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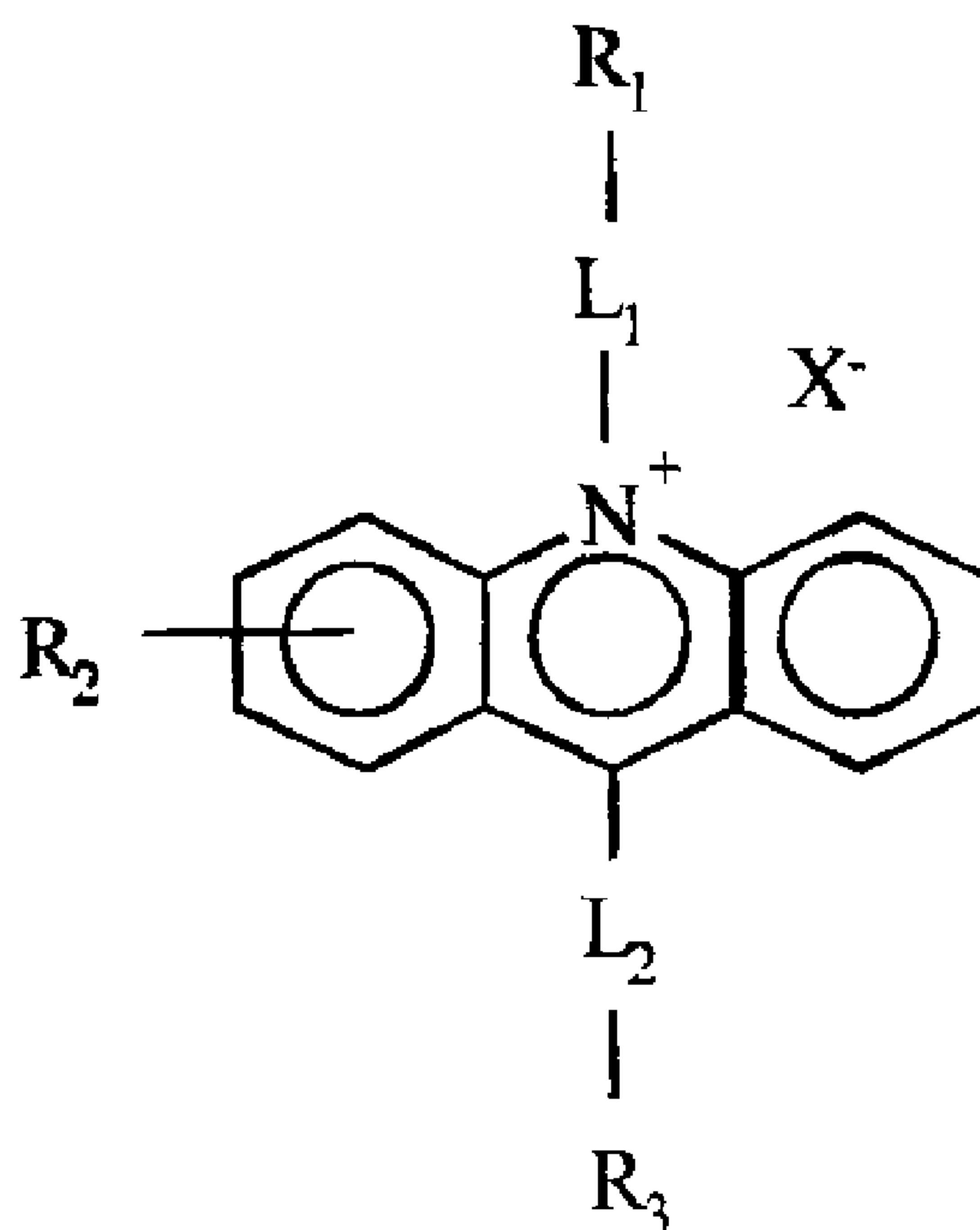
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(54) Title: CHEMILUMINESCENT COMPOUNDS



(57) Abrégé/Abstract:

The invention relates to chemiluminescent compounds of general formula: (I) wherein: either: R_1 is a reactive group capable of reacting with an amine or thiol moiety; L_1 is a hydrocarbon linker moiety comprising 2 - 12 carbon atoms, optionally substituted with

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hydroxy, halo, nitro or C₁-C₄ alkoxy; and R₂ is hydrogen, C₁-C₄ alkyl, C₁-C₄ haloalkyl, aryl, fused aryl, C₁-C₄ alkoxy, C₁-C₄ acyl, halide, hydroxy or nitro; or, alternatively: the combination R₁-L₁- comprises a C₁-C₄ alkyl group optionally substituted with hydroxy, halo, nitro or C₁-C₄ alkoxy; and R₂ comprises a group R₄-L₁-, where R₄ is a reactive group capable of reacting with an amine or thiol moiety; and L₁ is as defined above; L₂ is -C(=O)O-, -C(=O)-S- or -C(=O)N(SO₂R₅)-, wherein, in each case, the -C(=O) is linked to the ring carbon atom, and R₅ is C₁-C₈ alkyl, aryl, C₁-C₈ alkoxy or C₁-C₈ acyl; R₃ a substituted C₁-C₈ alkyl, C₂-C₈ alkenyl, C₁-C₈ alkynyl or aryl group wherein at least one of the said substituents is electron-withdrawing such that the pKa of the conjugate acid of the leaving group formed from R₃ and the -O-, -S- or -N(SO₂R₅) of the L₂ group is <= about 9.5; and X⁻ is an anion formed as the result of the synthesis and processing of the molecule; wherein the compound may contain one or more additional R₂ moieties on either or both outer rings, provided that only one of said R₂ moieties may comprise an R₄-L₁-group.

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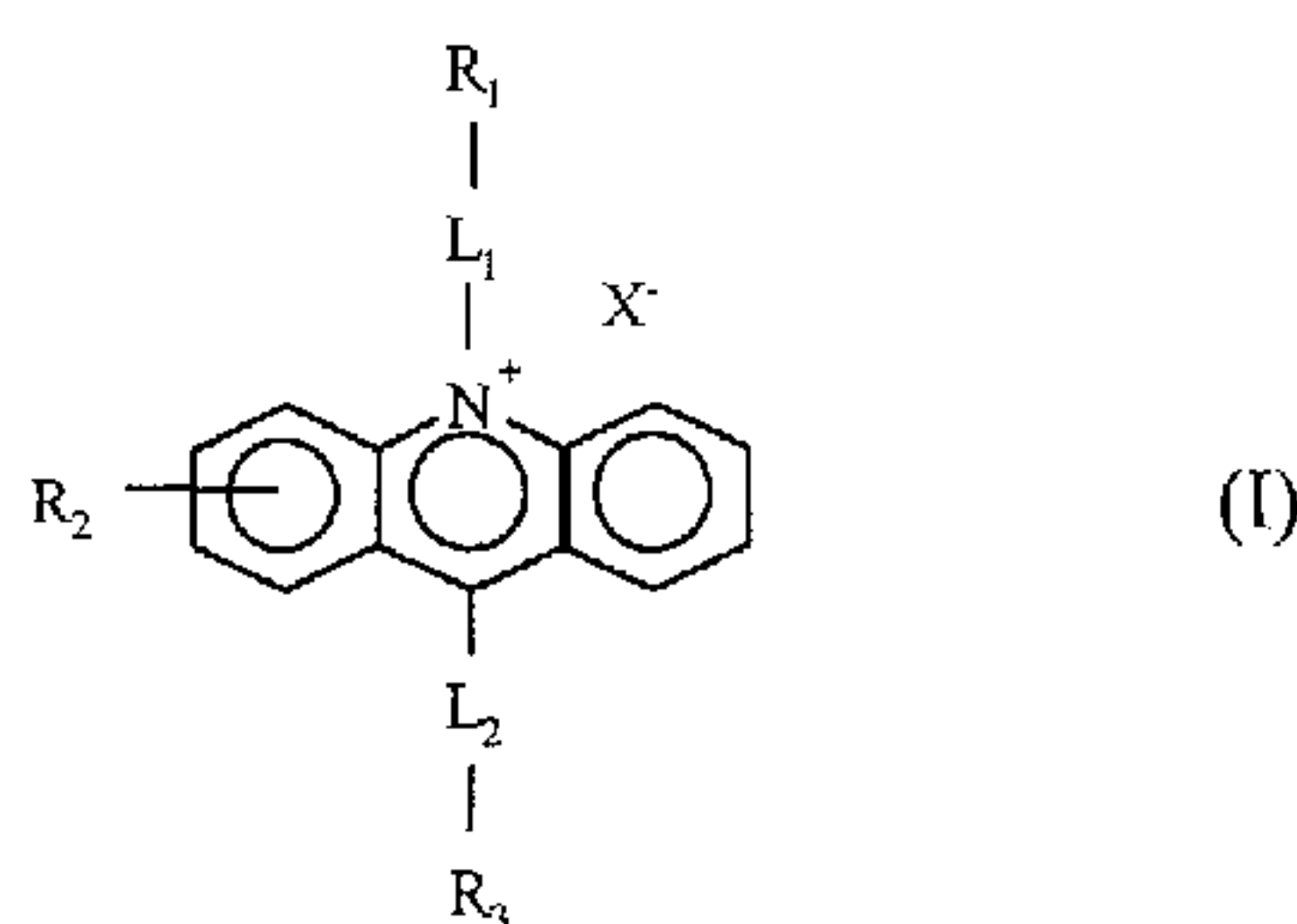
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(54) Title: CHEMILUMINESCENT COMPOUNDS



(I)

(57) Abstract: The invention relates to chemiluminescent compounds of general formula: (I) wherein: either: R₁ is a reactive group capable of reacting with an amine or thiol moiety; L₁ is a hydrocarbon linker moiety comprising 2 - 12 carbon atoms, optionally substituted with hydroxy, halo, nitro or C₁-C₄ alkoxy; and R₂ is hydrogen, C₁-C₄ alkyl, C₁-C₄ haloalkyl, aryl, fused aryl, C₁-C₄ alkoxy, C₁-C₄ acyl, halide, hydroxy or nitro; or, alternatively: the combination R₁-L₁- comprises a C₁-C₄ alkyl group optionally substituted with hydroxy, halo, nitro or C₁-C₄ alkoxy; and R₂ comprises a group R₄-L₁-, where R₄ is a reactive group capable of reacting with an amine or thiol moiety; and L₁ is as defined above; L₂ is -C(=O)O-, -C(=O)-S- or -C(=O)N(SO₂R₅)-, wherein, in each case, the -C(=O) is linked to the ring carbon atom, and R₅ is C₁-C₈ alkyl, aryl, C₁-C₈ alkoxy or C₁-C₈ acyl; R₃ a substituted C₁-C₈ alkenyl, C₁-C₈ alkynyl or aryl group wherein at least one of the said substituents is electron-withdrawing such that the pK_a of the conjugate acid of the leaving group formed from R₃ and the -O, -S or -N(SO₂R₅) of the L₂ group is ≤ about 9.5; and X⁻ is an anion formed as the result of the synthesis and processing of the molecule; wherein the compound may contain one or more additional R₂ moieties on either or both outer rings, provided that only one of said R₂ moieties may comprise an R₄-L₁-group.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

CHEMILUMINESCENT COMPOUNDS

The present invention relates to compounds which are of use as chemiluminescent labels in a variety of ligand binding assays, to assay methods employing these compounds and to kits including them. In particular, the invention relates to certain acridinium derivatives.

Chemiluminescence is the phenomenon whereby a chemical reaction leads to the emission of light. This occurs because the reaction product is formed in an electronically excited state and it therefore emits electromagnetic radiation (often in the visible spectrum) in order to return to its ground state.

The use of chemiluminescence energy transfer systems for the labelling of assay reagents has been described previously. In such labelling systems, there is provided a light emitting donor-acceptor pair the relative distance between which is dependent on the presence or absence of a ligand-binding event. For example, a ligand is coupled to a chemiluminescent emitter and the corresponding anti-ligand is coupled to an energy acceptor such that when the ligand and anti-ligand are present as a bound complex, the chemiluminescent emission is modulated by the close proximity of the acceptor. Such modulation is manifest either as a loss of chemiluminescence intensity overall or as a change in wavelength of the emission depending on the nature of the acceptor molecule.

Chemiluminescent acridinium salts have been widely used as labelling molecules in ligand binding assays such as immunoassays and nucleic acid hybridisation assays. Such assays involve coupling these molecules to the ligand or anti-ligand either directly by covalent bonds or by secondary ligand binding such as is exemplified by biotin-streptavidin binding where the labelling molecule is coupled to the streptavidin.

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The features required by molecules capable of acting as chemiluminescent acridinium labelling molecules are simply that 1) they are capable of coupling to (i.e.

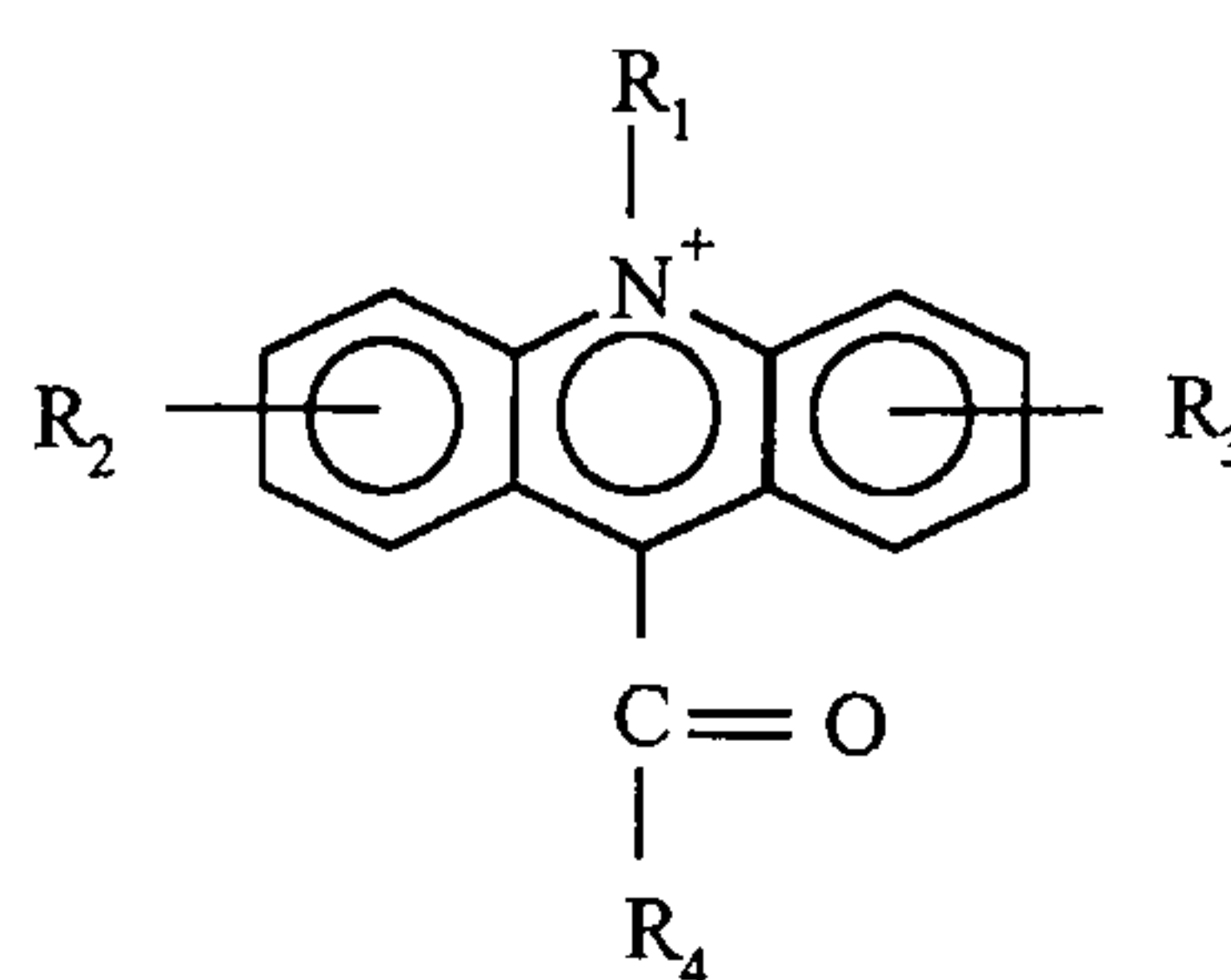
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labelling) the ligand without affecting the reactivity of the said ligand and 2) the labelling compound substantially retains its chemiluminescent activity. Any particular molecular structure that achieves these features is capable of acting as a labelling compound and, indeed, many such structures have been described.

