resulting sodium cryptate to the corresponding europium cryptate by the procedures described above.

APPLICATION OF THE COMPOUNDS AS FLUORESCENT TRACERS

As indicated earlier, the compounds of the invention are suitable as agents for labeling biological substances; to do this, the compound of the invention is coupled by covalent bonding with the biological substance to be assayed or detected. This coupling can be effected by the conventional coupling methods used in this field, for example the SMCC/SPDP or carbodiimide methods (see the book Laboratory Techniques in Biochemistry and Molecular Biology - Practise and Theory on Enzym- and Immunoassays, P. TIJSSEN, Elsevier, 1st ed. 1985).

The cryptates according to the invention are suitable as fluorescent tracers in all types of methods for the determination or detection of biological substances, and especially in the so-called methods of assay by competition or by excess, in the homogeneous phase or in the heterogeneous phase, which are well known to those skilled in the art and are described in particular by LANDON, Ann. Clin. Biochem. 1981, 18, p. 253, and SOINI, Clin. Chemi. 25, 353, 1979. They can also be used in the homogeneous method of detection and/or determination by luminescence which is described in international patent application WO 87/00927.

The biological substances to be detected or determined can be, for example, antibodies, antigens, toxins, enzymes, proteins, hormones, hormone receptors, steroids, avidin, biotin, streptavidin, microorganisms, haptens, nucleic acids, DNA and RNA fragments, lipids, carbohydrates, etc. Examples of such substances are cited in the description of European patent application 17 908, which is incorporated in the present description by way of reference.

The use of the compounds of the invention as fluorescent tracers is illustrated by the five experiments below:

The reagents SPDP and sulfo-SMCC were used in experiments A, D and E below, said reagents being respectively: "N-succinimidyl 3-(2-pyridylthio)propionate" and "sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate".

EXPERIMENT A: Formation of an antibody-cryptate conjugate by SMCC/SPDP

1) Activation of the europium cryptate obtained according to Example 3

50 µl of sulfo-SMCC at 13.1 mg/ml in a 20 mM phosphate buffer of pH 7 are added to 500 µl of a solution of this cryptate at 1.5 mg/ml in the same buffer.

The mixture is then incubated for 30 min at room temperature and agitated on a Vortex for 5 min. The solution is centrifuged to remove the precipitate. It is purified by passage over a Pharmacia Mono Q ion exchange column, forming a gradient between:

Buffer A: phosphate 20 mM pH 7 + 10% DMF

Buffer B: phosphate 20 mM pH 7 + 10% DMF + 1 molar NaCl

The cryptate is detected by measurement of its absorption at 307 nm (ε = 25,000 $M^{-1}cm^{-1}$ at 307 nm).

- the activated cryptate emerges in the dead volume;

- the excess sulfo-SMCC is eluted in the gradient.

2) Activation of the antibody

The monoclonal antibody E_1 used is the anti-prolactin antibody contained in the prolactin radio-immunometric assay kits manufactured by the company ORIS INDUSTRIE and known under the name "ELSA PROL".

30 µl of a 30 mM solution of SPDP in absolute ethanol are added to 1.2 ml of a solution of antibody E_1 at 14.8 mg/ml in a 50 mM phosphate buffer, containing 150 mM NaCl, of pH 7.1.

The mixture is incubated for 30 min at room temperature, with agitation.

The solution is passed over a Pharmacia G25 gel column equilibrated with the same phosphate buffer (ε = 210,000 $M^{-1}cm^{-1}$ at 280 nm).

The fraction eluted in the dead volume is recovered.

60 µl of a 400 mM solution of DTT (dithiothreitol in the phosphate buffer are added.

The mixture is incubated for 15 min at room temperature, with agitation, and the fraction emerging in the dead volume after passage over a G25 column equilibrated in the phosphate buffer is recovered.

3) Coupling of the activated antibody with the activated cryptate

50 µl of cryptate at 200 µmol/l are added to 60 µl of activated antibody at 2.6 mg/ml in a phosphate buffer.

The mixture is incubated for 12 hours at room temperature and centrifuged. The supernatant is dialyzed against a 50 mM phosphate buffer of pH 7.4.

