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(19) (CA) **CANADIAN PATENT** (12)

(54) Polysubstituted Aryl Acridinium Esters

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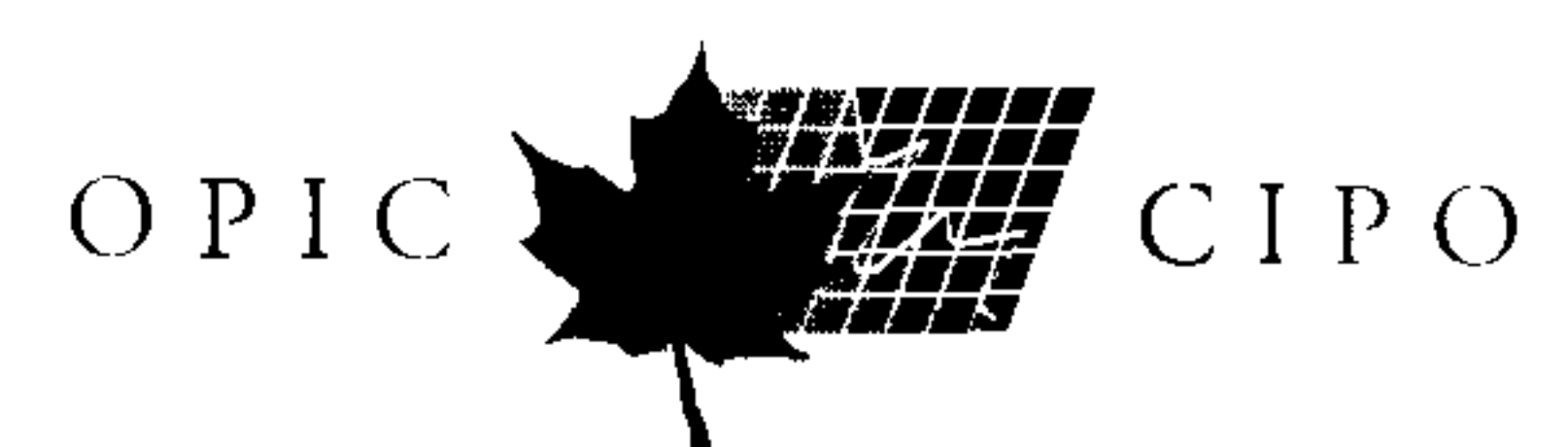
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(30) (US) U.S.A. 915,527 1986/10/06

(57) 17 Claims

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Canada



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Abstract

55 The present invention relates to novel acridinium esters which are
useful as luminescent labels in specific binding assays such as immunoassays or
nucleic acid hybridization assays. More particularly, polysubstituted aryl
acridinium esters are highly stable labels for use in a chemiluminescent
immunoassay.

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POLYSUBSTITUTED ARYL ACRIDINIUM ESTERS

Technical Field

5 The present invention relates to novel acridinium esters which are useful as luminescent labels in specific binding assays such as immunoassays or nucleic acid hybridization assays. More particularly, polysubstituted aryl acridinium esters have been found to be unexpectedly stable labels for use in a chemiluminescent immune assay.

Background Art

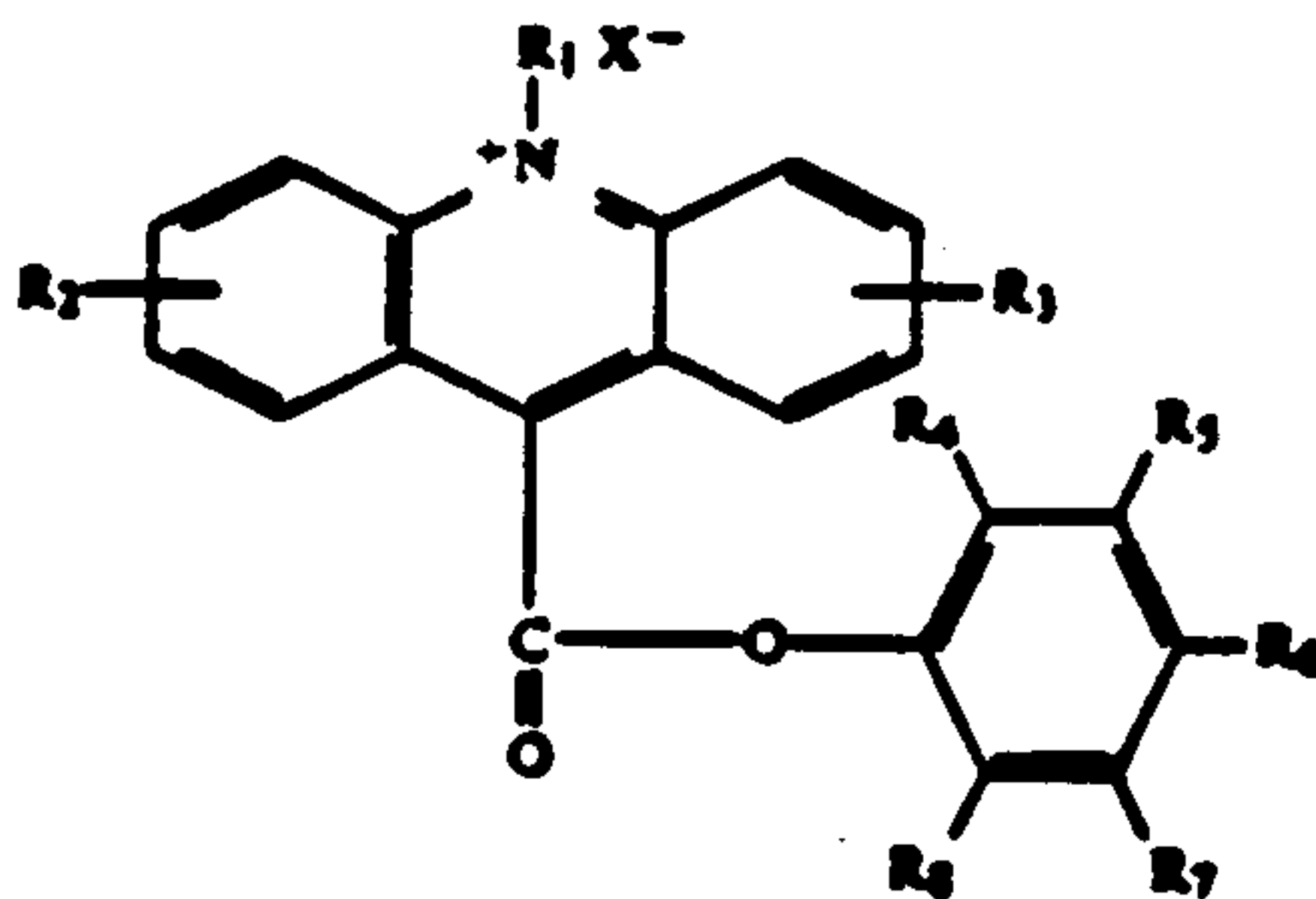
10 The use of unsubstituted aryl acridinium esters as chemiluminescent labels is disclosed by J.W. Woodhead et al in U.K. patent 2,008,247 and again in European published patent application No. 82,636. However, these compounds did not have any substituents on the ortho
15 positions of the phenoxy ring which constitutes part of the aryl ester component. Unfortunately the Woodhead compounds were not stable in pH 7.4 buffer media, and thus, not useful for commercial assays.

Disclosure of the Invention

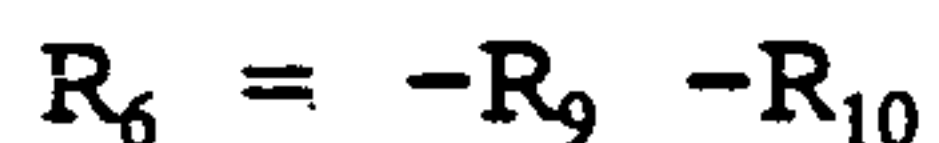
20 The present invention comprises novel polysubstituted aryl acridinium esters and luminescent labelled conjugates using these esters. As a result of these substitutions, the present compounds and conjugates have an unexpectedly better stability in pH 7.4 buffer
25 media, a threefold increase in light emitting efficiency when configured as a conjugate, and a twofold improvement in the signal-to-noise ratio when used in a solid phase specific binding assay.