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For example, EP-A-0082636 relates to acridinium compounds as chemiluminescent labels of the formula

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15 in which R₁ represents H, C₁-C₁₀ optionally substituted alkyl, alkenyl alkynyl or aryl; R₂ and R₃ are hydrogen, amino, substituted amino, carboxyl, hydroxyl alkoxy, nitro or halo; and R₄ is an optionally substituted phenoxy moiety.

20 Acridinium compounds of this type emit chemiluminescent radiation on treatment with basic hydrogen peroxide which oxidises the acridinium system and leads *via* a dioxetanone intermediate to the formation of a ketone group at the C-9 position. This product is formed in an excited state which subsequently relaxes to its corresponding ground state with emission of electromagnetic radiation.

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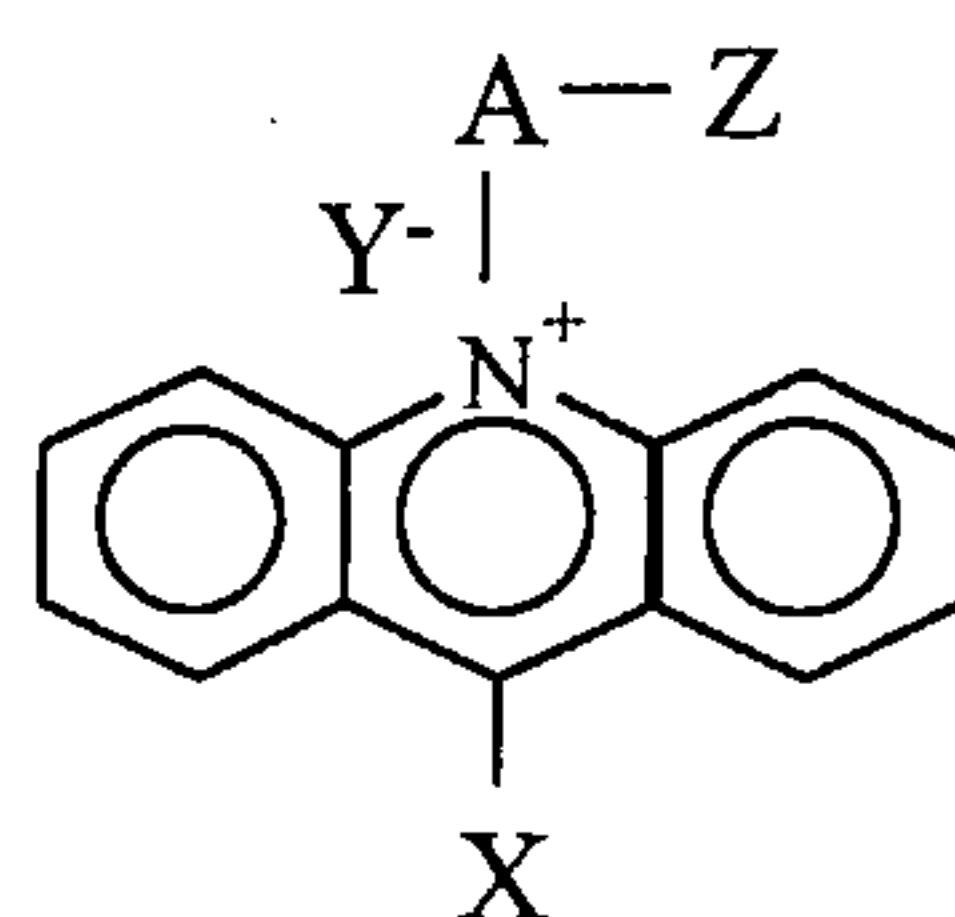
The compounds described in EP-A-0082636, however, bind to the ligand *via* a substituent of the phenoxy moiety R₄ and therefore after treatment with hydrogen peroxide, the radiation emitting acridone ring system is no longer bound to the ligand. This may have certain advantages in that the chemiluminescent radiation cannot be quenched non-specifically by the ligand but, on the other hand, the use of this type of compound may not be optimal in a chemiluminescence energy transfer system where specific quenching is required.

30

This potential problem was discussed by Patel and Campbell in *Clin. Chem.*, **29(9)**, 1604-1608 (1983). This document relates to a chemiluminescence energy transfer assay using the chemiluminescent compound aminobutylethyl-isoluminol and its isothiocyanate derivative. The authors state that attempts to improve the magnitude of the energy transfer using different chemiluminescent donors have failed. In particular they state "Nor has any energy transfer yet been detected between acridinium-labelled IgG...". They speculate that this could be because during the chemiluminescent reaction the excited N-methyl acridone is cleaved from the antigen. The authors also point out that the acridinium excitation reaction is best catalysed at high pH, which would disrupt the antibody-antigen complex.

EP-A-0324202 relates to chemiluminescent acridinium compounds of the formula:

15



20 in which A is a divalent organic moiety;

X is a substituent which can form a dioxetane together with the C-9 acridinium atom and hydrogen peroxide;

Y is a counter ion; and

Z is a functional group;

25 and wherein the benzene rings may carry one or more substituents, such as lower alkyl, lower alkoxy, halogen etc.

The difference between these compounds and those of EP-A-0082636 is said to be that they bind to a ligand *via* the group Z. Therefore, following the reaction with hydrogen peroxide, the chemiluminescent emitter remains attached to the ligand.

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However, the present inventors have come to realise that the problems associated with this technology are more complex than previously appreciated and that contrary to the teaching of EP-A-0324202, most of the compounds to which it relates are not suitable for use in chemiluminescence energy transfer systems.

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Indeed, such have been the problems with chemiluminescence energy transfer systems that, while energy transfer systems using fluorescence emitting labels are widely used, their chemiluminescent counterparts have not found commercial application. This is in spite of the fact that chemiluminescence can be detected with
10 far greater sensitivity than conventional fluorescence thus making it far more desirable as an analytical end-point.

One reason for the success of fluorescent systems and the lack of success of their chemiluminescent counterparts in resonance energy transfer systems is that initiation
15 of fluorescence is a physical, non-invasive process whereas initiation of chemiluminescence requires a change in chemical conditions which may both alter the optical properties of the acceptor and disrupt the very ligand/anti-ligand interaction which it is designed to monitor.

20 Although the acridinium compounds described in EP-A-0324202 are purported to be useful in energy transfer assays, the present inventors have come to realise that most of these compounds are not capable of permitting the chemiluminescent reaction to be initiated whilst maintaining the integrity of the binding complex being evaluated. This is certainly true of the sole example of an acridinium ester labelling compound,
25 in which the group X is a phenoxycarbonyl group (see Examples 21 and 25).

The problem arises because most of the compounds of EP-A-0324202, including the only acridinium ester compound exemplified, do not yield useful chemiluminescent emission except under the extreme alkaline conditions set forth in the only example,
30 in which chemiluminescence is initiated at $\text{pH} \geq 12$. However, most binding complexes are dissociated at the extremes of alkaline pH required to initiate the chemiluminescent reaction of the labelling molecules. This means that the use of

many of the acridinium compounds of EP-A-0324202 as chemiluminescent labels is very limited since they cannot workably be used in chemiluminescent resonance energy transfer systems which universally require the integrity of the binding complex to be maintained at the time of signal measurement. This was clearly not appreciated by the authors of EP-A-0324202 since the document does not mention this consideration. There is therefore no way of establishing from the teaching of EP-A-0324202 which compounds would in fact be workable as purported.

Another problem of the high pH values needed with prior art compounds is that the absorption spectrum of many of the commonly used quencher molecules is pH dependent and the elevated pH required for initiation of chemiluminescence from conventional acridinium labels renders such quenchers useless. Again, this problem was not appreciated by the authors of EP-A-0324202 and, indeed the document does not suggest any quenchers which could be used in combination with the compounds it describes.

Acridinium compounds have been described (EP-A-0682254) which are capable of undergoing chemiluminescent reactions at pH values lower than 12-14 but these compounds are used as enzyme substrates and not labelling compounds for linking to ligands. EP-A-0682254 teaches that suitable molecules can be prepared using the methods set out in documents such as EP-A-0324202 but specifically states that reactive groups required for the covalent coupling to substances of biological interest are not a feature of the compounds alluded to therein. Therefore, these previously described compounds are structurally completely distinct from the compounds of the present invention and they would not be suitable for use as labelling compounds. It is not possible to predict the effect on the pH for chemiluminescent initiation of adapting the molecules of EP-A-0682254 to include a coupling group and the coupling of such an adapted molecule to a substance of biological interest.

A further problem with chemiluminescence energy transfer reactions is that, in addition to the chemiluminescent labelling molecule, they require a suitable quencher which has an absorption spectrum which overlaps at least partially, but preferably

completely, with the emission spectrum of the labelling molecule. This might not seem to be a problem given that large numbers of quenchers are known but, in practice, it is no simple matter because the spectral range of both the labelling molecule and the quencher may vary considerably with the pH of their environment.

Therefore, a chemiluminescent transfer system requires a chemiluminescent labelling molecule which emits chemiluminescent radiation at a pH which is sufficiently low to avoid dissociation of a binding complex and which also emits chemiluminescent radiation at that pH having a spectrum which overlaps with that of a suitable quencher.

Thus there is a need for molecules which can be used to combine the exquisite sensitivity of detection of chemiluminescent molecules with the convenience of resonance energy transfer ligand binding assays.

Various embodiments of this invention provide an oligonucleotide probe comprising up to 40 nucleic acid bases having ends which are mutually complementary and a central section containing a 15 to 30 nucleic acid base sequence which is complementary to a target nucleic acid sequence, wherein the oligonucleotide probe is labelled at one end with a chemiluminescent acridinium ester labelling reagent of formula (I) and at the other end with a quencher such that attenuation of chemiluminescent light emission exists when a duplex of the complementary ends is intact. Attenuation may not exist when the duplex of complementary ends is rendered substantially non-intact by chemical or physical means such as when the duplex of complementary ends is disrupted by hybridisation of said central section of the oligonucleotide probe to said target sequence.

Various embodiments of this invention provide a method of detecting a target nucleic acid in a sample, the method comprising adding a probe of this invention to the sample wherein the probe comprises said central section that is complementary to said target nucleic acid, adding hydrogen peroxide to the medium while maintaining the pH at 6-10 and detecting emission of chemiluminescent radiation.

6a

Various embodiments of this invention provide a kit comprising a probe of this invention, hydrogen peroxide solution and a buffer adapted to maintain the pH of an analyte containing medium at pH 6-10.

Various embodiments of this invention provide the compound:

9-(2,6-Bis(trifluoromethyl)phenoxy carbonyl)-10-(10-succinimidyloxycarbonyldecyl)acridinium trifluoromethanesulfonate;
 9-(2,6-dibromophenoxy carbonyl)-10-(10-succinimidyloxycarbonyldecyl)acridinium trifluoromethanesulfonate;
 9-(2,6-bis(trifluoromethyl)phenoxy carbonyl)-10-(3-succinimidyloxycarbonylpropyl)acridinium trifluoromethanesulfonate;
 9-(2,6-dibromophenoxy carbonyl)-10-(3-succinimidyloxycarbonylpropyl)acridinium trifluoromethanesulfonate;
 9-(2,6-dinitrophenoxy carbonyl)-10-(10-succinimidyloxycarbonyldecyl)acridinium trifluoromethanesulfonate; or
 9-(2,6-dinitrophenoxy carbonyl)-10-(3-succinimidyloxycarbonylpropyl)acridinium trifluoromethanesulfonate.

Various embodiments of this invention provide a method of detecting or monitoring association or dissociation of first and second binding partners in a medium, wherein the first binding partner is labelled with a compound of this invention and the second binding partner is labelled with a quencher or is itself a quencher, such that when the first and second binding partners are associated, a chemiluminescent signal of the compound is modulated; the method comprising adding hydrogen peroxide to the medium while maintaining the pH at 6-10 and detecting emission of chemiluminescent radiation.

Various embodiments of this invention provide a method of detecting presence or absence in a medium of an analyte comprising a quencher for a compound of this invention, the method comprising:

6b

- a) adding to the medium a specific binding partner for the analyte, said specific binding partner labelled with the compound such that if analyte is present, a specific binding complex is formed;
- b) adding hydrogen peroxide to the medium while maintaining the pH at 6-10; and
- c) detecting emission of chemiluminescent radiation, wherein a modulated signal of the compound indicates the presence of the analyte.

Various embodiments of this invention provide a method of detecting presence or absence in a medium of an analyte which is not a quencher for a compound of this invention, the method comprising:

- a) adding to the medium a specific binding partner for the analyte, said specific binding partner labelled with a compound of this invention, or a quencher for a compound of this invention such that if analyte is present, a specific binding complex is formed;
- b) adding to the medium simultaneously or sequentially a secondary binding reagent labelled with either a quencher for the compound used in step (a) or the compound if a quencher is used in step (a); and
- c) detecting presence of the specific binding complex by a chemiluminescent method including the steps of adding hydrogen peroxide to the medium while maintaining the pH at 6-10 and detecting emission of chemiluminescent radiation, wherein a modulated signal of the compound indicates the presence of the analyte.

Various embodiments of this invention provide a method of detecting presence or absence in a medium of an analyte which is not a quencher for a compound of this invention, the method comprising:

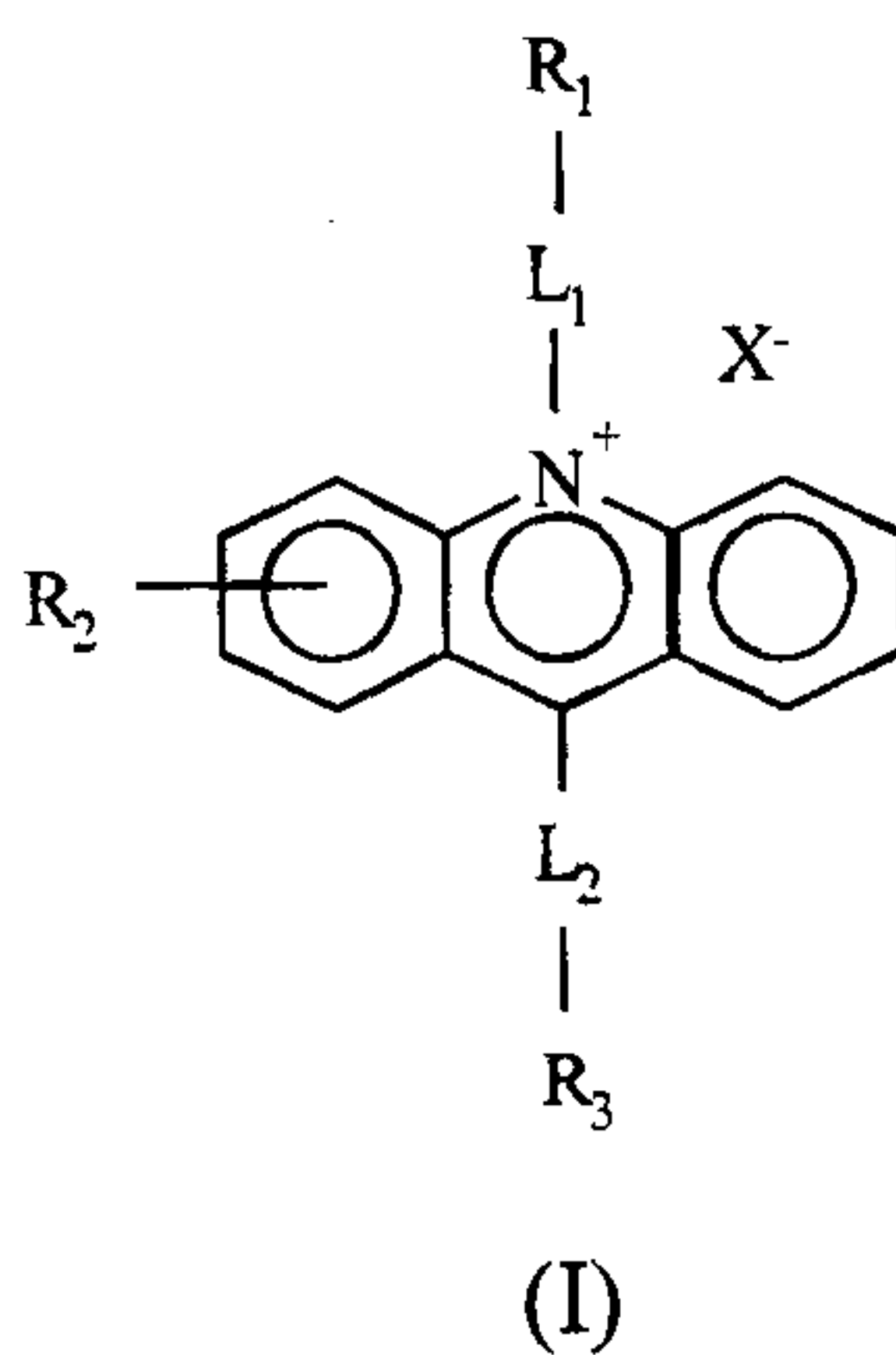
- a) adding to the medium a specific binding partner for the analyte labelled with the compound or with a quencher for the compound such that if analyte is present, a specific binding complex is formed;
- b) adding to the medium simultaneously or sequentially a second specific binding partner labelled with either a quencher or the compound to the first labelled specific binding partner; and

6c

- c) detecting the presence of specific binding complex by a chemiluminescent method including the steps of adding hydrogen peroxide to the medium while maintaining the pH at 6-10 and detecting emission of chemiluminescent radiation, wherein a modulated signal of the compound indicates the presence of the analyte.