EXPERIMENT B: Formation of an antibody-cryptate conjugate by carbodiimide

1) Succinilation of the antibody

83 µl of a solution containing 50 mg/ml of succinic anhydride in 1/3 DMSO 2/3 100 mM borate of pH 9 are added in three portions to 440 µl of antibody E_1 at 9.4 mg/ml in 100 mM borate of pH 9, immersed in melting ice.

The pH is kept at 9 with sodium hydroxide solution. The reaction is allowed to proceed for 30 min at 0°C.

2) Purification

This is carried out by high pressure chromatography on a TSK 3000 SW gel filtration column, the eluent being a 50 mM phosphate buffer of pH 7.4. Flow rate: 1 ml/min.

The succinilated antibody is recovered in 3 ml, which are concentrated to 370 µl.

3) Succinilated antibody/cryptate coupling

Solution 1: 25.5 mg of carbodiimide mixed with 1335 µl of 50 mM phosphate buffer of pH 7.4

Solution 2: 50 mg/ml of sulfo-N-hydroxysuccinimide in the same buffer (14.7 mg in 222.7 µl)

Solution 3: 0.96 mg/ml of cryptate according to Example 3 in the same buffer

The following are reacted for 1 hour at room temperature:

- 175 µl of solution of succinilated antibody
- 480 µl of solution 3
- 72.5 µl of solution 1
- 10 µl of solution 2

After exhaustive dialysis against 50 mM phosphate buffer of pH 7.4, the product is concentrated on an Amicon cone to give a conjugate at 0.86 mg/ml.

The product is frozen in the buffer with 1 mg/ml

of human serum albumin (HSA).

RESULTS: PROLACTIN ASSAY

The reagents of the ELSA-PROL prolactin assay kit are used.

The following are added to each tube containing the ELSA solid phase:

- 100 µl of standard
- 300 µl of E_1-cryptate conjugate obtained according to experiment A or B, in 50 mM phosphate buffer of pH 7.4 containing 1 mg/ml of HSA.

The mixture is incubated for 3 hours at 37°C.

The solid phase is washed with twice 3 ml of H_2O.

400 µl of buffer containing 5 mmol/l of sodium dodecylsulfate (SDS) are added.

300 µl of this solution are transferred to an ARCUS LKB apparatus for measurement of the fluorescence.

The results obtained are shown in Figure 1 attached. In this Figure, the intensity of the fluorescence has been plotted on the ordinate and the amount of prolactin in µU/ml on the abscissa.

These results show that the cryptates according to the invention are suitable as fluorescent tracers in an immunoassay.

Experiment C below illustrates the use of the cryptates according to the invention in the homogeneous method according to international patent application WO 87/00927.

EXPERIMENT C: Prolactin assay

I - Homogeneous assay of prolactin

A - Fixation of iodine to the antibody

The anti-prolactin monoclonal antibody G_2 is labeled with Wood's reagent [described in synthesis Anal. Biochem. 69, 339-349 (1975)].

2.93 mg of Wood's reagent are dissolved in 40 µl of 0.5 N sodium hydroxide solution.

400 µl of 200 mM carbonate buffer of pH 9.3 are added.

200 µl of antibody G_2 at 10 mg/ml in a 100 mM carbonate buffer of pH 9.3 are added to this solution and the mixture is incubated for 17 hours at 30°C.

It is purified on a Pharmacia PD10 column with a 100 mM carbonate buffer of pH 9.3.

The fractions eluted in the dead volume are collected; the conjugate is characterized by the following ratio of optical densities at 320 and 280 nm:

$$\frac{OD\ 320}{OD\ 280} \approx 1.4 \text{ to } 1.5$$

Its concentration is estimated on the assumption that 90% of the antibodies are recovered. The conjugate is divided up into aliquots and stored at -20°C.

B - Prolactin assay (curve A)

Prolactin standards at 0, 15, 30, 60 and 150 ng/ml are used. The assay is performed in EFLAB 12-well strips, catalog no. 96 02 107.

The following are added in succession:

- 100 µl of standard
- 100 µl of Wood's G_2 antibody at 5 µg/ml
- 100 µl of conjugate E_1 of experiment A at 0.375 µg/ml.