30 According to an aspect of the invention, a luminescent conjugate for use in luminescent assays comprises a polysubstituted aryl acridinium ester of the formula:

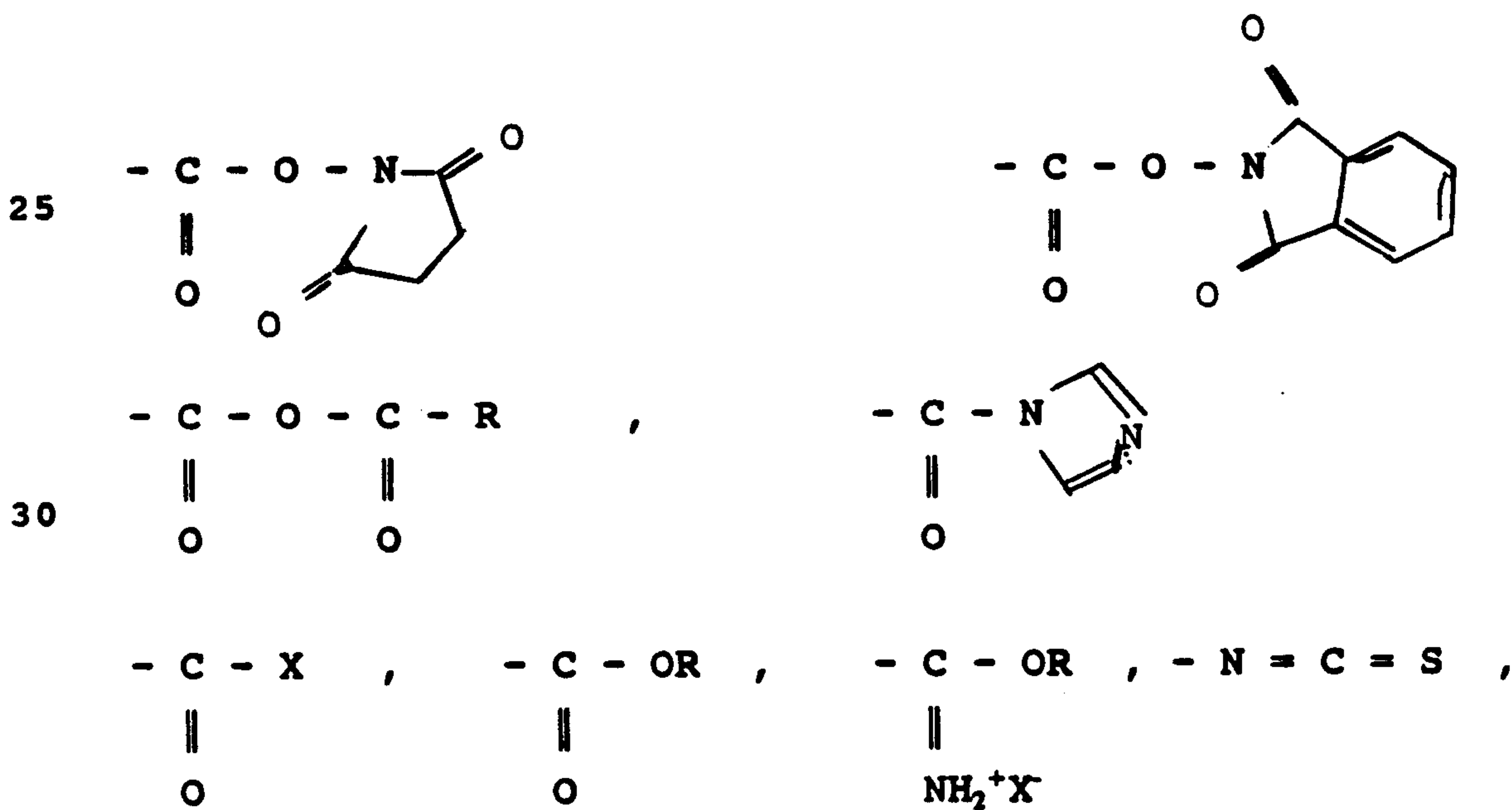
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where R_1 is an alkyl, alkenyl, alkynyl or aryl group; R_2 ,
 10 R_3 , R_5 or R_7 is a hydrogen, amino, carboxyl, hydroxyl,
 alkoxyl, nitro, or halide group; R_4 or R_8 is an alkyl,
 alkenyl, alkynyl, aryl, alkoxyl, amino, amido,
 sulfonamido, or sulfide group; and R_6 represents hydrogen,
 amino, carboxyl, hydroxyl, alkoxyl, nitro or halide group
 15 or the following substituent:

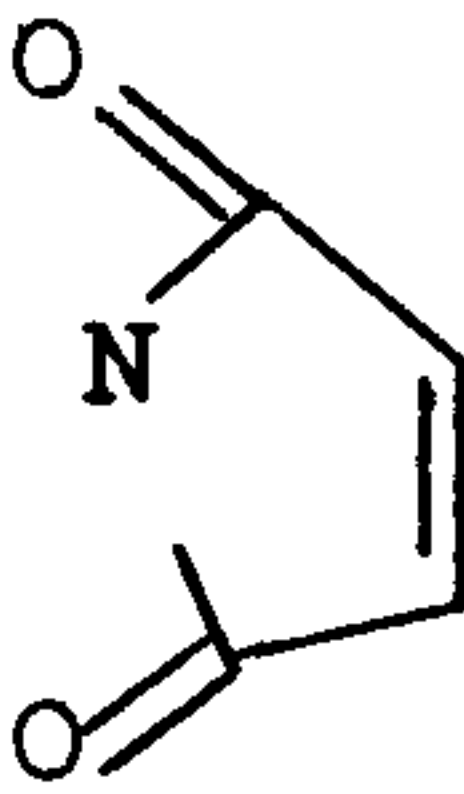


where R_9 is not required but optionally can be an alkyl,
 20 aryl, or aralkyl group, and R_{10} is selected from the
 following:

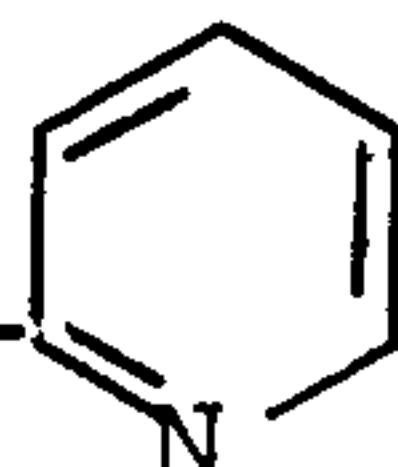


- N = C = O , - N₂⁺X⁻ , a halide , -N₃ , - C - OH

- OSO₂F , - OSO₂CF₃ , - OSO₂C₄F₉ , -OSO₂--CH₃

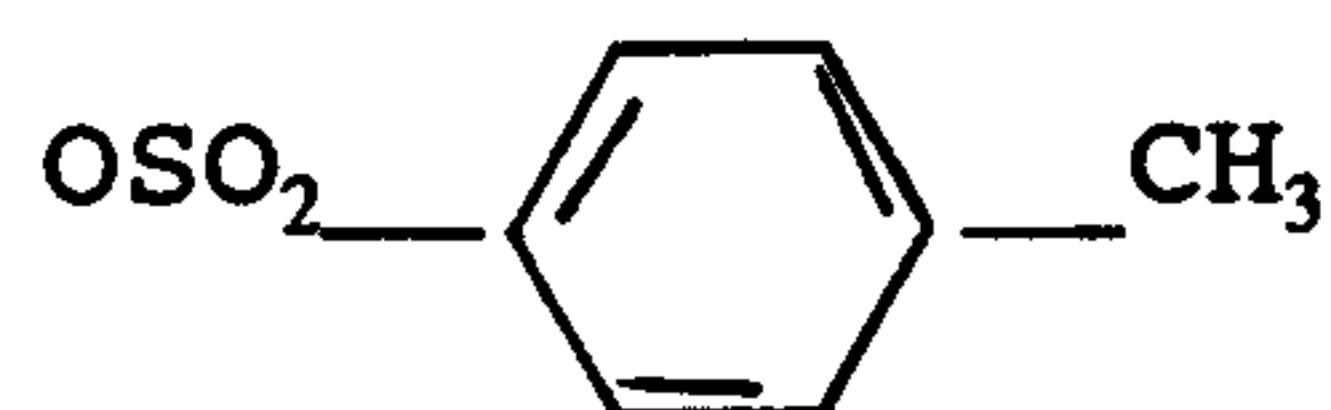
- NH₂ , - NHC(=O)-R-N

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-NHC(=O)-R-S-S-

15

X is CH₃SO₄⁻ , OSO₂F⁻ , a halide , OSO₂CF₃⁻ , OSO₂C₄F₉⁻ ,



20

R is alkyl, aryl, aralkyl; and finally R₅, R₆, and R₇ substituent positions on the phenoxy ring are interchangeable, wherein the R₆ substituent of the polysubstituted aryl acridinium ester is covalently coupled to a molecule with biological activity which molecule is capable of being covalently coupled to the R₆ substituent.

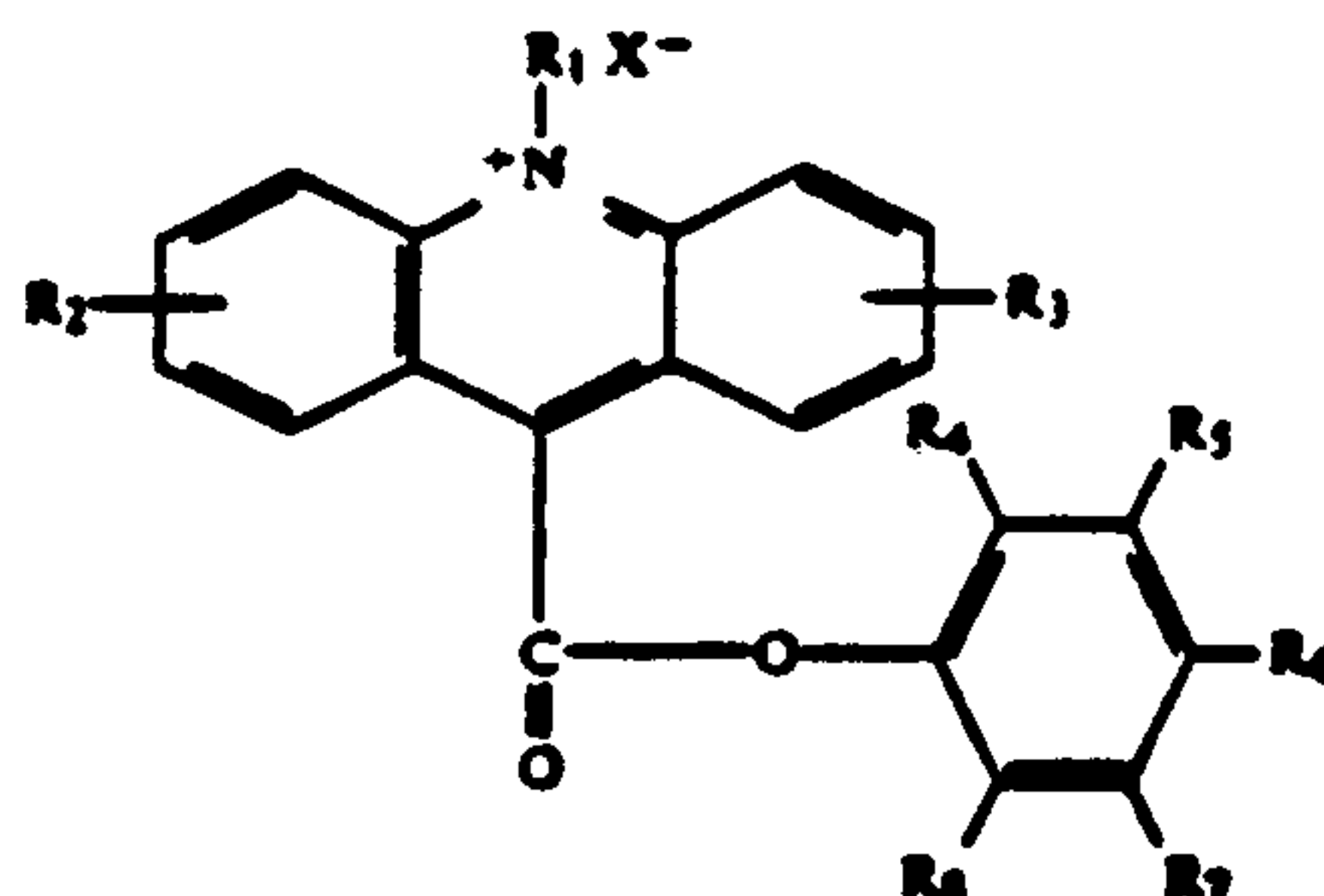
According to another aspect of the invention, a luminescent compound comprising a polysubstituted aryl acridinium ester selected from the group having the following structure:

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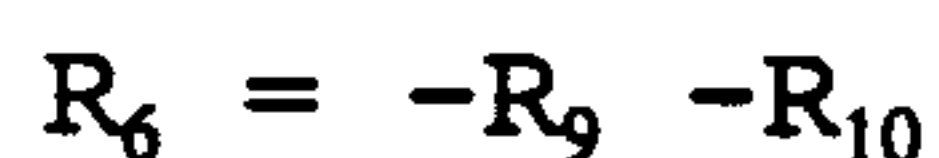
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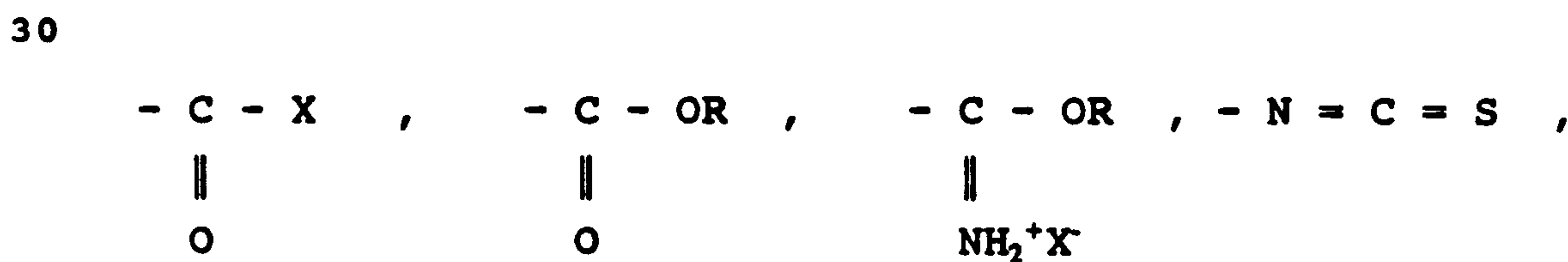
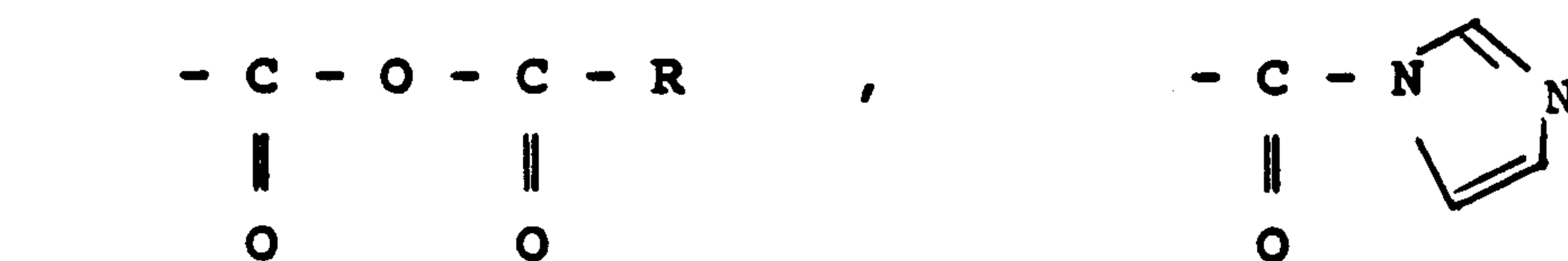