Various embodiments of this invention provide a method for detecting presence of inhibitors or promoters of a binding process, the method comprising mixing a first and a second specific binding partner labelled respectively with a compound of this invention and a quencher, adding hydrogen peroxide to the medium while maintaining the pH at 6-10 and detecting emission of chemiluminescent radiation, wherein modulation of a chemiluminescent signal of the compound indicates promotion of the binding process and the presence of an unmodulated signal indicates inhibition of the binding process.

In a first aspect of the present invention there is provided a compound of general formula (I)



wherein:

either:

R_1 is a reactive group capable of reacting with an amine or thiol moiety;

L_1 is a hydrocarbon linker moiety comprising 2 - 12 carbon atoms, optionally substituted with hydroxy, halo, nitro or C_1 - C_4 alkoxy; and

R_2 is hydrogen, C_1 - C_4 alkyl, C_1 - C_4 haloalkyl, aryl, fused aryl, C_1 - C_4 alkoxy, C_1 - C_4 acyl, halide, hydroxy or nitro;

or, alternatively:

the combination R_1-L_1 - comprises a C_1-C_4 alkyl group optionally substituted with hydroxy, halo, nitro or C_1-C_4 alkoxy; and

5 R_2 comprises a group R_4-L_1 -, where R_4 is a reactive group capable of reacting with an amine or thiol moiety; and L_1 is as defined above;

L_2 is $-C(=O)O-$, $-C(=O)-S-$ or $-C(=O)N(SO_2R_5)-$,

wherein, in each case, the $-C(=O)$ is linked to the ring carbon atom, and

R_5 is C_1-C_8 alkyl, aryl, C_1-C_8 alkoxy or C_1-C_8 acyl;

10

R_3 is a substituted C_1-C_8 alkyl, C_2-C_8 alkenyl, C_2-C_8 alkynyl or aryl group wherein at least one of the said substituents is electron-withdrawing such that the pK_a of the conjugate acid of the leaving group formed from R_3 and the $-O$, $-S$ or $-N(SO_2R_5)$ of the L_2 group is $\leq$ about 9.5; and

15

X^- is an anion formed as the result of the synthesis and processing of the molecule; wherein the compound may contain one or more additional R_2 moieties on either or both outer rings, provided that only one of said R_2 moieties may comprise an R_4-L_1 -group.

20

The substituents on the R_3 group are chosen such that the pK_a of the conjugate acid of the leaving group formed by R_3 and the $-O$, $-S$ or $-N(SO_2R_5)$ of the L_2 group is $\leq$ about 9.5, which in practice means that at least one of the substituents on R_3 is electron withdrawing. This is a particularly important feature as it renders the molecule significantly chemiluminescent at pH 8 or less. This provides the compounds of the invention with an important advantage over the prior art compounds and, in particular the compounds exemplified in EP-A-0324202. The only ester compound exemplified in EP-A-0324202 (Examples 21 and 25) does not yield significant chemiluminescent emission at pH 8 and, indeed requires a pH of at least 12 for useable chemiluminescent emission.

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In contrast to prior art compounds, the chemiluminescence emission of the optimised acridinium labels of the present invention can be initiated at pH values compatible with commonly used quenchers and compatible with the stability requirements of ligand-binding complexes.

5

Thus, although EP-A-0324202 purports to relate to compounds which are useful as chemiluminescent labels, the problems outlined above are not identified. Furthermore, there is no example of the use of any compound in an energy-transfer assay and, indeed the general formula set out in the document encompasses
10 numerous compounds which would be completely unusable in such an assay. The document also contains no teaching as to which quenchers might be used in combination with the compounds. The only example of a chemiluminescent acridinium ester in EP-A-0324202 contains a phenoxy substituent at the position equivalent to R₃ of the present invention and is reacted at a pH in excess of 12 for
15 chemiluminescent emission, at which pH any binding complex would dissociate and many quenchers would be ineffective.

EP-A-0324202 describes intermediate ester compounds in Examples 1 to 8. Thus, numerous compounds are encompassed by EP-A-0324202 and yet the authors of that
20 document show no appreciation of the importance of the structure of the compound for its purpose in energy transfer assays. Unsubstituted compounds, electron withdrawing compounds and electron donating compounds are given equal weight when, in fact, the present inventors have found that only electron withdrawing compounds are able to be used in energy transfer assays. The only measurement
25 conditions described in EP-A-0324202 are pH $\geq$ 12, which results in dissociation of ligand binding complexes making energy transfer impossible. There is no teaching, or even appreciation, in the document as to which type of substituent is workable and the only acridinium ester precursor to be converted to a chemiluminescent final product is the compound of Example 1, which is an unsubstituted phenyl ester.
30 Example 25 demonstrates that this compound is chemiluminescent when treated with basic hydrogen peroxide but there is no example of the linkage of a chemiluminescent compound to a ligand or of a chemiluminescence energy transfer

assay. Indeed, as already mentioned, the particular compound exemplified could not be used in this way. It is therefore completely impossible to ascertain from this prior art document whether a particular compound would be useful in a chemiluminescence energy transfer assay. In some embodiments of the present invention, however, compounds in which R₃ is 4-chlorophenyl, 4-methoxyphenyl, 4-acetylphenyl or 1,1,1,3,3,3-hexafluoro-2-propyl may, in some circumstances be excluded from the definition of general formula (I).

In the context of the present invention, the term “ligand” refers to any moiety which is capable of binding specifically to a second moiety, referred to herein as an “anti-ligand” to form a complex. Examples of ligands and anti-ligands include antibodies and antigens or antigenic determinants; nucleic acid sequences; enzymes and enzyme substrates; and receptors and receptor-binding molecules.

“Labelling molecule” refers to a molecule which can be chemically bound to a ligand or anti-ligand and which emits a detectable signal enabling the ligand or anti-ligand to be detected. The labelling molecules of the present invention are chemiluminescent labelling molecules, which are capable of emitting chemiluminescent radiation on initiation of a suitable chemical reaction.

“Quencher” refers to a molecule which can be chemically bound to a ligand or anti-ligand and has an absorption spectrum which overlaps wholly or partially with the chemiluminescent or fluorescent emission spectrum of a labelling molecule such that when the quencher is brought into close proximity (typically less than 10nm) with the labelling molecule, the chemiluminescent or fluorescent emission spectrum is modulated.

“C₁-C₈ alkyl” refers to a straight or branched saturated hydrocarbon chain having up to eight carbon atoms. Examples include methyl, ethyl, propyl, isobutyl, t-butyl, n-hexyl and n-octyl. The term “C₁-C₄ alkyl” has a similar meaning but has from one to four carbon atoms.

“C₂-C₈ alkenyl” refers to a straight or branched hydrocarbon chain having from 2 to 8 carbon atoms and containing at least one carbon-carbon double bond. Examples include ethenyl, 2-propenyl and 3-hexenyl.

5 “C₂-C₈ alkynyl” refers to a straight or branched hydrocarbon chain having from 2 to 8 carbon atoms and containing at least one carbon-carbon triple bond. Examples include ethynyl, 2-propynyl and 3-hexynyl.

10 “Haloalkyl” refers to an alkyl group having one or more substituents selected from the group consisting of chloro, fluoro, bromo and iodo.

“Aryl” refers to a 5 to 10 membered single or fused aromatic ring system which may contain one or more heteroatoms, for example nitrogen, oxygen or sulphur. Examples include phenyl, pyridyl, furanyl and naphthalenyl.

15

“Halo” refers to fluoro, chloro, bromo or iodo and “halide” to fluoride, chloride, bromide and iodide.

20 It will be appreciated that the means of coupling labelling molecules to biologically important molecules are well-established and that there are many variations of R₁ and L₁ which will permit the present invention to be practised. However, it has been found that the favourable results are achieved when L₁ comprises 2 to 10 carbon atoms. More preferably, L₁ is a fully saturated chain consisting of methylene units.

25 R₁ and R₄ groups which have been found to be particularly useful in linking the compound to a biologically important molecule include active esters, such as succinimidyl esters and imidate esters, maleimides and active halides such as chlorocarbonyl, bromocarbonyl, iodocarbonyl, chlorosulphonyl and fluorodinitrophenyl.

30

Similarly, it is well-established that there are numerous substitutions that could be represented by R₂ which allow the chemiluminescent activity to be retained and

which can be used to alter the emission wavelength of the chemiluminescent label. This affects the choice of quencher used in an assay. The present inventors have found, however, that the compounds which are most useful include those in which R_2 is hydrogen or C_1 - C_4 alkyl, especially methyl or ethyl as these are suitable for use in combination with the acceptor methyl red. Compounds in which R_2 is hydrogen are particularly preferred.

In preferred compounds of the present invention, L_2 is $-C(=O)O-$.

As already mentioned above, R_3 must be chosen such that the pK_a of the conjugate acid of the leaving group formed by R_3 and the $-O$, $-S$ or $-N(SO_2R_5)$ is $\leq$ about 9.5 and this means that it contains at least one electron-withdrawing group.

However, if the R_3 moiety forms a leaving group which is too reactive, this will mean that the compound is not always sufficiently stable to be useful as a labelling compound. For optimum efficiency as a labelling compound for chemiluminescent transfer assays, it is therefore preferred that the pK_a of the conjugate acid of the leaving group formed by R_3 and the $-O$, $-S$ or $-N(SO_2R_5)$ of the L_2 group is ≥ 3 .

Therefore, in addition to electron withdrawing groups, the R_3 substituent can contain groups that are not electron withdrawing and may be even electron donating provided that the effect of the electron withdrawing group that is present predominates. The structures of R_3 are readily predictable by calculation of the pK_a values of the leaving groups resulting from the chemiluminescent reaction and therefore the scope of such groups can be appreciated by those skilled in the art.

However, by way of example, useful R_3 groups include alkyl or aryl groups substituted with one or more halides or alkyl halides. Particularly preferred compounds include those in which R_3 is a phenyl group substituted independently at the 2 and 6 positions with such groups and particularly with nitro, fluorine, chlorine, bromine or trifluoromethyl. Examples of such groups include 2,6-dibromophenyl, 2,6-bis(trifluoromethyl)phenyl and 2,6-dinitrophenyl.

X^- may be any one of a number of suitable anions; however, halide or halide-containing anions such as iodide, fluorosulfate, trifluoromethanesulfonate or trifluoroacetate are preferred.

5

Compounds where R_2 is hydrogen or lower alkyl are particularly appropriate for use with methyl red as the energy acceptor as these compounds have the particular advantage that their spectrum of chemiluminescence emission lies completely within the absorption spectrum of the quencher methyl red at pH 9. This means that a chemiluminescence energy transfer system in which one of the preferred compounds set out above is used as the emitter and methyl red is the acceptor can be used to make quantitative measurements of the extent and rate of a reaction between a ligand and an anti-ligand and therefore the presence of substances or events that affect the said reaction. This may be done simply by measuring the extent of the change in the chemiluminescent signal.

15

As already mentioned, it is no simple matter to select a quencher which is suitable for use with any particular chemiluminescent emitter because the spectral range of both emitter and quencher may vary with the pH of their environment and the spectral overlap of these compounds of formula (I) with methyl red is particularly desirable.

20

Particularly preferred compounds of the invention include:

25

9-(2,6-bis(trifluoromethyl)phenoxy carbonyl)-10-(10-succinimidyloxycarbonyl decyl)acridinium trifluoromethanesulfonate;

9-(2,6-dibromophenoxy carbonyl)-10-(10-succinimidyloxycarbonyldecyl)acridinium trifluoromethanesulfonate;

9-(2,6-bis(trifluoromethyl)phenoxy carbonyl)-10-(3-succinimidyloxycarbonyl propyl)acridinium trifluoromethanesulfonate; and

30

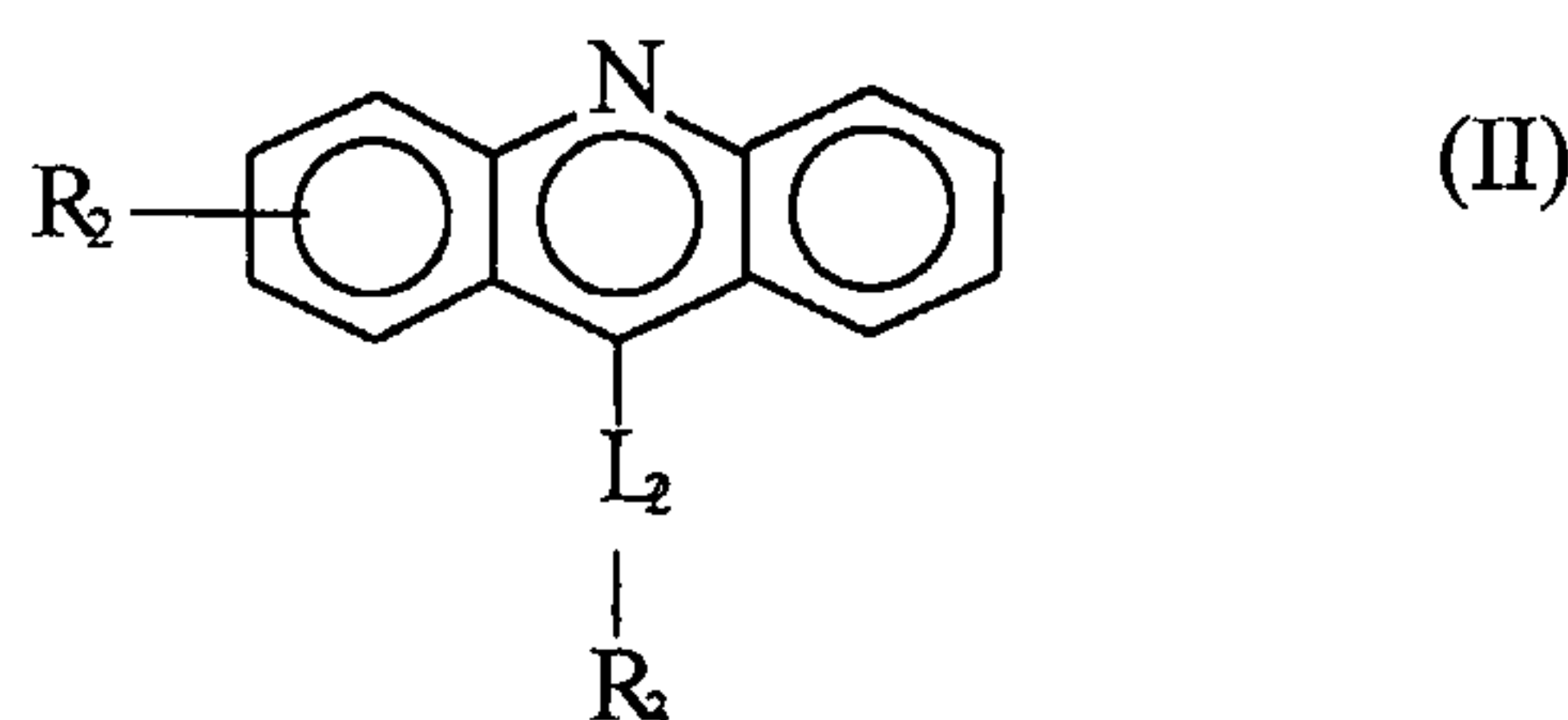
9-(2,6-dibromophenoxy carbonyl)-10-(3-succinimidyloxycarbonylpropyl)acridinium trifluoromethanesulfonate;

9-(2,6-dinitrophenoxycarbonyl)-10-(10-succinimidylloxycarbonyldecyl)acridinium trifluoromethanesulfonate; and

9-(2,6-dinitrophenoxycarbonyl)-10-(3-succinimidylloxycarbonylpropyl)acridinium trifluoromethanesulfonate.