- The mixture is incubated for 15 minutes at room temperature.
- The results are read off on an ARCUS apparatus.
- A standard curve is drawn by plotting the ΔF value as a function of the value of the standards (Figure 2), where

$$\Delta F = \frac{\text{Fluorescence of the standard measured} - \text{Fluorescence of the standard 0}}{\text{Fluorescence of the standard 0}}$$

I - Prolactin assay by radioimmunometry (curve B)

The ELSA-PRL kit (ORIS company) is used.

- The following are added to the ELSA tube:
  - 300 µl of the radioactive tracer,
  - 100 µl of standard.
- After agitation, the mixture is incubated for 3 hours at 37°C.
- The tubes are washed twice with water.
- The tubes are counted on a counter.
- The B/T value is plotted for each standard (Figure 2), where

  B = radioactivity counted for the standard

  T = radioactivity counted for 300 µl of the radioactive tracer.

The results obtained (see Figure 2) show that the cryptates according to the invention are suitable as tracers in the homogeneous method according to international patent application WO 87/00927. It may also be noted that, by virtue of the cryptates according to the invention, this homogeneous method is particularly advantageous compared with the assay method using a radioactive element, which requires a much longer incubation time, washing steps and the handling of radioactive elements.

EXPERIMENT D - Formation of a DNA-cryptate conjugate by SMCC/SPDP

1/ Functionalization of DNA to form DNA-NH_2

A modified nucleotide, 5-amino(12)dUTP (product marketed by CALBIOCHEM), is introduced into a given DNA by nick translation using the procedure recommended by CALBIOCHEM. The aminated DNA is then purified by three successive washes on a "Centricon" filter (marketed by AMICON) with 10 mM phosphate buffer of pH 7.4.

2/ Coupling of the DNA-NH_2 with SPDP

55 µl of a solution containing 9 µg of DNA-NH_2

(measured by UV) in 0.1 M borate buffer of pH 9.0 are added to 5 µl of a 20 mM solution of SPDP. The reaction lasts 4 hours at 45°C. The unreacted SPDP is removed by three washes on a "Centricon" with a 50 mM phosphate buffer of pH 7.0.

3/ Deprotection of the DNA-SPDP and coupling with the cryptate

90 µl of a solution of 6 µg (estimated by optical density) of DNA-SPDP in 50 mM phosphate buffer of pH 7.0 are treated just before coupling with 10 µl of a 10 mM solution of DTT in the same buffer. Deprotection lasts 20 minutes at room temperature. The DTT, which inhibits the next coupling, is removed by three washes on a "Centricon" with the previous phosphate buffer. The solution of DNA-SH is concentrated on a "Centricon" to a final volume of 90 µl.

90 µl of a solution of DNA-SH are brought into contact with 50 µl of the europium cryptate of Example 3, activated with SMCC according to section 1 of experiment A (14 mM), in 20 mM phosphate buffer of pH 7.0 containing 10% of DMF. Coupling takes place at 30°C over 1 hour. The DNA-cryptate conjugate is purified by three successive precipitations with ethanol. The degree of coupling is evaluated by measurement of the absorption at 260 nm and the fluorescence at 620 nm.

4/ A DNA probe coupled with a cryptate in this way can be used for the detection and assay of a complementary nucleic acid sequence in accordance with one of the strategies described in the review by J. MATTHEWS et al. in Analytical Biochemistry 169, 1-25 (1988), entitled "Analytical strategies for the use of DNA probes".

EXPERIMENT E - Formation of a streptavidin-cryptate covalent conjugate

1/ Streptavidin-SPDP coupling

20 µl of a 20 mM solution of SPDP in a 0.5 M

carbonate buffer of pH 9.5 are added to 180 µl of a solution containing 1 mg of streptavidin in the same buffer.

The reaction lasts one hour at room temperature. The excess SPDP is removed by four successive washes on a "Centricon" with 0.1 M phosphate buffer of pH 7.4.

2/ Deprotection of the streptavidin-SPDP and coupling with the cryptate

40 µl of 100 mM DTT in 0.1 M phosphate buffer of pH 7.4 are added to 360 µl of a solution of streptavidin-SPDP (≈ 1 mg) in the same buffer.