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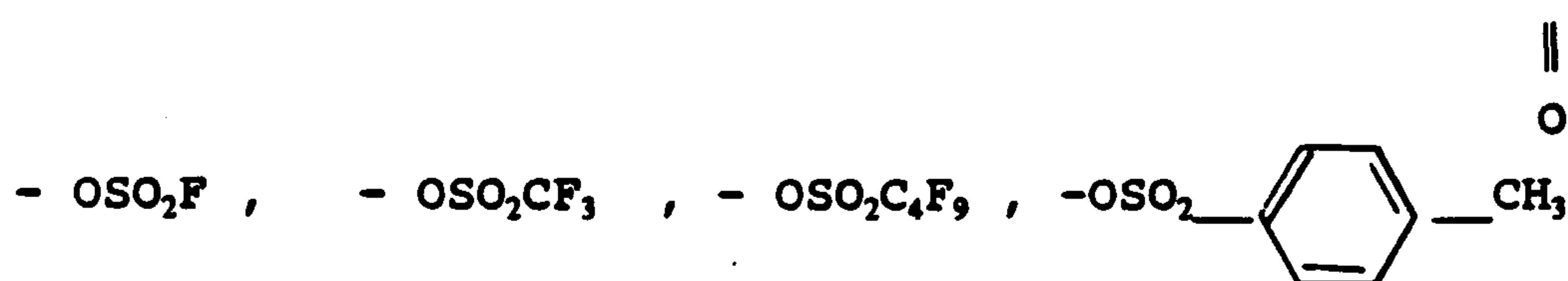
where R_1 is an alkyl, alkenyl, alkynyl or aryl group; R_2 , R_3 , R_5 or R_7 is a hydrogen amino, carboxyl, hydroxyl, alkoxy, nitro, or halide group; R_4 or R_8 is an alkyl group; and R_6 represents hydrogen, amino, carboxyl, hydroxyl, alkoxy, nitro or halide group or the following substituent:



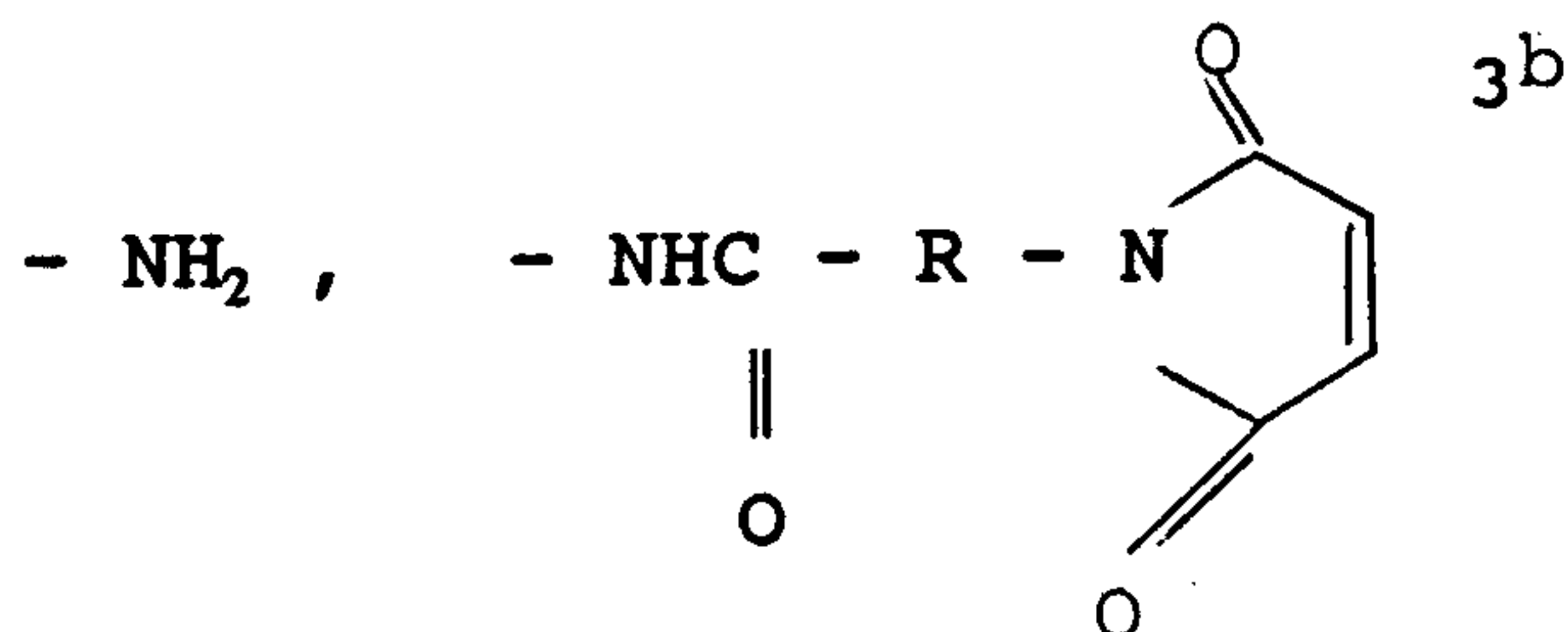
where R_9 is not required but if present is an alkyl, aryl, or aralkyl group, and R_{10} is selected from the following:



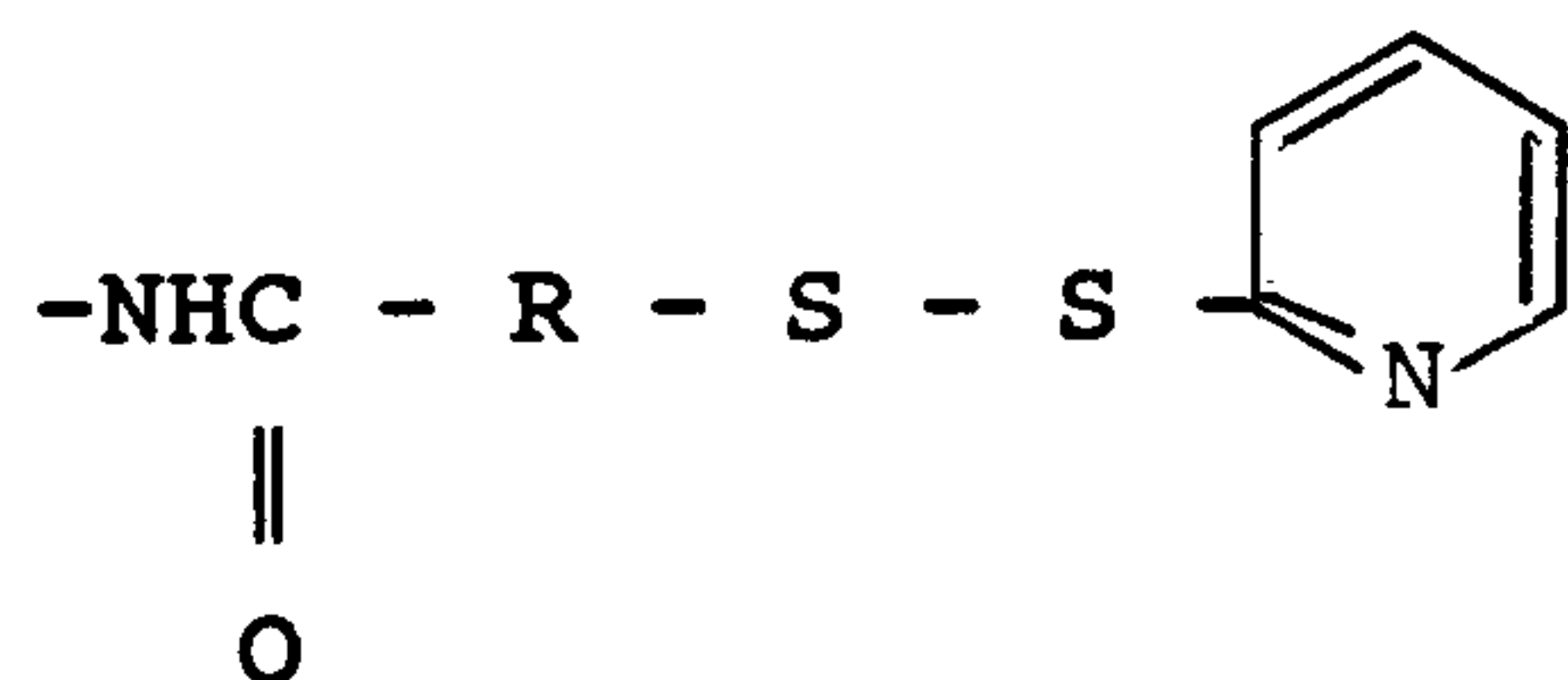
- $N = C = O$, - $N_2^+ X^-$, a halide , - N_3 , - $C - OH$



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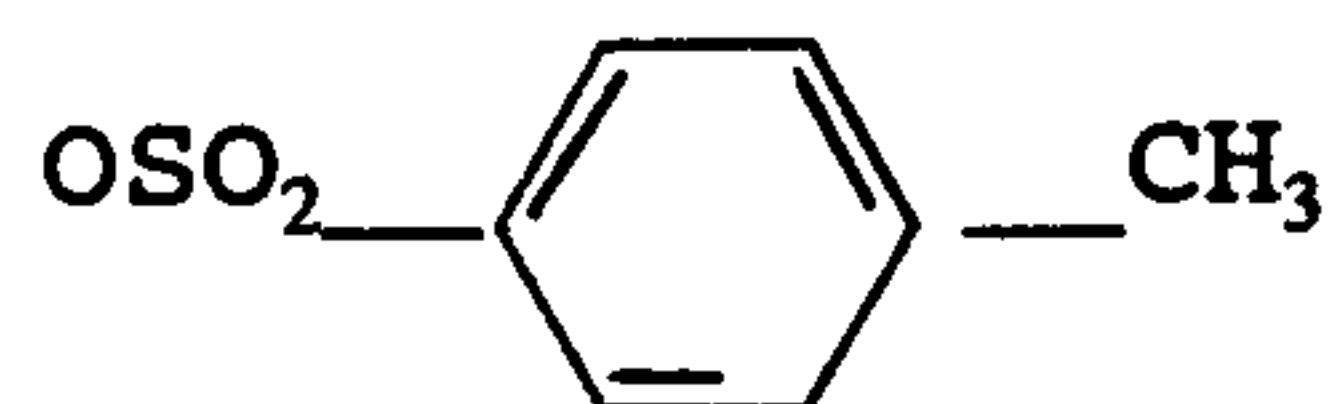
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10

X is CH₃SO₄⁻ , OSO₂F⁻ , a halide, OSO₂CF₃⁻ , OSO₂C₄F₉⁻ ,

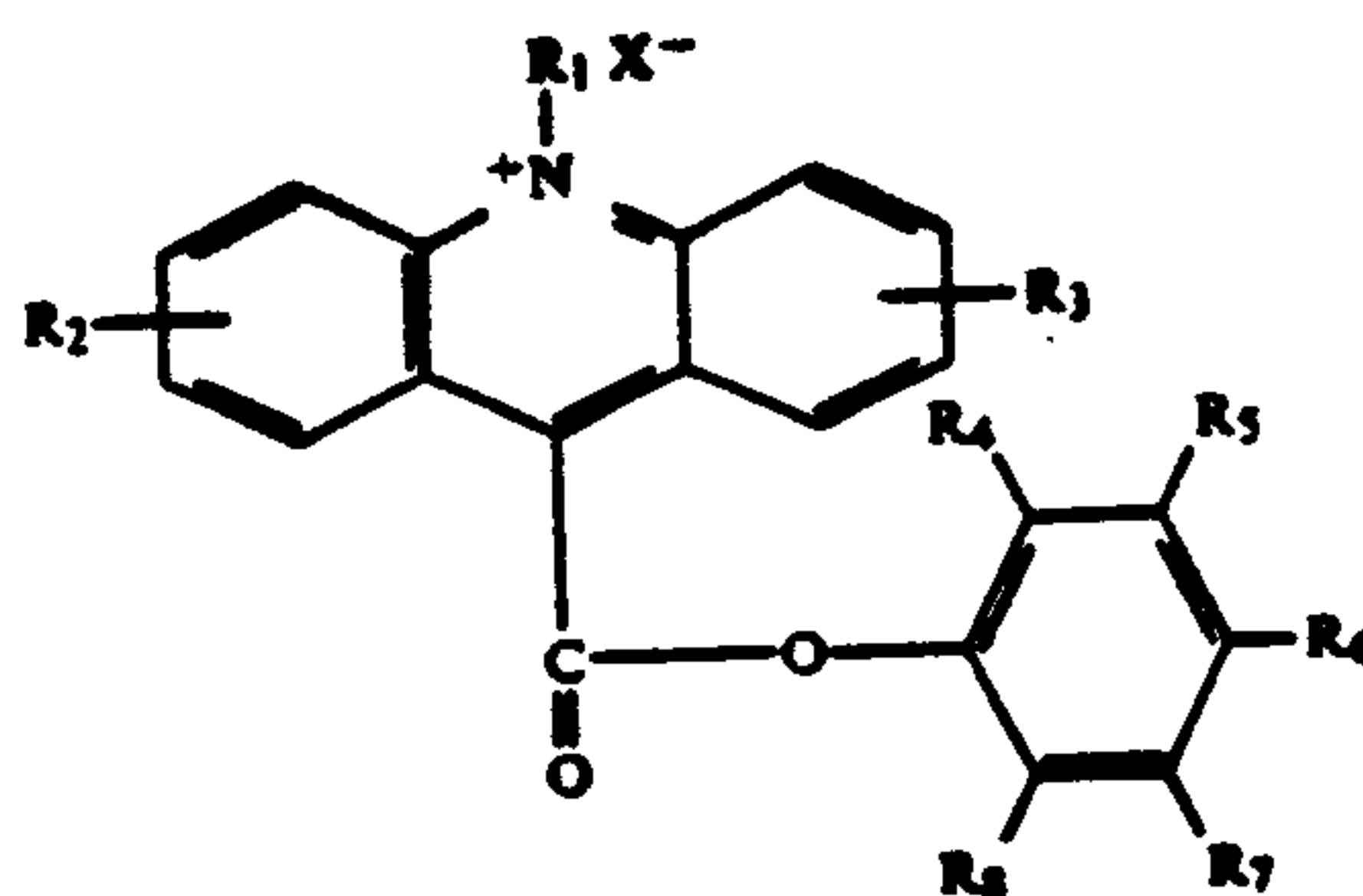
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R is alkyl, aryl, aralkyl; and finally R₅, R₆, and R₇ substituent positions on the phenoxy ring are interchangeable.