5

Compounds of the present invention may be prepared by well known methods. For example a compound of general formula (I) may be prepared by treating a compound of general formula (II):



15 wherein R_2 , L_2 and R_3 are as defined in general formula (I) with a compound of general formula (III)



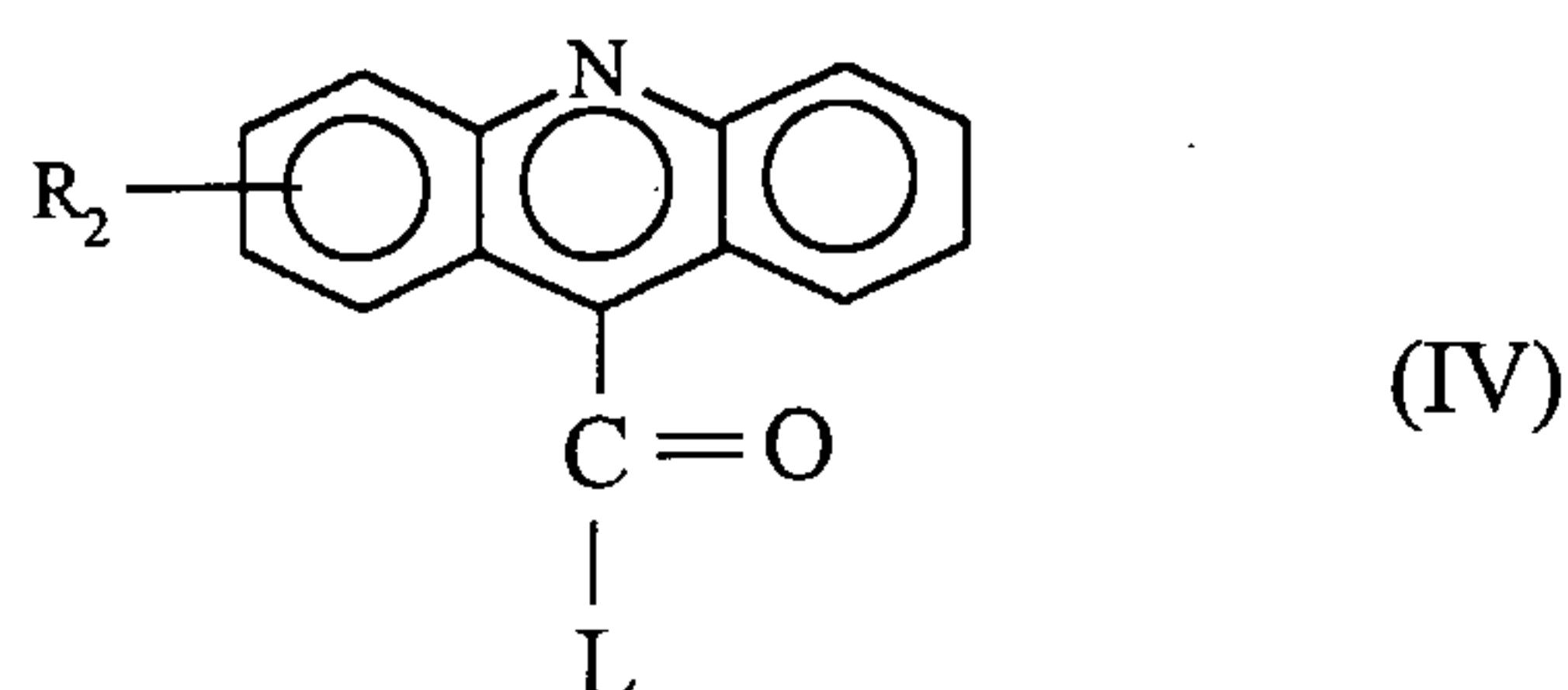
20 wherein L_1 , R_1 and X are as defined in general formula (I), with X preferably being a leaving group such as triflate or iodide.

Typically, the reaction will be carried out in a polar organic solvent such as nitrobenzene and at elevated temperature of about 80 to 150°C.

25 Compounds of general formula (III) are well known and are readily available or can be prepared from readily available materials by known methods. For example, when the R_1 moiety of general formula (III) is a succinimidyl ester, it can be prepared by the reaction of N-hydroxysuccinimide with the corresponding carboxylic acid.

30 Compounds of general formula (II) can be prepared by reacting a compound of general formula (IV)

14



wherein R_2 is as defined in general formula (I) and L is a leaving group;

with a compound of general formula (V):



wherein R_3 is as defined in general formula (I) and Z is O or S.

The reaction may be conducted in a polar organic solvent such as pyridine and preferably at a temperature of from 10 to 50°C, but typically at room temperature.

Compounds of general formula (IV) are well known in the art and can be prepared by standard methods from compounds which are readily available. For example, acridine-9-carbonyl chloride can be prepared from acridine-9-carboxylic acid. Compounds of formula (V) are also readily available.

As discussed above, compounds of formula (I) emit chemiluminescence at a much lower pH than the compounds known in the prior art and this makes them particularly useful in a number of analytical methods.

Compounds of general formula (I) are particularly useful in any analytical method which uses chemiluminescence quenching to monitor association or dissociation of molecular species. For example, the cleavage of an enzyme substrate can be monitored by incorporating a compound of general formula (I) and a quencher respectively at either side of the point of cleavage such that the presence of active

enzyme is determined by loss of quenching. Such methods are useful for identifying inhibitors or promoters of a given enzyme.

Similarly, chemiluminescent emitter/quencher pairs incorporating compounds of
5 general formula (I) are useful in studying non-covalent binding processes such as are
exemplified by immunoassay, protein binding, receptor binding, nucleic acid
hybridisation and the like. Accordingly, such methods can be used to identify and/or
quantify substances which affect such processes or are part of such processes. The
bases of such determinations are well established and can be used together with the
10 teaching herein disclosed to provide improved assay methods with the knowledge
that one binding partner is labelled with a compound of general formula (I) and a
further respective binding partner is labelled with a quencher or is itself a quencher.
In certain circumstances, a binding partner may incorporate both emitter and
quencher as exemplified below.

15

Therefore, in a further aspect of the invention there is provided a method of detecting
or monitoring the association or dissociation of a first and a second binding partner
in a medium, wherein the first binding partner is labelled with a compound of general
formula (I) and the second binding partner is labelled with a quencher or is itself a
20 quencher, such that when the first and second binding partners are associated, the
chemiluminescent signal of the compound of general formula (I) is modulated; the
method comprising adding hydrogen peroxide to the medium while maintaining the
pH at about 6-10 and detecting the emission of chemiluminescent radiation.

25 The first and second binding partners may be molecular species which associate or
dissociate *via* a covalent reaction which may be catalysed, for example by an
enzyme. Alternatively, the first and second binding partners may be a ligand and an
anti-ligand, for example complementary nucleic acids, an antibody and an antigen or
antigenic determinant or a receptor and receptor binding molecule.

30

The method described above may also be adapted for the detection of an analyte in a
medium. The analyte and the binding partners may be molecular species which

associate or dissociate *via* a covalent reaction which may be catalysed, for example by an enzyme. Alternatively, they may be ligands and anti-ligands, for example complementary nucleic acids, an antibody and an antigen or antigenic determinant or a receptor and receptor binding molecule.

5

There is also provided a method of detecting the presence or absence in a medium of an analyte comprising a quencher for a compound of general formula (I), the method comprising:

- 10 a) adding to the medium a specific binding partner for the analyte labelled with a compound of general formula (I) such that if analyte is present a specific binding complex is formed;
- b) adding hydrogen peroxide to the medium while maintaining the pH at about 6-10; and
- 15 c) detecting the emission of chemiluminescent radiation, wherein a modulated signal of the compound of general formula (I) indicates the presence of the analyte.

When the analyte is not a quencher for a compound of general formula (I), the invention provides a method for detecting an analyte in a medium, the method comprising:

- 20 a) adding to the medium a specific binding partner for the analyte labelled with a compound of general formula (I) or with a quencher for a compound of general formula (I) such that if analyte is present, a specific binding complex is formed;
- 25 b) adding to the medium simultaneously or sequentially a secondary binding reagent; and
- c) detecting the presence of specific binding complex by a chemiluminescent method including the steps of adding hydrogen peroxide to the medium while
- 30 maintaining the pH at about 6-10 and detecting the emission of chemiluminescent radiation, wherein a modulated signal of the compound of general formula (I) indicates the presence of the analyte.

In the method described above, the presence of analyte bound to the said first specific binding partner may be determined and/or quantified by either a competitive-binding assay or a "sandwich" assay. For the former assay, the secondary binding reagent is an analyte analogue labelled with either a quencher or a compound of general formula (I) as appropriate to the labelled first labelled specific binding partner. For the latter assay the secondary binding reagent is a second specific binding partner labelled with either a quencher or a compound of general formula (I) as appropriate to the first labelled specific binding partner. The general design of competitive and non-competitive binding assays is well-known to those skilled in the art and established principles can be applied to the present invention save that the specific teachings of the present invention are followed in regard to the specific needs of the improved compounds and associated chemiluminescence quenching system as disclosed herein.

In a further example of an assay of this aspect of the invention, the analyte is a nucleic acid and the assay employs an oligonucleotide probe as specific binding partner which is a single stranded nucleic acid comprising up to about 40 bases of which the two ends (about 4-8 bases) are mutually complementary and the central section contains a sequence of about 15 to 30 bases which is complementary to all or part of the target sequence of the analyte. The oligonucleotide probe may be labelled with a compound of general formula (I) at one end and a quencher at the other. In the absence of analyte, the mutually complementary ends of the oligonucleotide probe will bind to one another bringing the two ends of the molecule into contact such that any chemiluminescent signal emitted by the molecule of general formula (I) will be modulated by the quencher. In the presence of analyte, however, the central section of the molecule will bind to the analyte more strongly than the binding of the two ends of the molecule. Therefore, in the presence of analyte, the quencher will be separated from the compound of general formula (I) so that no quenching of the chemiluminescent radiation will occur.

In a further modification, the method can be adapted to detect the presence of inhibitors or promoters of a binding process. The method comprises mixing a first and a second specific binding partner labelled respectively with a compound of general formula (I) and a quencher, adding hydrogen peroxide to the medium while
5 maintaining the pH at about 6-10 and detecting the emission of chemiluminescent radiation, wherein modulation of the chemiluminescent signal of the compound of general formula (I) indicates promotion of the binding process and the presence of an unmodulated signal indicates inhibition of the binding process.

10 As mentioned above, one example is the use of the method to monitor the cleavage of an enzyme substrate, in which case the first and second binding partners will be molecular species (peptides) covalently attached to each other, labelled at sites respectively either side of a cleavage site with a compound of general formula (I) and a quencher such that cleavage of the substrate may be determined by loss of
15 quenching.

In a further example, each of two complementary strands of nucleic acid is labelled respectively with a compound of general formula (I) and a quencher. The relative labelling positions must be selected such that the emitter and quencher are in
20 sufficiently close proximity to one another to interact when the complementary strands are bound in a duplex. Therefore, chemiluminescence is only observed when the strands are substantially separated, for example by a process such as enzyme activity, temperature change, competition for binding with unlabelled oligonucleotides, chaotropic agents and the like.

25 Preferred compounds of general formula (I) for use in this assay method are as described above for the first aspect.

When the compound of general formula (I) is one of these preferred compounds, it
30 may be used in combination with methyl red as a quencher since the chemiluminescent emission spectrum of the preferred compounds falls entirely within the absorption spectrum of methyl red at pH 6-10.

The assay methods described above are more sensitive than the corresponding assay using fluorescent detection methods. Therefore, a further advantage of chemiluminescent detection methods is that they may obviate the need for steps such as nucleic acid amplification, which is often used in conjunction with fluorescent detection methods simply to increase the amount of analyte present sufficiently for it to fall within the detection limits of the assay.

The assay methods of the present invention are simple to carry out and are suitable for the detection of a wide range of analytes.

In a further aspect of the invention, there is provided a reagent comprising a ligand or anti-ligand labelled with a compound of general formula (I). The ligand or anti-ligand may be a labelled nucleic acid or a peptide or protein, for example an antibody, antigen, antigenic determinant, enzyme, enzyme substrate, receptor or receptor binding molecule. The reagent is suitable for use in the assay methods described above.

In some cases the ligand or anti-ligand may further be labelled with a quencher. For example, the reagent may comprise a nucleic acid molecule comprising up to about 40 bases wherein the ends are mutually complementary and the central section contains a sequence of about 15 to 30 bases which is complementary to a target sequence and wherein the molecule is labelled at one end with a compound of general formula (I) and at the other end with a quencher.

Alternatively, the ligand or anti-ligand may comprise a nucleic acid duplex each strand of which is labelled respectively with a compound of general formula (I) and a quencher such that attenuation of the light emission exists when the duplex is substantially intact but not when the duplex is rendered substantially non-intact by chemical or physical means.

A further example of such a reagent comprises an enzyme substrate labelled at sites respectively either side of a cleavage site with a compound of general formula (I) and a quencher such that cleavage of the substrate may be determined by loss of quenching.

5

Preferred compounds of general formula (I) are as described above and when one of these preferred compounds is used, the quencher may be methyl red.

10 The reagent may form part of a kit and therefore the invention further provides a kit comprising a reagent as described above, hydrogen peroxide solution and a buffer adapted to adjust the pH of the analyte containing medium to pH 6-10, preferably about pH 9.

15 As mentioned above, the reagent may comprise a quencher in addition to the compound of general formula (I), for example when the ligand or anti-ligand is a nucleic acid molecule or an enzyme substrate.

20 Alternatively, the reagent may comprise either a specific binding partner for an analyte or a secondary binding partner and the kit may further comprise a second reagent labelled with a quencher. Thus, if the reagent comprise a specific binding partner for an analyte, the second reagent will comprise a secondary binding partner, and *vice versa*.

25 When the kit is adapted for use in a competitive binding assay, the secondary binding reagent comprises an analyte analogue labelled with a quencher or a compound of general formula (I) as appropriate to the labelled first labelled specific binding partner. For sandwich assays, the secondary binding reagent comprises a second specific binding partner labelled with either a quencher or a compound of general formula (I) as appropriate to the first labelled specific binding partner.

30

The reagent and the second reagent may be provided either as a mixture or as separate components of the kit.

The invention will now be described in greater detail with reference to the following non-limiting examples.

In a plot which shows the absorption spectra of the quenchers Dabcyl and methyl red at pH 9 plotted on the same axes as the emission spectrum of acridinium ester, at this pH, the emission spectrum of the acridinium ester overlaps completely with that of methyl red.

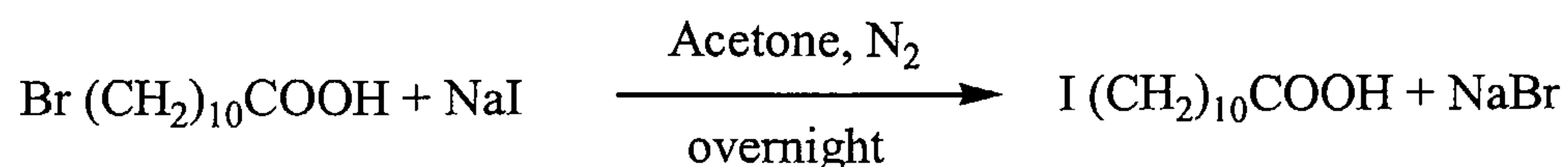
In a plot of amount of target sequence in a test solution against chemiluminescence intensity obtained from a nucleic acid probe having complementary end sequences labelled respectively with a compound of general formula (I) and methyl red, as the amount of target sequence in the solution increases, the hybridisation of the two end sequences of the probe to one another decreases and therefore the amount of quenching of the chemiluminescent signal also decreases.

In the following examples, the compounds which are synthesised and tested are all compounds in which R_1 is a reactive group capable of reacting with an amine or thiol moiety and L_1 is a linker. However, compounds which can be linked to a ligand *via* the R_2 moiety (i.e. compounds in which R_2 is R_4-L_1) are also useful in the present invention. The principle of linking chemiluminescent compounds of this type to a ligand *via* an outer ring substituent (R_2 in general formula (I)) is well known in the prior art, for example from US 5283334 (McCapra), and it has been demonstrated that such compounds are chemiluminescent.