The reaction lasts 15 minutes at room temperature, with agitation, and the reaction mixture is then washed three times on a "Centricon" with the previous buffer.

The streptavidin-SH (≈ 1 mg), in 625 µl of 0.1 M phosphate buffer of pH 7.4, is coupled with 43 µg of the europium cryptate of Example 3, activated with SMCC according to section 1 of experiment A, in 175 µl of 20 mM phosphate buffer of pH 7 containing 10% of DMF. The coupling lasts 2 h 30 min at room temperature. The unreacted cryptate-SMCC is removed by four washes on a "Centricon" with 0.1 M phosphate buffer of pH 7.4.

The degree of coupling is estimated by measurement of the absorption at 280 nm and the fluorescence at 620 nm.

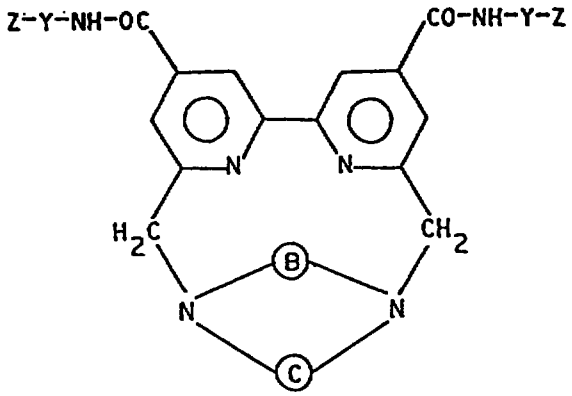
The conjugate obtained is purified by gel filtration on a TSKG3000 SW column with a 1 M NaCl buffer containing 0.1% of TFA. Detection is effected by measurement of the absorption at 280 nm and the fluorescence at 620 nm.

3/ This type of streptavidin-cryptate conjugate can be used as a fluorescent tracer for biotinylated molecules (nucleic acids, oligonucleotides, antibodies, antigens, haptens or other biological substances) which themselves

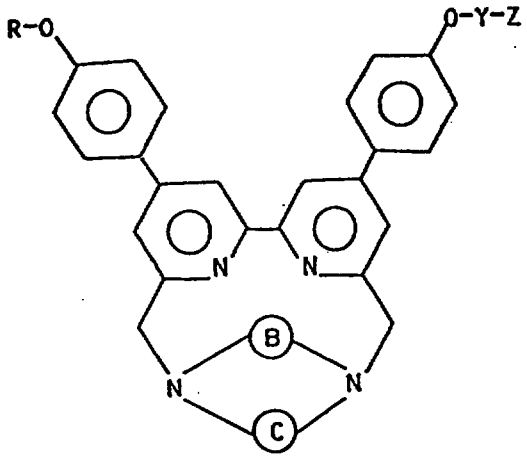
recognize biomolecules for which they have a great affinity in a mixture, in a conventional assay or detection method.

CLAIMS

1. Rare earth cryptates, characterized in that they consist of at least one rare earth salt complexed by a macropolycyclic compound having one of formulae I or II below:

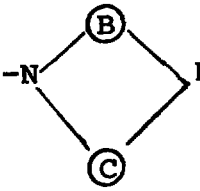


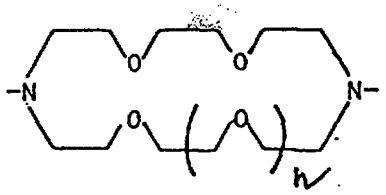
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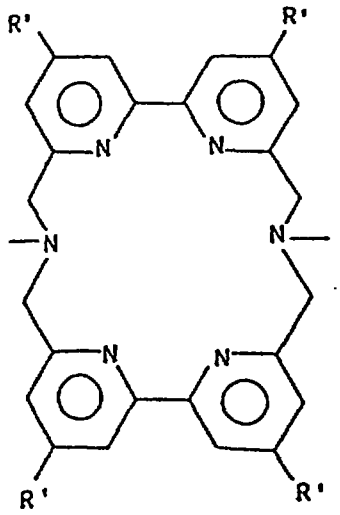
II

in which:

- the ring of the formula  is one of the following rings:

1) 

n = 0 or 1
[N_2O_4] macrocycle or (22) ring
[N_2O_3] macrocycle or (21) ring

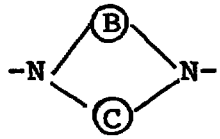
2) 

bis-bipyridine macrocycle

- Y is a spacer group or arm consisting of a divalent organic radical selected from linear or branched C_1 to C_{20} alkylene groups, which may or may not contain one or more double bonds and/or may or may not be interrupted by one or more heteroatoms such as oxygen, nitrogen, sulfur or phosphorus, from C_5 to C_8 cycloalkylene groups or from C_6 to C_{14} arylene groups, said alkylene, cycloalkylene or arylene groups being unsubstituted or substituted by alkyl, aryl or sulfonate groups;
- Z is a functional group capable of bonding covalently with a biological substance;
- R is a methyl group or represents the group -Y-Z; and
- R' is hydrogen or a group -COOR", in which R" is a C_1 to C_{10} alkyl group and preferably represents the methyl, ethyl or tert-butyl group, or R' is a group -CO-NH-Y-Z.

2. Cryptates according to claim 1 of formula I, characterized in that Y is $-CH_2-CH_2-$ and Z is $-NH_2-$.

3. Cryptates according to one of claims 1 or 2, characterized in that

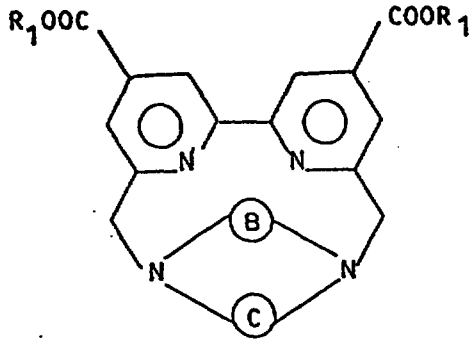


is the bis-bipyridine macrocycle.

4. Cryptates according to claim 1 of formula II, characterized in that Y is $-CH_2-$, Z is CN and R is the methyl group.

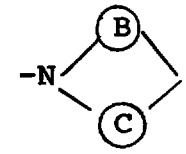
5. Cryptates according to claim 1 of formula II, characterized in that Y is $-CH_2-CH_2-$, Z is NH and R is the methyl group.

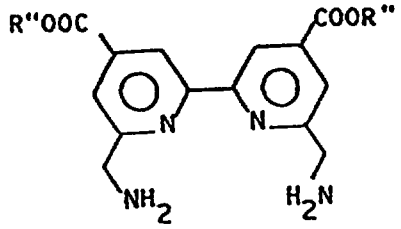
6. Method of obtaining the cryptates of formula I according to claim 1, characterized in that it consists in subjecting the compound of the formula:



3

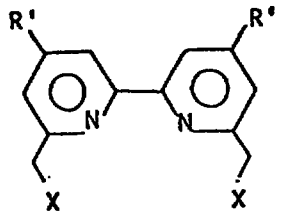
in which R_1 is a C_1-C_{10} alkylene group, to aminolysis by reaction with the amine of the formula NH_2-Y-Z, in which Y and Z are as defined in claim 1, the reaction being carried out under a nitrogen atmosphere at room temperature, and in converting the resulting compound to a rare earth cryptate by ion exchange.

7. Method according to claim 6, of obtaining the compounds of formula I in which  is the bis-bipyridine macrocycle, characterized in that the compound of formula 3 is obtained by reacting one molecule of



5

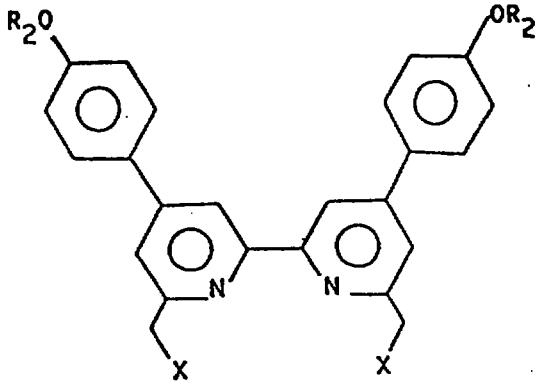
in which R" is as defined in claim 1, with two molecules of the compound of the formula



6

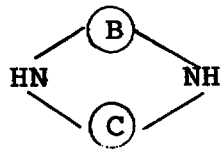
in which R' is as defined in claim 1 and X is a halide ion, the reaction being carried out in an anhydrous organic solvent at the reflux temperature of the solvent.

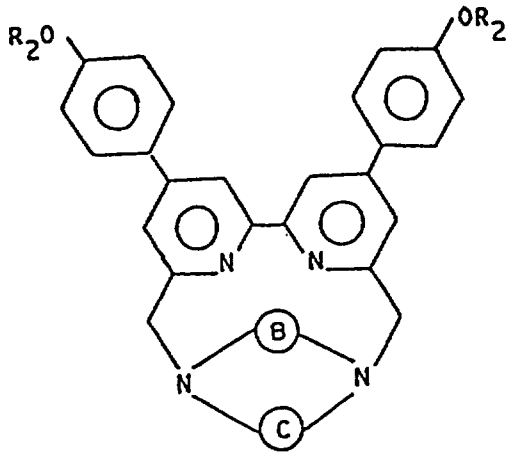
8. Method of obtaining the cryptates of formula II, according to claim 1, characterized in that it consists in reacting the halogenated compound 9:



R_2 = H or CH_3

9

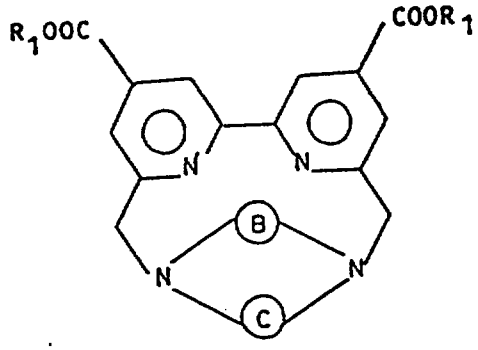
with the macrocycle  , in solution in an anhydrous organic solvent and at the reflux temperature of said solvent, to form the macropolycyclic complex 10:



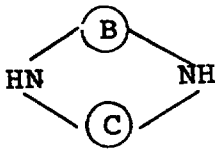
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which is then treated with the halogenated compound of the formula XYZ, in which Y and Z are as defined in claim 1 and X is a halide ion.

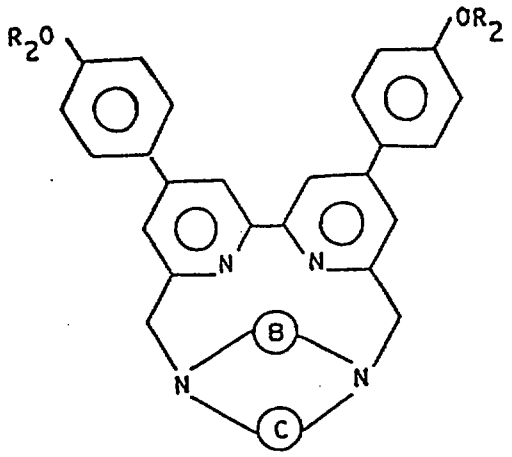
9. As synthesis intermediates, the macropolycyclic complexes of the formula



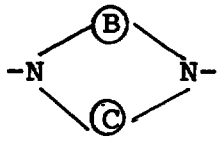
3

in which R_1 and  are as defined in claims 1 and 6 respectively.

10. As synthesis intermediates, the compounds of the formula



10

in which  is as defined in claim 1 and R_2 is H or CH_3.

11. Intermediates according to one of claims 9 or 10, characterized in that they are in the form of free cryptants.

12. Intermediates according to one of claims 9 or 10, characterized in that they are in the form of alkali metal ion cryptates or rare earth cryptate.

13. Application of the cryptates according to any one of claims 1 to 5 as fluorescent tracers.

14. Application of the cryptates according to any one of claims 1 to 5 as fluorescent tracers in methods

* C L M S *

for the detection and/or determination of biological substances, and especially in homogeneous luminescence methods.