Example 1 – Preparation of 9-(2,6-dibromophenoxy carbonyl)-10-(10-succinimidyl oxy carbonyldecyl)acridinium trifluoromethanesulfonate

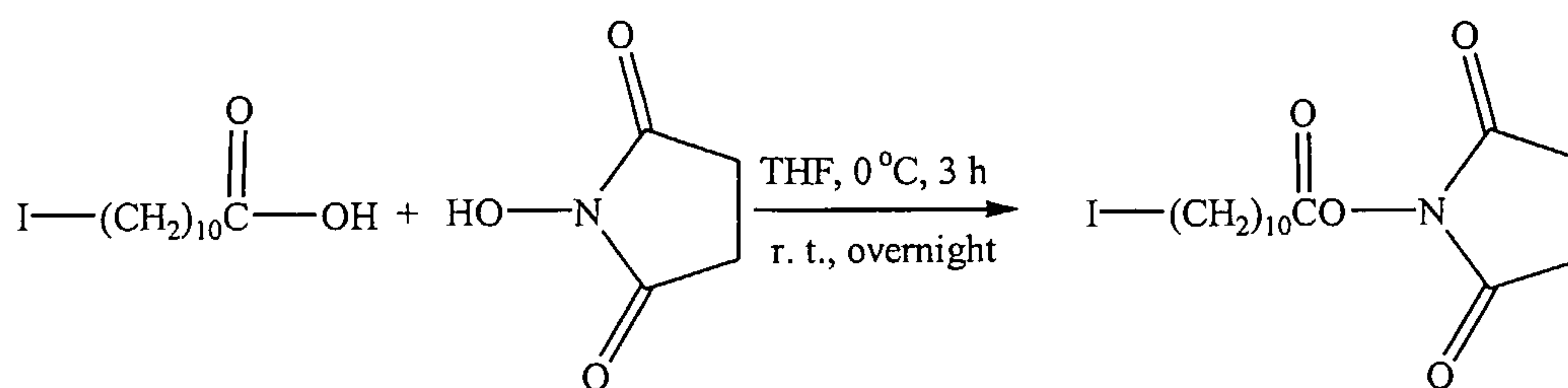
a. 11-iodoundecanoic acid^[8, 9]

5



To a 50 ml flask, 11-bromoundecanoic acid (2.192 g, 8.27 mmol) and NaI (3.0370 g, 20.3 mmol) were added and the mixture was dried over an oil pump for 1 h. Acetone (20 ml) was added and the mixture was stirred for 20 h. The resulting mixture was poured into water (20 ml) and extracted with diethyl ether (25 ml × 4), then the combined organic extracts were washed with saturated NaSO₃, dried with MgSO₄, filtered and evaporated to give a crude pale red solid product (2.3609 g, 91.5% yield). The solid was purified by column chromatography (silica, hexane + ether + formic acid, 100:100:0.1). The fractions of R_f = 0.49 were collected and evaporated to dryness to yield a white solid (2.230g, 83%, m. p, 63-64 °C^[8]). ¹HNMR, (CDCl₃, δ, ppm), 3.12 (2H, t, J = 7 Hz), 2.28 (2H, t, J = 7 Hz), 1.75 (2H, quintet, J = 7 Hz), 1.56 (2H, quintet, J = 7 Hz), 1.24-1.18 (12H, m); ¹³CNMR (CDCl₃, δ, ppm), 179.9 (C=O), 34.3, 33.9, 30.8, 29.7, 29.6, 29.5, 29.4, 28.9, 25.0, 7.5 (10 × CH₂); IR (ν, cm⁻¹) 1693 (C=O); MS (EI) 313 (M + H⁺, 3%), (CI) 330 (M + NH₄⁺, 100%).

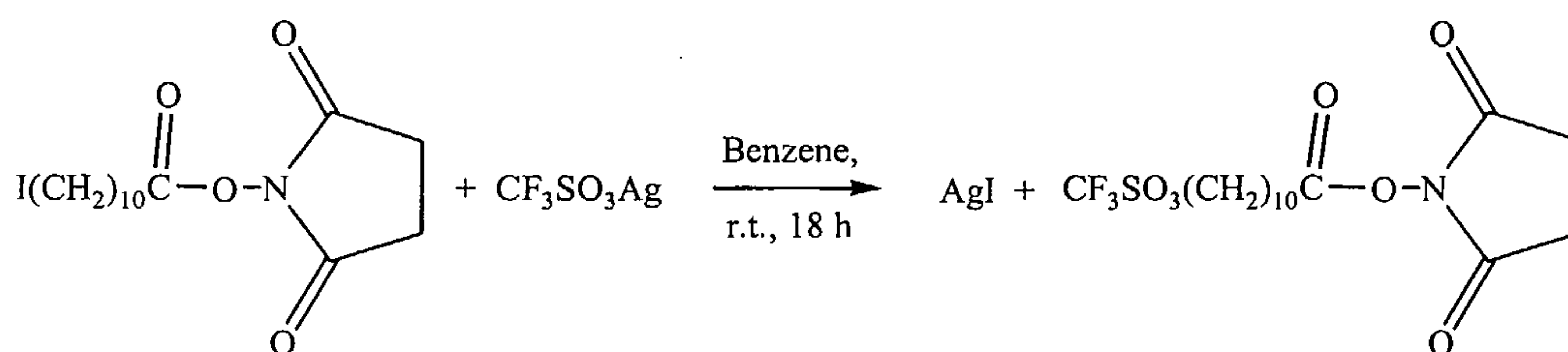
b. Succinimidyl 11-iodoundecanoate^[10]



To a 50ml flask, 11-iodoundecanoic acid (556 mg, 1.78 mmol), *N*-hydroxysuccinimide (440 mg, 3.83 mmol) and dry THF (10 ml) were added and the system was flushed with nitrogen. The mixture was cooled in an ice bath for 10 min. then dicyclohexylcarbodiimide (DCC, 770 mg, 3.76 mmol) in THF (10 ml) was

introduced. The mixture was maintained at 0 °C for 3 h, then at room temperature overnight. The resulting mixture was filtered to remove most of the side product dicyclohexylurea (DCU), the filtrate was concentrated under reduced pressure and the residue was then loaded onto a silica gel column for chromatography. The column was eluted with toluene + ethyl acetate (4:1) and the fractions with $R_f = 0.59$ were combined and evaporated to give a white solid (639 mg, mp 83 °C). The impurities were removed by crystallization from hexane + EtOAc at 65 °C, to yield the pure product (387 mg, 53%). ^1H NMR, (CDCl_3 , δ , ppm), 3.16 (2H, t, $J = 7$ Hz), 2.81 (4H, s), 2.57 (2H, t, $J = 7$ Hz), 1.79, (2H, quintet, $J = 7$ Hz), 1.71 (2H, quintet, $J = 7$ Hz), 1.41-1.22 (12H, m); ^{13}C NMR (CDCl_3 , δ , ppm) 169.6, 169.1 (2 x C=O), 34.0, 31.3, 30.9, 29.7, 29.6, 29.4, 29.1, 28.9, 26.0, 25.0, 7.7 (12 x CH_2); IR (ν , cm^{-1}) 1725; MS (CI) 427 ($\text{M} + \text{NH}_4^+$, 15%), 301 (15%), 225 (16%), 149 (30%), 132 (100%).

c. Succinimidyl 11-(trifluoromethanesulfonyloxy)undecanoate^[11-13]



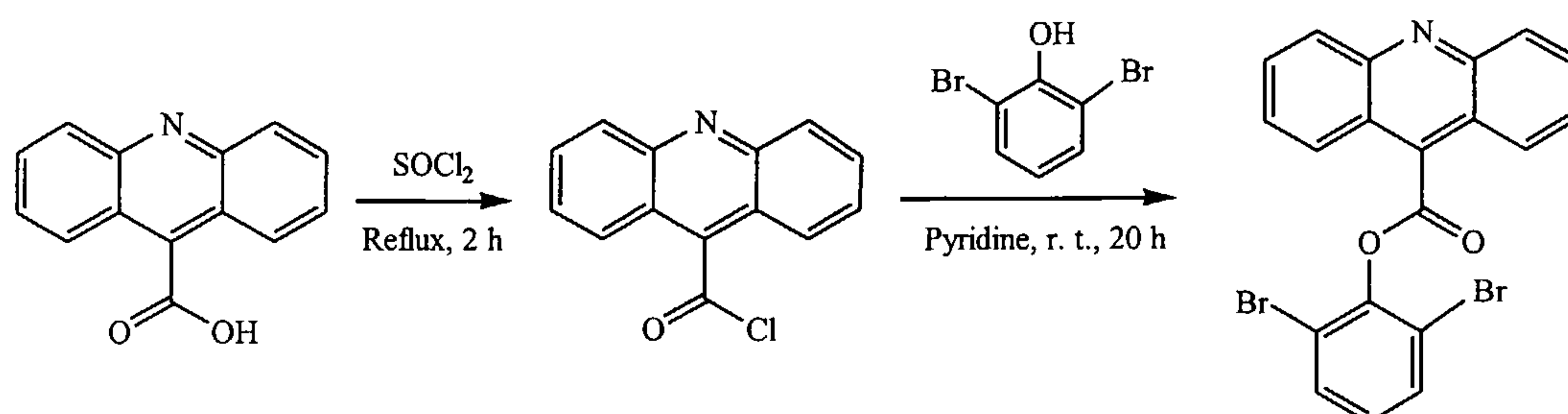
To a 5 ml flask, succinimidyl 11-undecanoate (188 mg, 0.460 mmol) and silver triflate (214 mg, 0.836 mmol) were added, and the flask was flushed with N_2 for 5 min. Benzene (1.5 ml) was introduced with a syringe, whereupon some yellow solid appeared immediately. The mixture was stirred for 18 h at room temperature, then was loaded onto a short chromatography column (silica gel, 2g), which was eluted with dichloromethane (DCM). The fractions with $R_f = 0.48$ were combined and evaporated to give a white gel (133 mg, yield, 67%).

^1H NMR (CDCl_3 , δ , ppm), 4.44 (2H, t, $J = 6$ Hz), 2.75 (4H, s), 2.51 (2H, t, $J = 7$ Hz), 1.73 (2H, m), 1.63 (2H, quintet, $J = 7$ Hz), 1.37-1.17, (12H, m); ^{19}F NMR (CDCl_3 , δ , ppm), -74.8; ^{13}C NMR (CDCl_3 , δ , ppm), 169.6, 169.0 (C=O), 119.0 (quartet,

CF₃SO₃), 78.1, 31.3, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 26.0, 25.4, 24.9 (12 x CH₂); IR (ν, cm⁻¹), 1725.

d. 2,6-dibromophenyl acridine-9-carboxylate^[3-5]

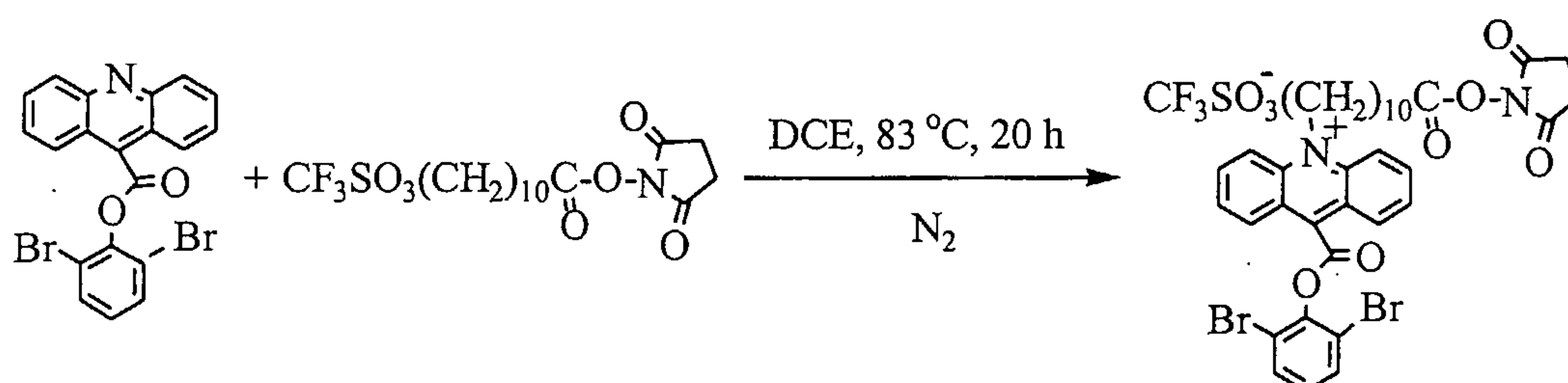
5



In a round bottom flask (50 ml) equipped with a CaCl₂ drying tube and a magnetic stirrer bar, acridinium-9-carboxylic acid chloride (2.036g, 8.42 mmol) in pyridine was heated to (70 °C). After the solid had dissolved completely, the solution was cooled to room temperature, 2,6-dibromophenol (2.325 g, 9.23 mmol) was added and the mixture was stirred vigorously overnight. The resulting mixture was stripped of pyridine to leave a brown solid. DCM (10 ml) was used to extract the product and the extract was subjected to column chromatography, eluted with toluene + EtOAc (4:1). The fractions with R_f 0.51 were collected and evaporated to give a pale yellow solid. The solid was re-dissolved in DCM (40 ml), washed with dilute NaOH (5 x 40 ml), dried over MgSO₄, filtered and evaporated to give a pale yellow powder (2.080 g, 54% yield), mp 159-160 °C.

e. 9-(2,6-dibromophenoxy carbonyl)-10-(10-succinimidyl oxycarbonyl decyl)acridinium trifluoromethanesulfonate^[6, 7]

20



To a 5 ml flask, 2,6-dibromophenyl acridine-9-carboxylate (93.2 mg, 0.204 mmol), succinimidyl 11-(trifluoromethanesulfonyloxy)undecanoate (94.1 mg, 0.218 mmol), and a stirrer bar were added, then the flask was equipped with an air condenser and flushed with N₂. Tetrachloroethane (1.5 ml) was introduced by a syringe, whereupon the mixture turned to bright yellow. The flask was placed into an oil bath (140 °C) for 4 h, during which the mixture gradually turned to orange and eventually a brown-red solution was obtained. The whole mixture was stripped of solvents and then extracted with DCM and precipitated with EtOEt. The precipitate was re-dissolved in DCM and precipitated repeatedly until TLC showed no presence of starting materials. A yellow solid (28 mg, 15% yield, mp 165-166 °C) was obtained and characterized. ¹HNMR, (CDCl₃, δ, ppm), 8.84 (2H, d, J = 8 Hz), 8.66 (2H, d, J = 9 Hz), 8.39 (2H, dd, J = 8, 7 Hz), 7.89 (2H, dd, J = 9, 7 Hz), 7.58 (2H, d, J = 8 Hz), 7.06 (1H, t, J = 8 Hz), 5.47 (2H, t, J = 8 Hz), 2.65 (4H, s), 2.41 (2H, t, J = 7 Hz), 2.03 (2H, m), 1.64-1.49 (4H, m), 1.31-1.07 (10H, m); ¹⁹FNMR (CDCl₃, δ, ppm), -78.2; ¹³CNMR (CDCl₃, δ, ppm), 169.7, 169.1, 161.5 (3 x C=O), 147.1, 145.8, 141.7, 140.8, 133.8, 130.3, 129.9, 129.3, 123.9, 119.8, 117.7, 52.9, 31.3, 30.1, 29.6, 29.5, 29.4, 29.3, 29.0, 26.9, 26.0, 24.9; IR (ν, cm⁻¹) 1774, 1728 (C=O); MS (ES⁻), 149 (100%, CF₃SO₃); (ES⁺), 739 (80%, M-CF₃SO₃), 519 (100%).

Example 2 – Preparation of 9-(2,6-dibromophenoxy carbonyl)-10-(3-succinimidyl oxy carbonyl propyl)acridinium trifluoromethanesulfonate

a. Succinimidyl 4-Iodobutanoate

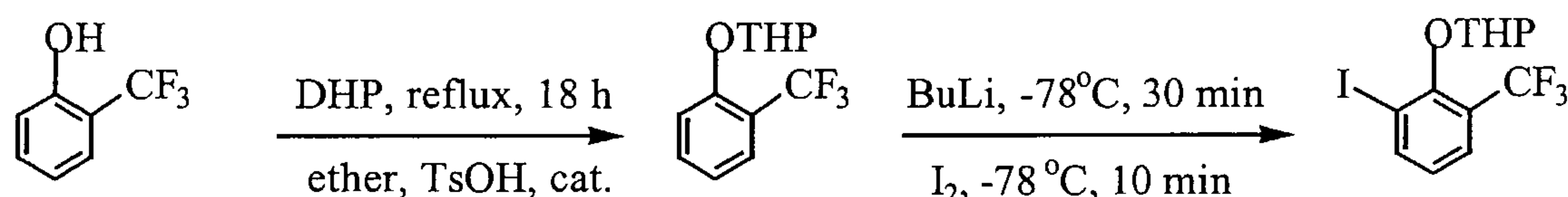
4-Iodobutyric acid (115 mg, 0.54 mmol) in tetrahydrofuran (THF, 5 ml) was cooled to 0 °C. To the solution, N-hydroxysuccinimide (65 mg, 0.57 mmol) in THF (1 ml) and dicyclohexylcarbodiimide (125 mg, 0.61 mmol) in THF (2 ml) were added respectively. The mixture was stirred at 0 °C for 3 h and then at room temperature overnight. The suspension obtained was filtered. The filtrate was concentrated and purified by silica gel chromatography (Toluene/ethyl acetate (Tol-EtOAc), 4:1). The desired product (132 mg) was obtained as a pale yellow solid, mp 86-87°C.

b. 9-(2,6-Dibromophenoxy carbonyl)-10-(3-succinimidyloxy carbonyl)propyl)acridinium iodide

2,6-Dibromophenyl acridine-9-carboxylate synthesised as in Example 1d (43 mg, 0.094 mmol) and succinimidyl 4-iodobutanoate (50 mg, 0.16 mmol) in nitrobenzene (2 ml) in a glass tube were heated at 120 °C on an oil bath for 2 hours. The brown solution obtained was washed with petroleum ether (40-60) (5 x 2 ml) to give a brown solid (about 2 mg). The washings were combined and evaporated on a rotary evaporator and then under vacuum for 2h to get an orange solution. The mixture was reused. The experiment was carried out under the conditions described in Example 1e.

Example 3 – Preparation of 9-(2,6-bis(trifluoromethyl)phenoxy carbonyl)-10-(10-succinimidyloxycarbonyldecyl)acridinium trifluoromethanesulfonate

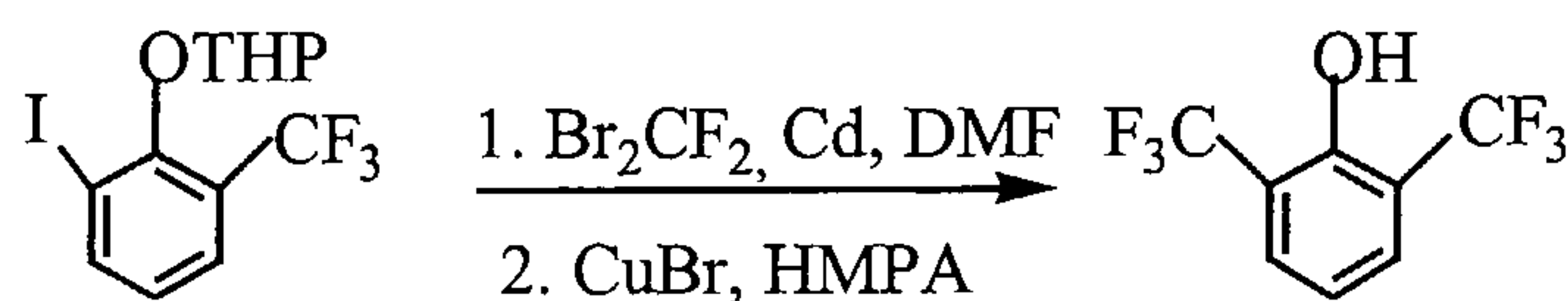
a. 2-trifluoromethyl-6-iodophenol^[1, 2]



To a 50 ml flask, 2-trifluoromethylphenol (1.00 g, 6.17 mmol) and 3,4-dihydropyran (2 ml) were added, and then a few crystals of toluenesulfonic acid monohydrate (TsOH.H₂O) were added. After ether (25 ml) was introduced, the flask was placed in an oil bath and refluxed for 19 h. The resulting mixture was poured into saturated NaHCO₃ (20 ml), the aqueous layer was extracted with diethyl ether (25 ml x 2), and the combined extracts were washed with saturated NaCl, dried over MgSO₄ and stripped of solvents to give a colourless oil, 2-(trifluoromethyl)phenol tetrahydropyranyl ether (1.35 g, 5.50 mmol, 89% yield). ¹HNMR (CDCl₃, δ, ppm), 7.50 (1H, d, J = 8Hz), 7.38 (1H, t, J = 8Hz), 7.18 (1H, d, J = 8Hz), 6.94 (1H, t, J = 8 Hz), 5.48 (1H, approx. t, J = 3 Hz), 3.77 (1H, dt, J = 3, 11 Hz), 3.55 (1H, m), 1.96 (1H, m), 1.89-1.73 (2H, m), 1.71-1.46 (3H, m). The oil was dissolved in THF (8.5 ml) and treated with BuLi (3 ml of 2.5 M, 7.5 mmol); after stirring at -78 °C for 30mins, the reaction mixture was treated with a solution of iodine (2.2013 g, 8.67

mmol) in THF (8.5 ml), stirred for 10 min, and then allowed to warm to room temperature. The mixture was poured into 20% Na₂SO₃ and vigorously shaken. The aqueous layer was extracted with diethyl ether and the combined extract was washed with saturated NaCl and then dried over MgSO₄. After stripping of solvent, a yellow-red oil was obtained. ¹HNMR (CDCl₃, δ, ppm), 7.88 (1H, dd, J = 8, 1 Hz), 7.46 (1H, dd, J = 8, 1 Hz), 6.80 (1H, t, J = 8 Hz), 5.25 (1H, dd, J = 5, 2 Hz), 4.04 (1H, m), 3.43 (1H, m), 2.01 (1H, m), 1.91-1.79 (2H, m), 1.58-1.42 (3H, m). This showed the oil to be 2-(trifluoromethyl)-6-iodophenol tetrahydropyranyl ether. The oil was subjected to short-path distillation, during which decomposition of the tetrahydropyranyl ether took place, giving the product as 2-(trifluoromethyl)-6-iodophenol (1.667 g, yield, 94% yield if assumed to be pure, but in reality contaminated by about 15% of dihydropyran hydrate); ¹HNMR (CDCl₃, δ, ppm), 7.88 (1H, d, J = 8 Hz), 7.47 (1H, d, J = 8 Hz), 6.79 (1H, t, J = 8 Hz), 5.85 (1H, s).

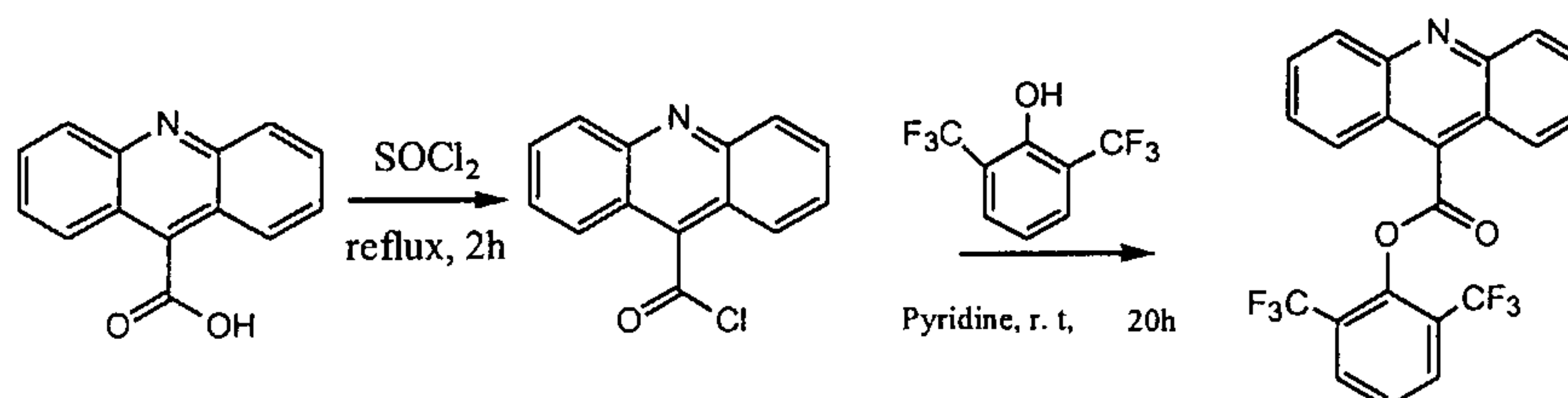
b. 2,6-bis(trifluoromethyl)phenol^[1,2]



To a 50 ml flask equipped with a stirrer bar, Cd powder (3.057 g, 27.2 mmol) and dimethyl formamide (DMF, 10 ml) were added. The flask was flushed with N₂ and then cooled to 0 °C in an ice bath. CBr₂F₂ (1ml + 1ml + 0.5ml, 27.17 mmol in total) was added slowly by syringe, then the mixture was allowed to warm up to room temperature and stirred for an additional 30min. The mixture was diluted with hexamethylphosphoramide (HMPA, 10 ml) and cooled to 0 °C, CuBr (1.8036 g, 12.57 mmol) was added and the whole was stirred for 10min. To the resultant mixture containing F₃CCu was added 2-trifluoromethyl-6-iodophenol (937.1 mg, 3.25 mmol) and the mixture was heated over an oil bath (65 °C) with stirring for 2h. The whole mixture was poured into a mixture of 3M HCl (30 ml) and ether (30 ml), the solids were removed from the mixture by filtration and the two liquid layers were separated. The aqueous phase was extracted with further ether (25 ml × 5), and the combined extract was washed with saturated NaCl and then dried over MgSO₄. The

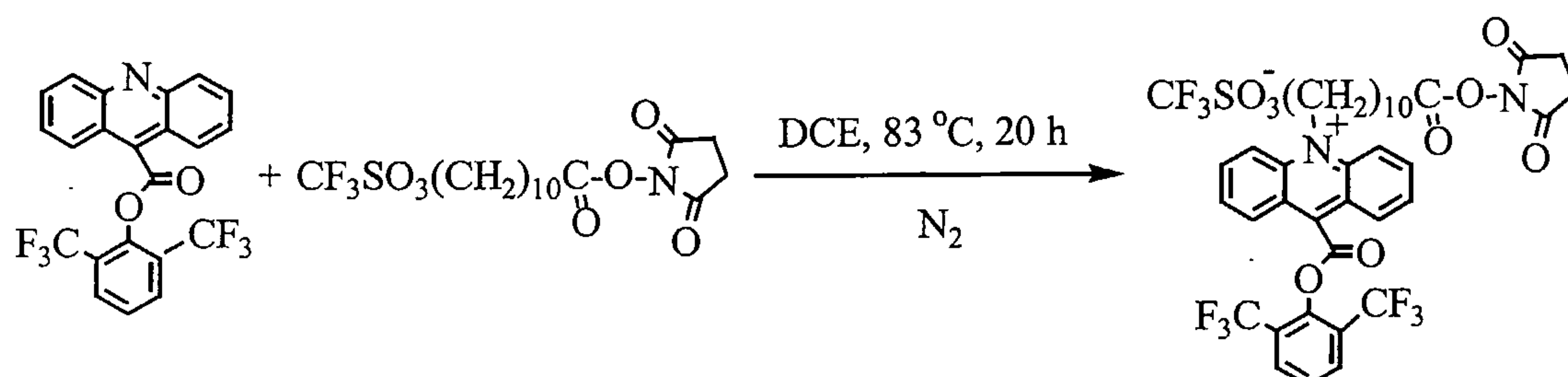
solvent was removed by rotary evaporation and the residue was subjected to short path distillation. A fraction with bp 70 °C (1 mbar) was collected; yield 283.4 mg, 38%. ¹HNMR (CDCl₃, δ, ppm), 7.70 (2H, d, J = 8 Hz), 7.06 (1H, t, J = 8 Hz); ¹³CNMR (CDCl₃, δ, ppm), 153.0, 131.0, 123.8 (q due to coupling to 3 F nuclei), 120.3, 120.0. EI-MS, m/z, 230 (M⁺, 18%), 213 (28%), 179 (23%), 163 (30%), 135 (100%).

c. 2,6-bis(trifluoromethyl)phenyl acridine-9-carboxylate^[3-5]



In a 5ml flask equipped with a stirrer bar, a condenser, and a CaCl₂ drying tube, acridine-9-carboxylic acid chloride (131.6 mg, 0.544 mmol), 2,6-bis(trifluoromethyl)phenol (130 mg, 0.565 mmol) and pyridine (3 ml) were mixed and stirred vigorously at 40 °C for 1 day. The solvent was evaporated from the mixture, and the residual solid was subjected to column chromatography (silica, toluene:EtOAc 4:1). The fractions with R_f = 0.49 were collected and evaporated to give an orange solid (137.5 mg, 58% yield), mp 171 °C; ¹HNMR (CDCl₃, δ, ppm), 8.51 (2H, d, J = 9 Hz), 8.32 (2H, d, J = 9 Hz), 7.96 (2H, d, J = 8 Hz), 7.79 (2H, dd, J = 9.7 Hz), 7.61 (2H, dd, J = 9, 7 Hz), 7.56 (1H, t, J = 8 Hz); ¹³CNMR (CDCl₃, δ, ppm), 164.6 (C=O), 148.6, 146.3, 131.9, 131.0, 130.0, 128.5, 127.8, 126.5, 126.2, 125.8, 123.8, 122.8 (q due to coupling to 3 F nuclei); IR (ν, cm⁻¹), 1758 (C=O); MS: (EI), m/z, 435 (M⁺, 10%), 206 (82%), 178 (100%); (CI), m/z, 436 (M+H⁺, 39%), 180 (100%).

d. 9-(2,6-bis(trifluoromethyl)phenoxyacarbonyl)-10-(10-succinimidyl
oxycarbonyldecyl)acridinium trifluoromethanesulfonate^[6, 7]



5

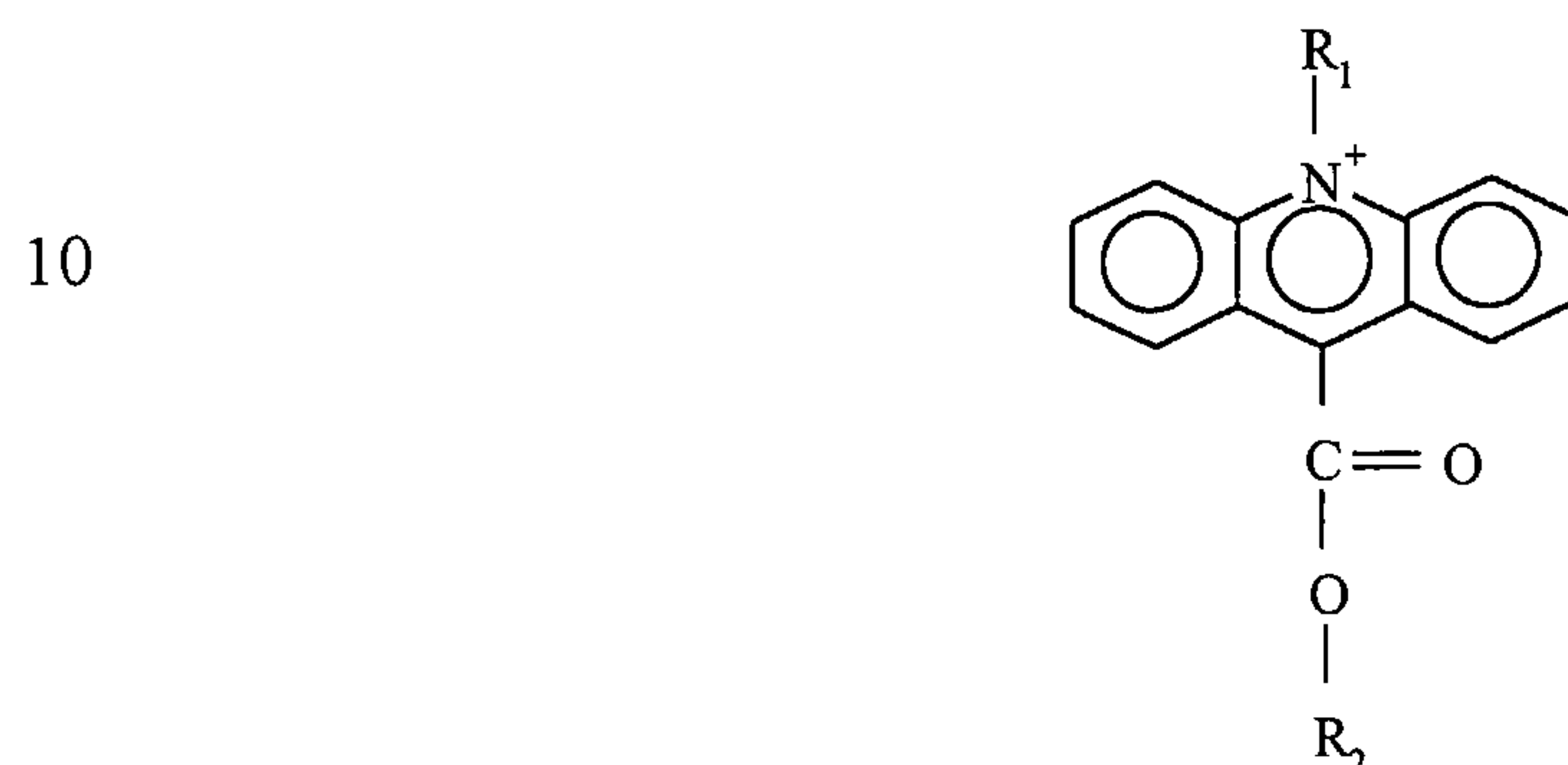
To a 5 ml flask previously flushed with nitrogen, 2,6-bis(trifluoromethyl)phenyl
 acridinium-9-carboxylate (74.9 mg, 0.172 mmol) and succinimidyl 11-
 (trifluoromethanesulfonyloxy)undecanoate (prepared according to Example 1c, 74.8
 mg, 0.174 mmol) were added. The flask was re-flushed with N₂, then dry
 10 dichloroethane (1.6 ml) was introduced by syringe. The mixture was maintained at
 80 °C for 20 h and afterwards stripped of solvents on a rotary evaporator. The
 mixture was extracted with dichloromethane (1 ml), then the extract was subjected to
 column chromatography on silica gel, eluted by dichloromethane (DCM)/CH₃CN
 (3:1). The component with R_f = 0.19 was collected and evaporated to give a yellow
 15 solid (15.9 mg, 10.7% yield), mp 164-165 °C; ¹HNMR (CDCl₃, δ, ppm) 8.88 (2H, d,
 J = 9 Hz), 8.77 (2H, d, J = 9 Hz), 8.58 (2H, dd, J = 9, 7 Hz), 8.15 (2H, d, J = 8 Hz),
 8.07 (2H, dd, 9, 7 Hz), 7.81, (1H, t, J = 8 Hz), 5.69 (2H, t, J = 8 Hz), 2.85 (4H, s),
 2.60 (2H, t, J = 7 Hz), 2.29-2.18 (2H, m), 1.84-1.69 (4H, m), 1.51-1.26 (10H, m);
¹³CNMR (CDCl₃, δ, ppm), 169.7, 169.1, 161.8, 144.9, 141.7, 140.6, 132.4, 130.0,
 20 129.0, 128.5, 126.0, 125.7, 124.3, 119.8, 53.3, 31.3, 30.1, 29.6, 29.5 (2 signals), 29.3,
 29.0, 26.9, 26.0, 24.9 (the CF₃ signals were not seen as the signal to noise ratio was
 too low); MS (ES+) 717 (C₃₇H₃₅F₆N₂O₆⁺, 25%), (ES-) 149 (CF₃SO₃⁻, 100%).

Methods similar to those of Examples 1 to 3 have been used to prepare 9-(2,6-
 25 dinitrophenoxyacarbonyl)-10-(10-succinimidyl oxycarbonyldecyl)acridinium
 trifluoromethanesulfonate and 9-(2,6-dinitrophenoxyacarbonyl)-10-(3-succinimidyl
 oxycarbonylpropyl)acridinium trifluoromethanesulfonate.

Example 4 – Effect of Variation of X Group on Chemiluminescence

This example shows that not all compounds having X groups as taught in EP-A-0324202 are capable of undergoing a chemiluminescent reaction at moderate pH.

Compounds of the following general formula were synthesised according to established methods:



In the above formula:

In Compound (a), R₁ is methyl, R₂ is phenyl;

In Compound (b), R₁ is methyl, R₂ is 3-methylphenyl;

In Compound (c), R₁ is methyl, R₂ is 2,6-difluorophenyl;

20 In Compound (d), R₁ is (succinimidylloxycarbonyl)propyl, R₂ is 2,6-dibromophenyl

Chemiluminescence from these compounds was measured using a luminometer (LuminoTM, Stratec Elektronik GmbH, Pfortzheim, Germany) following onboard initiation of the reaction by injection (200μl) of buffered hydrogen peroxide of pH 9 or pH 12. pH 9 was achieved using disodium hydrogen orthophosphate (0.02M/l), pH 12 was achieved as set forth in EP-A-0324202 by diluting 100μl of 5M aqueous sodium hydroxide and 20μl of 30% aqueous hydrogen peroxide in 20ml water. In both cases, 30% hydrogen peroxide was used.

30 The measurement time was 5 seconds, over which time photon counts were integrated and began immediately the reagent was injected. The amounts of each compound used and the luminometer counting factor were chosen so that each

compound exhibited comparable peak heights at pH 12. Nominally, concentrations in the range 1-10ng/ml were used with the pure compounds being first dissolved in acetonitrile at a concentration of 1mg/ml and then further diluted in mM/l hydrochloric acid. 10µl volumes were used for luminometry.

5

Table 1

	Compound			
	(a)	(b)	(c)	(d)
pH 12	100	100	100	100
pH 9	7	3	100	100

10

Table 1 shows the chemiluminescent emission intensity at pH 9 as a percentage of that obtained using the conventional reaction conditions (pH 12) set out in EP-A-0324202. It can be seen that, although all four compounds possess X groups falling within the scope of EP-A-0324202, only compounds (c) and (d) are capable of emitting significant chemiluminescence at moderate pH. These two compounds have phenyl moieties which possess electron withdrawing groups as required by the present invention.

15

Example 5 – Effect of pH on Stability of Binding Complexes

20

A stem loop oligonucleotide of the following sequence possessing respective end modifications comprising methyl red (MR) and a primary amine linker was synthesised to order and purchased commercially (Oswel Research Products, Romsey, UK).

NH₂-5'-CCGGTCCGGGCAATGACAGCAAAAACAGGGACCGGT-MR

25

The primary amine was labelled with the acridinium ester Compound (d) from Example 2 using succinimidyl ester chemistry by established methods. (Nelson *et al*, *Nonisotopic Probing, Blotting and Sequencing*, pp. 391-428, Academic Press, 1995) Clearly, neither Compound (a) nor Compound (b) could be used as the label because, as seen in Table 1 above, they do not emit significant chemiluminescence at pH 9.

However, Compound (d) has the property of retaining 100% of its pH 12 emission intensity when the pH is reduced to 9.

The chemiluminescence emitted by the oligonucleotide labelled with Compound (d) was measured at pH 9 and pH 12 using the method set out in Example 2 and the results are shown in Table 2.

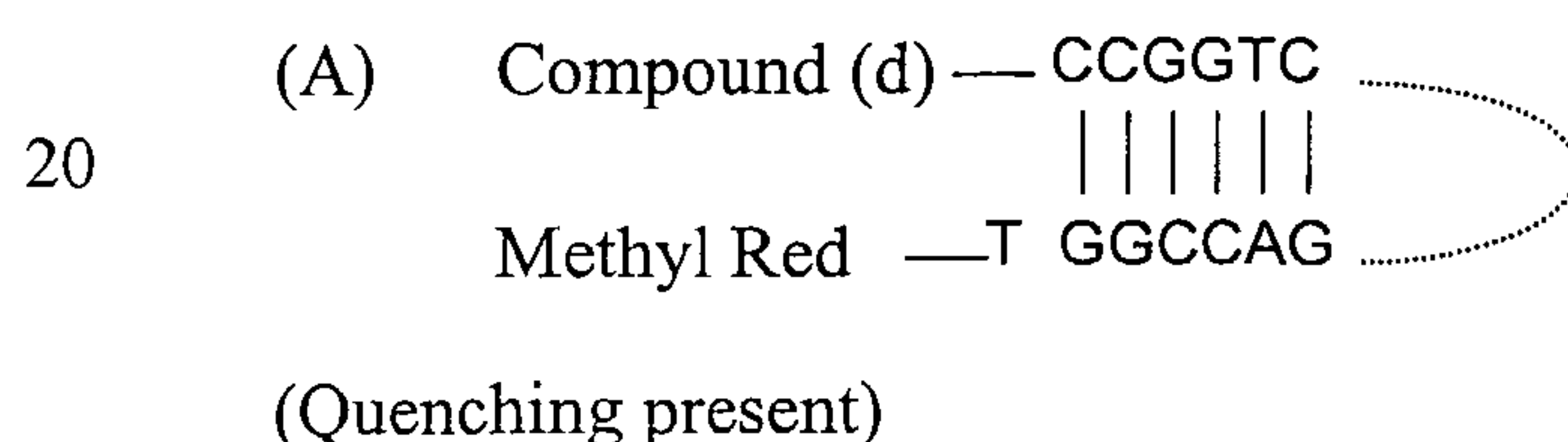
Table 2

	Unquenched maximal RLU* (0% quenching)	Actual RLU observed	% quenching
pH 9	1153347	24832 (2% of maximum)	98
pH 12	1326487	1299306 (98% of maximum)	2

*RLU = relative light units.

Table 2 shows that at pH 9, the chemiluminescent signal emitted by Compound (d) is 98% quenched by methyl red but at pH 12, the quenching is only 2%.

The oligonucleotide labelled with Compound (d) exists in free solution under mild conditions (pH 9) as a stem-loop structure due to the formation of a stable intramolecular binding complex of Structure (A)



Thus, at pH 9, minimal background chemiluminescence emission is seen due to chemiluminescence quenching arising from the close proximity of the quencher, methyl red, to the emitter, Compound (d).

In contrast, when chemiluminescence is initiated at pH 12 as set out in EP-A-0324202, significant chemiluminescent emission is observed. This is because, at pH

12, hybridisation between the complementary nucleic acids at the 5' and 3' terminals of the oligonucleotide is disrupted and so the labelled oligonucleotide adopts structure (B).

5 (B) Compound (d) — CCGGTC-----GACCGG T – Methyl Red
(Quenching absent)

In structure (B), the emitter Compound (d) is not sufficiently close to the quencher, methyl red, for quenching to occur.

10

From the results of this experiment, it is clear that chemiluminescence quenching cannot be used as the basis of an assay system under the conditions taught in EP-A-0324202 since the binding complex is non-specifically dissociated under these conditions. The same is true for systems other than nucleic acid systems, for
15 example those based on antibody-antigen binding.

By contrast, the binding complex remains intact at pH 9, permitting quenching to occur. Thus, only acridinium labels which undergo a chemiluminescent reaction at moderate pH such as those described herein can be used as the basis of a
20 homogeneous energy transfer binding assay.

Example 6 – Choice of a Quencher for use with Compounds of Formula (I)

EP-A-0324202 suggests energy transfer acceptors such as fluorescein and Lucifer
25 Yellow which re-emit the absorbed energy at a different wavelength from that of the donor and thus such acceptors cannot be used as quenchers. No indication is given in the document of any other useful quenchers for the claimed compounds. However, fluorescence quenchers have been described and it might be expected that commonly used fluorescent quenchers could be employed. This example demonstrates that the
30 well established quencher DabcylTM cannot, as might have been expected, be used to quench the emission from acridinium emitters under all circumstances. This is because the absorption spectrum of the quencher at the pH required to bring about

the chemiluminescent reaction shifts relative to the emission spectrum of the acridinium label.

The emission spectrum of the primary emitter of the acridinium ester chemiluminescence (N-methylacridone) is well established. The absorption spectra of methyl red and DabcylTM were measured in a spectrofluorimeter. These spectra were overlaid with that of the emission spectrum of N-methylacridone to yield the a composite spectrum. ~~shown in Figure 1. It can be seen that t~~ There is only a modest overlap between the emission spectrum of N-methylacridone and the absorption spectrum of DabcylTM at pH 9. Since efficient resonance energy transfer quenching requires maximal spectral overlap, it can be seen that such DabcylTM quenching is inefficient at the pH required to initiate the chemiluminescent reaction of the preferred acridinium esters. By contrast, the preferred compounds of the present invention exhibit 100% spectral overlap with the methyl red quencher at pH 9. Thus, only a methyl red quencher and not the conventionally used DabcylTM is an efficient quencher of acridinium esters which are capable of undergoing a chemiluminescent reaction at pH 9. This is somewhat unexpected since DabcylTM has been stated to be a useful quencher in many energy transfer assays.

Example 7 – Chemiluminescent Properties of Compounds of Formula (I)

This example demonstrates that the compounds of the present invention can be used as the basis of a homogeneous energy transfer quenching binding assay since such compounds can exhibit chemiluminescence at moderate pH and are capable of acting as part of efficient quenching systems in conjunction with carefully selected quenchers, such as methyl red. The example also demonstrates that, by contrast, acridinium esters which require elevated pH to bring about chemiluminescence are incapable of being used for such purpose as is the case with numerous compounds which fall within the scope of EP-A-0324202.

The labelled stem loop oligonucleotide described in Example 3 (1-10fM) in 10µl hybridisation buffer (0.1M/l lithium succinate, pH 5.1; 8.5% w/v lithium lauryl sulphate; 0.125M/l lithium hydroxide; 1.5mM/l EDTA and 1.5mM/l EGTA) was

admixed respectively with solutions containing 0, 1, 10, 50, 100, 500 and 1000pM of a partially complementary oligonucleotide target sequence (gcccggttactgtcgttttgtcc) in hybridisation buffer (10µl). The mixtures were incubated at 60°C for 60 min and allowed to equilibrate to room temperature prior to luminometry using the appropriate pH initiation solutions. The principle of these interactions is well known such that the complementary binding of the loop region to its target disrupts the stem binding causing the donor and acceptor moieties to become separated. Thus, in absence of target, the resulting mixture consisted of Structure A at pH 9 and Structure B at pH 12 (see Example 3). Increasing concentrations of target resulted in the formation of increasing amounts of Structure C, which is distinguishable from structure A on the basis of loss of quenching.

(C)

Compound (d) - 5' - CCGGTCCGGGCAATGACAGCAAAAACAGGGACCGGT-MR
GCCCGTTACTGTCGTTTTGTCC

(MR = Methyl Red)

Thus, at pH 9, chemiluminescence intensity is proportional to the concentration of target sequence (Figure 2). By contrast, there is no relationship between chemiluminescence intensity and target concentration at pH 12 due to binding complex dissociation. This confirms that only acridinium esters capable of yielding chemiluminescence at moderate pH can be used in such assays.

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SEQUENCE LISTING

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<140> 2,536,658

<141> August 26, 2004

<150> PCT/GB2004/003660

<151> August 26, 2004

<150> GB 0320236.3

<151> August 29, 2003

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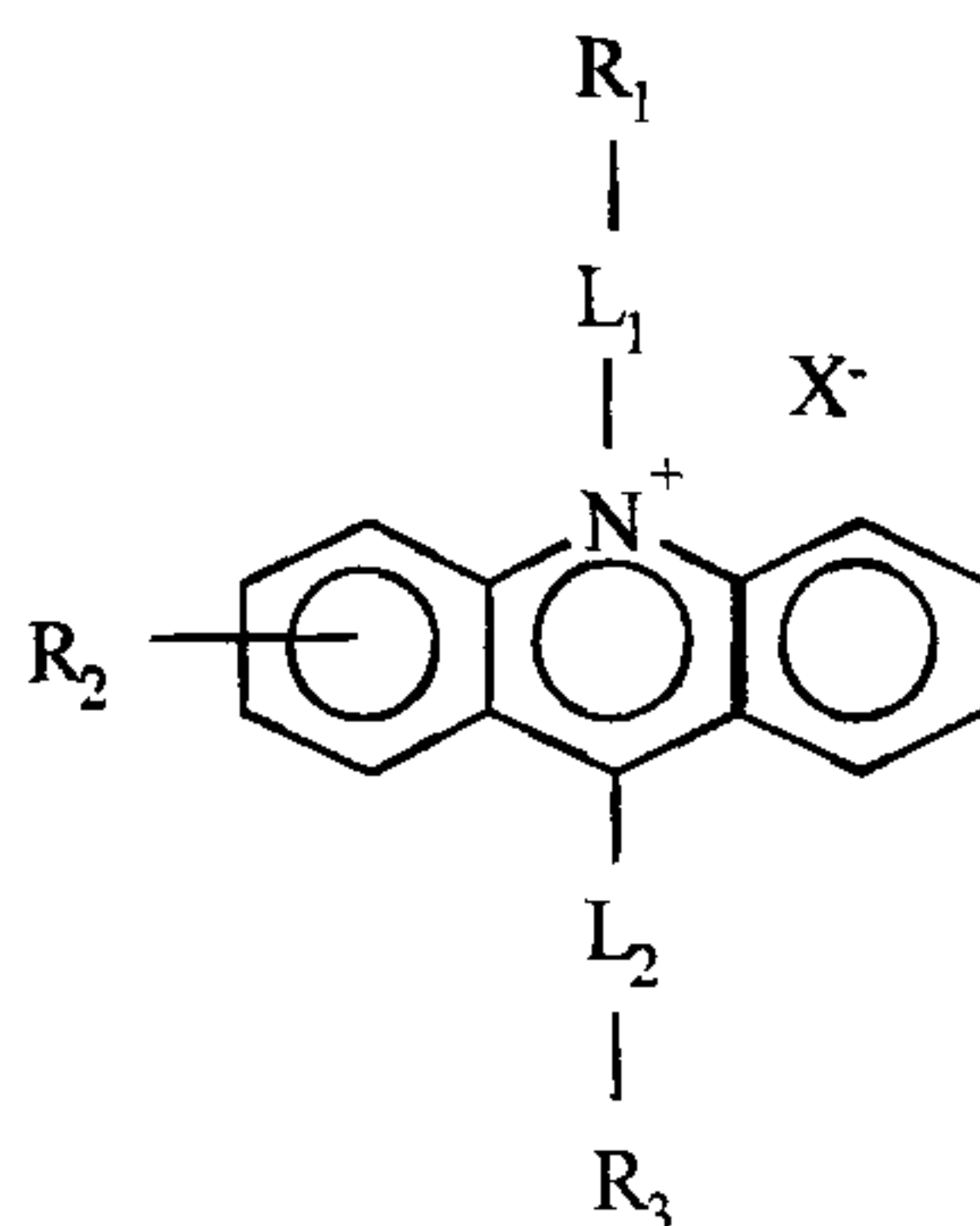
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CLAIMS

1. An oligonucleotide probe comprising up to 40 nucleic acid bases having ends which are mutually complementary and a central section containing a 15 to 30 nucleic acid base sequence which is complementary to a target nucleic acid sequence,

wherein the oligonucleotide probe is labelled at one end with a chemiluminescent acridinium ester labelling reagent of formula (I) and at the other end with a quencher such that attenuation of chemiluminescent light emission exists when a duplex of the complementary ends is intact,

wherein said chemiluminescent acridinium ester labelling reagent of formula (I) is:



(I)

wherein:

either:

R_1 is a reactive group capable of reacting with an amine or thiol moiety;

L_1 is a hydrocarbon linker moiety comprising 2 – 12 carbon atoms, optionally substituted with hydroxy, halo, nitro or C_1 - C_4 alkoxy; and

R_2 is hydrogen, C_1 - C_4 alkyl, C_1 - C_4 haloalkyl, aryl, fused aryl, C_1 - C_4 alkoxy, C_1 - C_4 acyl, halide, hydroxy or nitro;

or, alternatively:

the combination R_1 - L_1 - comprises a C_1 - C_4 alkyl group optionally substituted with hydroxy, halo, nitro or C_1 - C_4 alkoxy; and

R_2 comprises a group R_4 - L_1 -, where R_4 is a reactive group capable of reacting with an amine or thiol moiety; and L_1 is as defined above;

L_2 is $-C(=O)O-$, $-C(=O)-S-$ or $-C(=O)N(SO_2R_5)-$,

wherein, in each case, the $-C(=O)$ is linked to the ring carbon atom, and

R_5 is C_1-C_8 alkyl, aryl, C_1-C_8 alkoxy or C_1-C_8 acyl;

R_3 is a phenyl group substituted independently at the 2 and 6 positions with nitro, fluorine, chlorine, bromine or trifluoromethyl groups, wherein at least one of the said substituents is electron-withdrawing such that the pK_a of the conjugate acid of the leaving group formed from R_3 and the $-O$, $-S$ or $-N(SO_2R_5)$ of the L_2 group is ≤ 9.5 ; and

X^- is an anion formed as the result of the synthesis and processing of the molecule;

wherein the compound may contain one or more additional R_2 moieties on either or both outer rings, provided that only one of said R_2 moieties may comprise an R_4-L_1 - group.

2. The probe of claim 1, wherein said attenuation does not exist when the duplex of complementary ends is rendered substantially non-intact by chemical or physical means.

3. The probe of claim 1, wherein said attenuation does not exist when the duplex of complementary ends is disrupted by hybridisation of said central section of the oligonucleotide probe to said target sequence.

4. The probe of claim 1, 2 or 3, wherein R_1 , or R_4 of said reagent of formula (I) is an active ester, a maleimide or an active halide.

5. The probe of any one of claims 1 to 4, wherein R_1 or R_4 of said reagent of formula (I) is a succinimidyl ester, an imidate ester, chlorocarbonyl, bromocarbonyl, iodocarbonyl, chlorosulfonyl or fluorodinitrophenyl.

6. The probe of claim 1, 2 or 3 wherein, independently or in any combination:

R_1 is an active ester, an imidate, or an active halide;

L₁ comprises 3 to 10 carbon atoms;
R₂ is hydrogen or C₁-C₄ alkyl;
L₂ is -C(=O)O-; and
X⁻ is a halide or halide-containing anion

of said reagent of formula (I).

7. The probe of any one of claims 1 to 3 and 6, wherein, independently or in any combination:

R₁ is a succinimidyl ester, an imidate ester, chlorocarbonyl, bromocarbonyl, iodocarbonyl, chlorosulfonyl or fluorodinitrophenyl moiety;
L₁ comprises 3 to 10 methylene units;
R₂ is hydrogen; and
X⁻ is iodide, fluorosulfonate, trifluoromethanesulfonate or trifluoroacetate

of said reagent of formula (I).

8. The probe of claim 1, 2 or 3 wherein, independently or in any combination:

R₁ is a maleimide moiety;
L₁ comprises 3 to 10 methylene units;
R₂ is hydrogen; and
X⁻ is iodide, fluorosulfonate, trifluoromethanesulfonate or trifluoroacetate

of said reagent of formula (I).

9. The probe of any one of claims 1 to 8, wherein R₃ of said reagent of formula (I) is chosen such that the pK_a of the conjugate acid of the L₂-R₃ leaving group is ≥ 3 .

10. The probe of claim 1, 2 or 3, wherein said reagent of formula (I) is:

9-(2,6-Bis(trifluoromethyl)phenoxy carbonyl)-10-(10-succinimidyloxy carbonyl decyl)acridinium trifluoromethanesulfonate;

9-(2,6-dibromophenoxy carbonyl)-10-(10-succinimidyloxy carbonyl decyl)acridinium trifluoromethanesulfonate;

9-(2,6-bis(trifluoromethyl)phenoxy carbonyl)-10-(3-succinimidyloxy carbonyl propyl)acridinium trifluoromethanesulfonate;

9-(2,6-dibromophenoxy carbonyl)-10-(3-succinimidyloxy carbonyl propyl)acridinium trifluoromethanesulfonate;

9-(2,6-dinitrophenoxy carbonyl)-10-(10-succinimidyloxy carbonyl decyl)acridinium trifluoromethanesulfonate; or

9-(2,6-dinitrophenoxy carbonyl)-10-(3-succinimidyloxy carbonyl propyl)acridinium trifluoromethanesulfonate.

11. The probe of any one of claims 1 to 10, wherein said quencher is methyl red.

12. The compound:

9-(2,6-Bis(trifluoromethyl)phenoxy carbonyl)-10-(10-succinimidyloxy carbonyl decyl)acridinium trifluoromethanesulfonate;

9-(2,6-dibromophenoxy carbonyl)-10-(10-succinimidyloxy carbonyl decyl)acridinium trifluoromethanesulfonate;

9-(2,6-bis(trifluoromethyl)phenoxy carbonyl)-10-(3-succinimidyloxy carbonyl propyl)acridinium trifluoromethanesulfonate;

9-(2,6-dibromophenoxy carbonyl)-10-(3-succinimidyloxy carbonyl propyl)acridinium trifluoromethanesulfonate;

9-(2,6-dinitrophenoxy carbonyl)-10-(10-succinimidyloxy carbonyl decyl)acridinium trifluoromethanesulfonate; or

9-(2,6-dinitrophenoxy carbonyl)-10-(3-succinimidyloxy carbonyl propyl)acridinium trifluoromethanesulfonate.

13. A method of detecting or monitoring association or dissociation of first and second binding partners in a medium, wherein the first binding partner is labelled with the compound of claim 12, and the second binding partner is labelled with a quencher or is itself a quencher, such that when the first and second binding partners are associated, a chemiluminescent signal of the compound of claim 12 is modulated; the method comprising adding hydrogen peroxide to the medium while maintaining the pH at 6-10 and detecting emission of chemiluminescent radiation.

14. The method as claimed in claim 13, wherein the first and second binding partners are molecular species which associate or dissociate via a covalent reaction.

15. The method as claimed in claim 13, wherein the first and second binding partners are a ligand and an anti-ligand.

16. A method of detecting presence or absence in a medium of an analyte comprising a quencher for the compound of claim 12, the method comprising:

- a) adding to the medium a specific binding partner for the analyte, said specific binding partner labelled with the compound of claim 12 such that if analyte is present, a specific binding complex is formed;
- b) adding hydrogen peroxide to the medium while maintaining the pH at 6-10; and
- c) detecting emission of chemiluminescent radiation, wherein a modulated signal of the compound of claim 12 indicates the presence of the analyte.

17. A method of detecting presence or absence in a medium of an analyte which is not a quencher for the compound of claim 12, the method comprising:

- a) adding to the medium a specific binding partner for the analyte, said specific binding partner labelled with the compound of claim 12 or a quencher for the compound of claim 12 such that if analyte is present, a specific binding complex is formed;

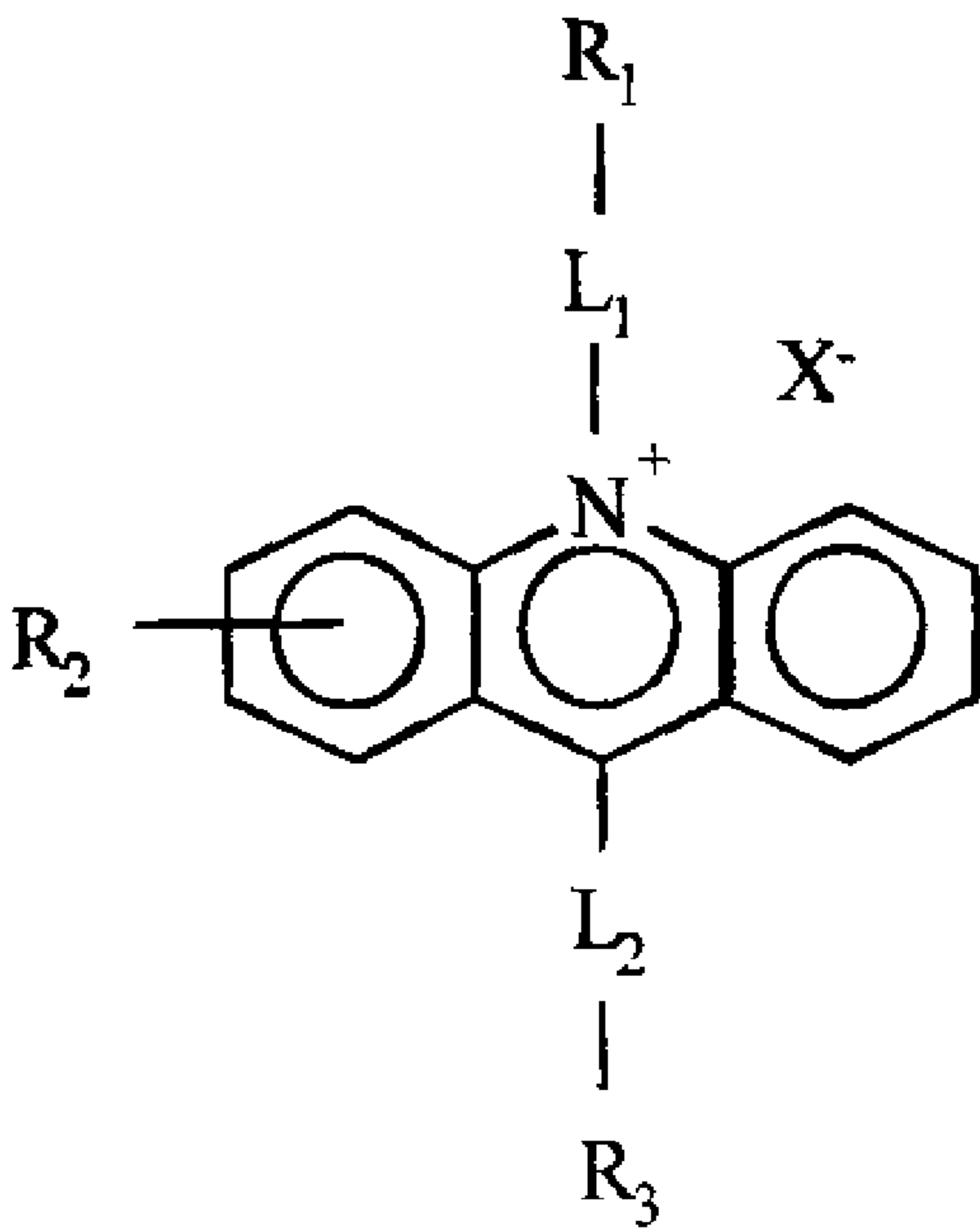
- b) adding to the medium simultaneously or sequentially a secondary binding reagent labelled with either a quencher for the compound of claim 12 used in step (a) or the compound of claim 12 if a quencher is used in step (a); and
- c) detecting presence of the specific binding complex by a chemiluminescent method including the steps of adding hydrogen peroxide to the medium while maintaining the pH at 6-10 and detecting emission of chemiluminescent radiation, wherein a modulated signal of the compound of claim 12 indicates the presence of the analyte.

18. A method of detecting presence or absence in a medium of an analyte which is not a quencher for the compound of claim 12, the method comprising:

- a) adding to the medium a specific binding partner for the analyte labelled with the compound of claim 12 or with a quencher for the compound of claim 12 such that if analyte is present, a specific binding complex is formed;
- b) adding to the medium simultaneously or sequentially a second specific binding partner labelled with either a quencher or the compound of claim 12 to the first labelled specific binding partner; and
- c) detecting the presence of specific binding complex by a chemiluminescent method including the steps of adding hydrogen peroxide to the medium while maintaining the pH at 6-10 and detecting emission of chemiluminescent radiation, wherein a modulated signal of the compound of claim 12 indicates the presence of the analyte.

19. A method for detecting presence of inhibitors or promoters of a binding process, the method comprising mixing a first and a second specific binding partner labelled respectively with the compound of claim 12 and a quencher, adding hydrogen peroxide to the medium while maintaining the pH at 6-10 and detecting emission of chemiluminescent radiation, wherein modulation of a chemiluminescent signal of the compound of claim 12 indicates promotion of the binding process and the presence of an unmodulated signal indicates inhibition of the binding process.

20. The method as claimed in claim 19 for monitoring cleavage of an enzyme substrate or hybridisation of complementary nucleic acids.
21. The method as claimed in any one of claims 13 to 20, wherein the quencher is methyl red.
22. A method of detecting a target nucleic acid in a sample, the method comprising adding the probe of any one of claims 1 to 11 to the sample wherein the probe comprises said central section that is complementary to said target nucleic acid, adding hydrogen peroxide to the medium while maintaining the pH at 6-10 and detecting emission of chemiluminescent radiation.
23. The method of any one of claims 13 to 22, wherein adding hydrogen peroxide and detecting chemiluminescent emission occurs at about pH 9.
24. A kit comprising the probe of any one of claims 1 to 11, hydrogen peroxide solution and a buffer adapted to maintain the pH of an analyte containing medium at pH 6-10.



(I)