According to another aspect of the invention, a luminescent compound comprising a polysubstituted aryl acridinium ester selected from the group having the following structure:

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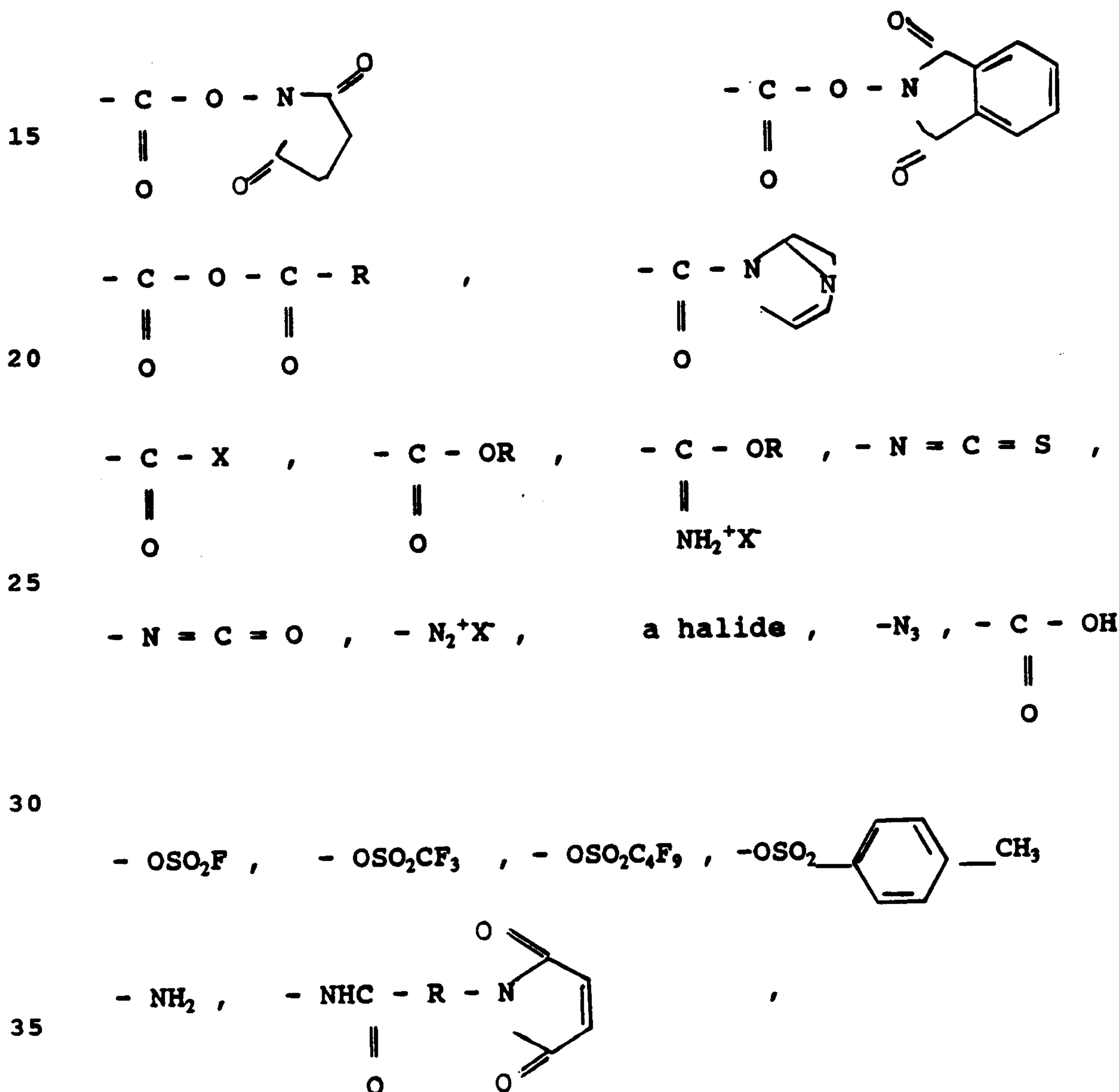
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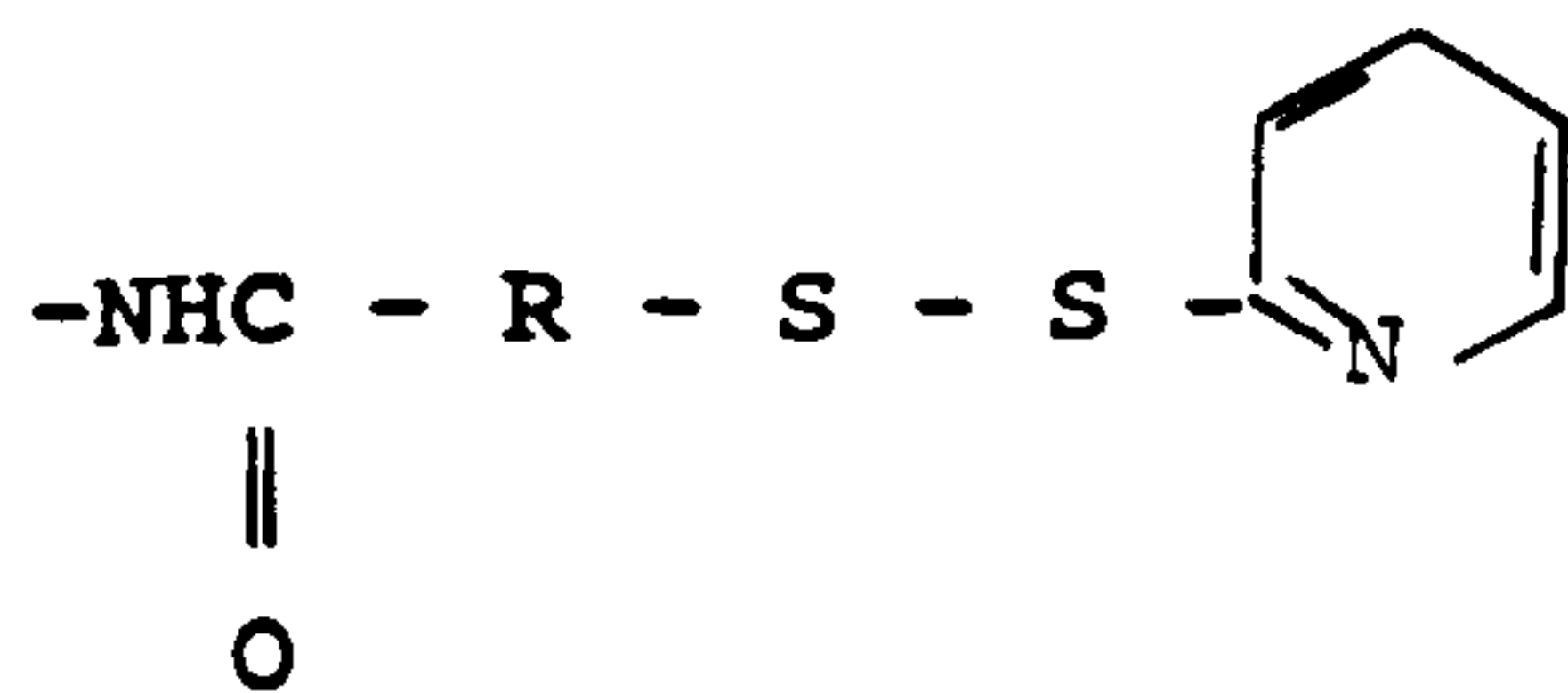
where R₁ is an alkyl, alkenyl, alkynyl or aryl group; R₂, R₃, R₅ or R₇ is a hydrogen, amino, carboxyl, hydroxyl,

alkoxyl, nitro, or halide group; R_4 or R_8 is an alkyl,
 alkenyl, alkylanyl, aryl, alkoxy, amino, amido,
 5 sulfonamido, or sulfide group; and R_6 represents hydrogen,
 amino, carboxyl, hydroxyl, alkoxyl, nitro or halide group
 or the following substituent:



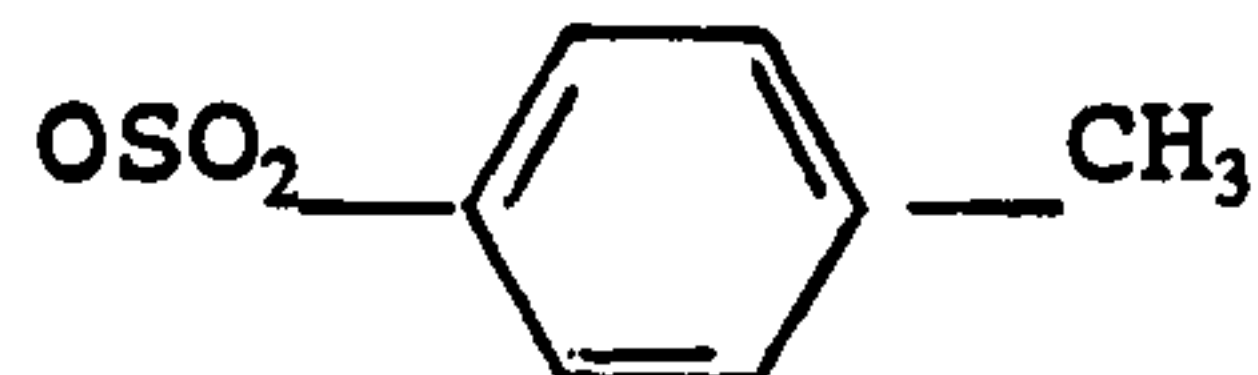
where R_9 is not required but optionally can be an alkyl,
 10 aryl, or aralkyl group, and R_{10} is selected from the
 following:





3 d 13 4 1 4 9 8

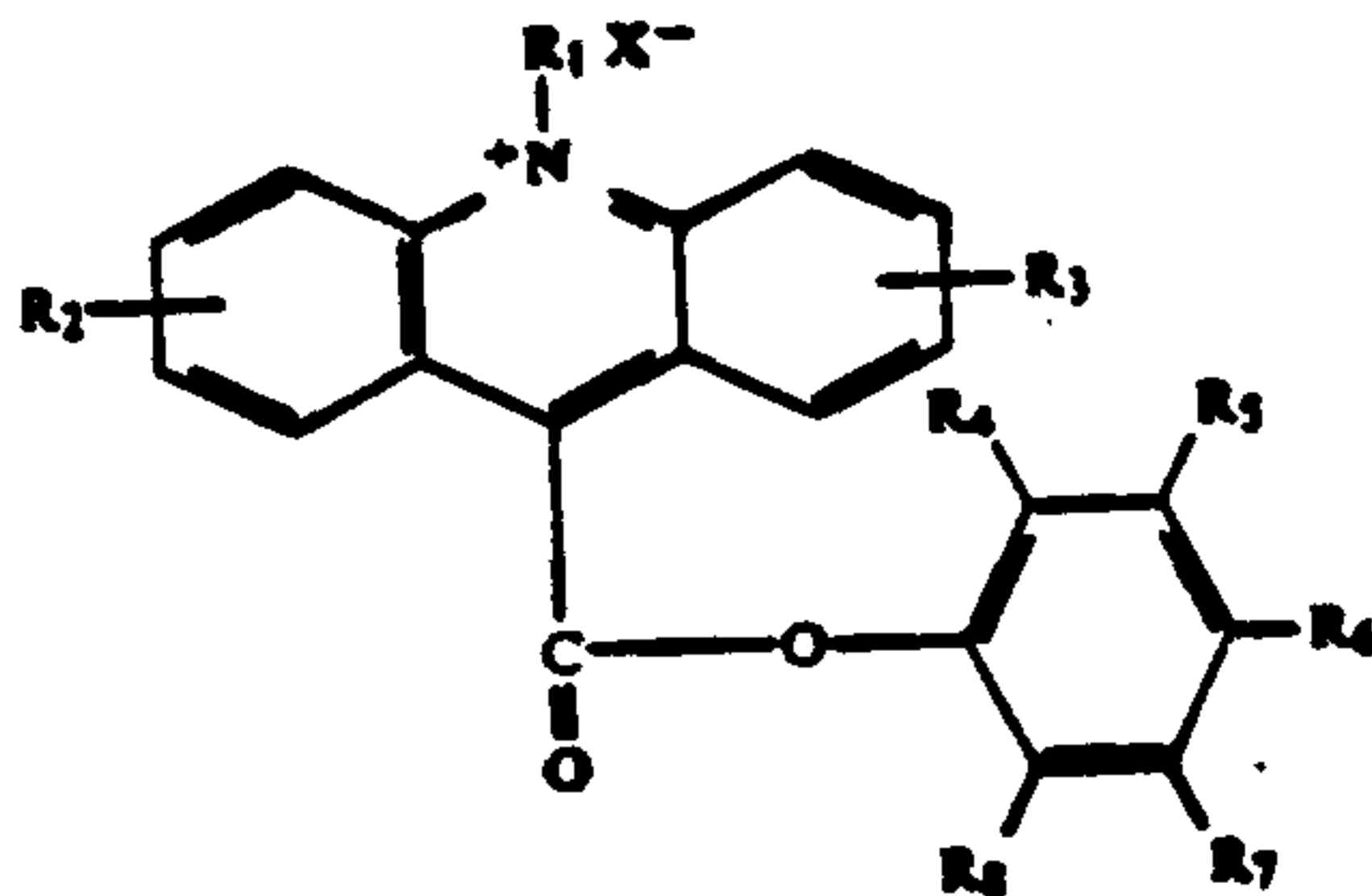
5 X is CH_3SO_4^- , OSO_2F^- , a halide, $\text{OSO}_2\text{CF}_3^-$, $\text{OSO}_2\text{C}_4\text{F}_9^-$,



R is alkyl, aryl, aralkyl; and finally R_5 , R_6 , and R_7
 10 substituent positions on the phenoxy ring are
 interchangeable.

According to another aspect of the invention, a
 luminescent compound comprising a polysubstituted aryl
 acridinium ester selected from the group having the
 15 following structure:

20

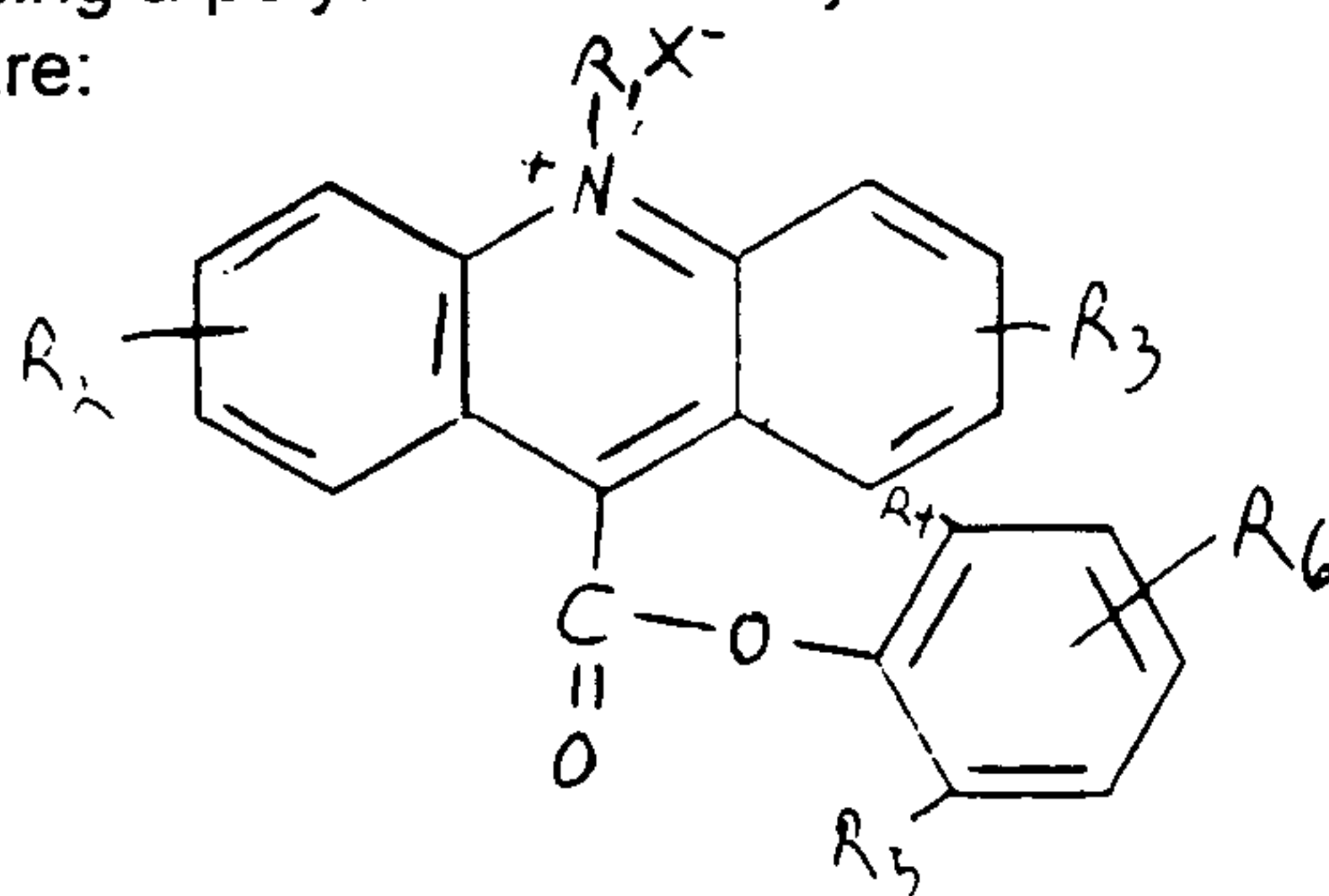


where R_1 is an alkyl, alkenyl, alkynyl or aryl group; R_2 ,
 25 R_3 , R_5 , R_6 or R_7 is a hydrogen, amino, carboxyl, hydroxyl,
 alkoxyl, nitro, or halide group; R_4 or R_8 is an alkyl,
 alkenyl, alkynyl, aryl, alkoxy, amino, amido,
 sulfonamido, or sulfide group; and finally R_5 , R_6 , and R_7
 30 substituent positions on the phenoxy ring are
 interchangeable.

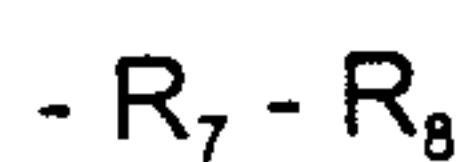
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In accordance with another aspect of the invention,

A luminescent compound comprising a polysubstituted aryl acridinium ester selected from the group having the following structure:

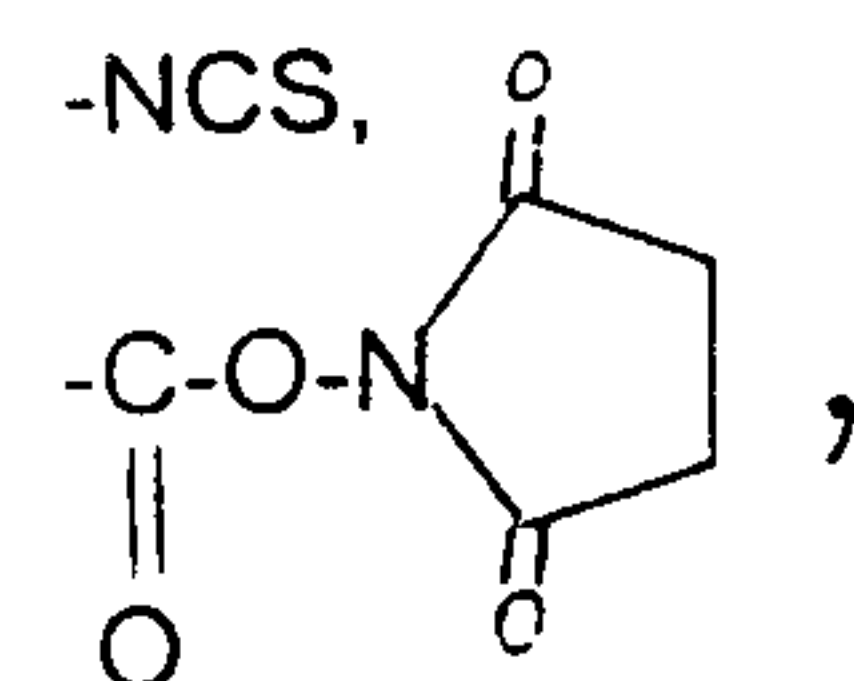
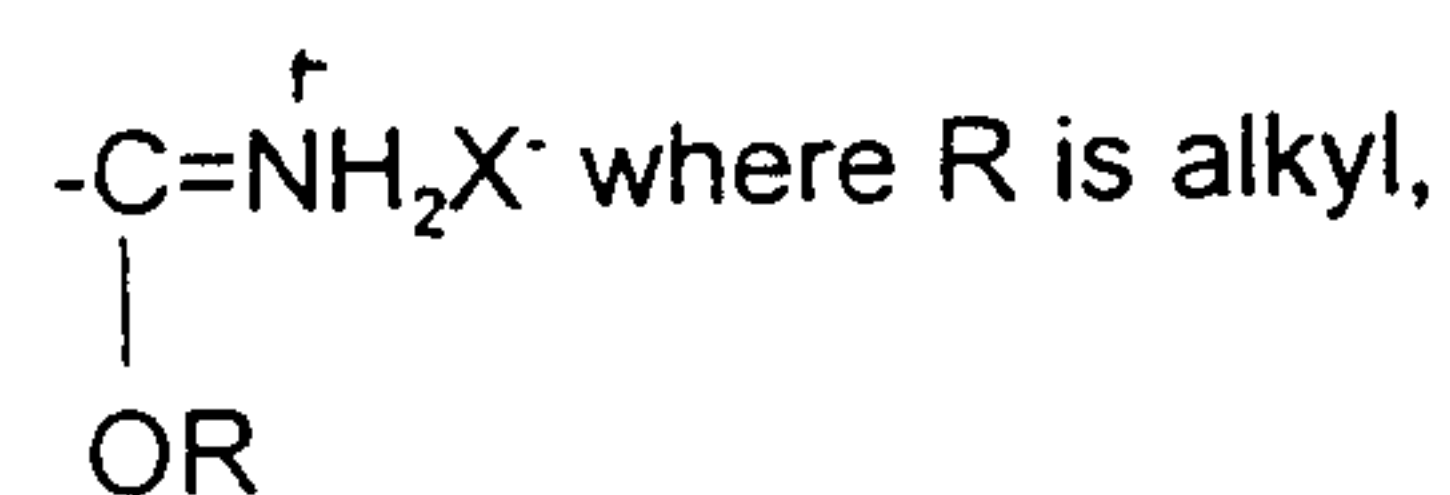


where R_1 is alkyl; R_2 and R_3 are each a hydrogen, carboxyl or amino group; R_4 and R_5 are each an alkyl, aryl or alkoxy group and R_6 is a hydrogen, nitro or halide group or a group of the following formula:



where R_7 is an alkyl, aryl or aralkyl group or a direct bond and R_8 is selected from the following:

CO_2R where R is alkyl, aryl or hydrogen,



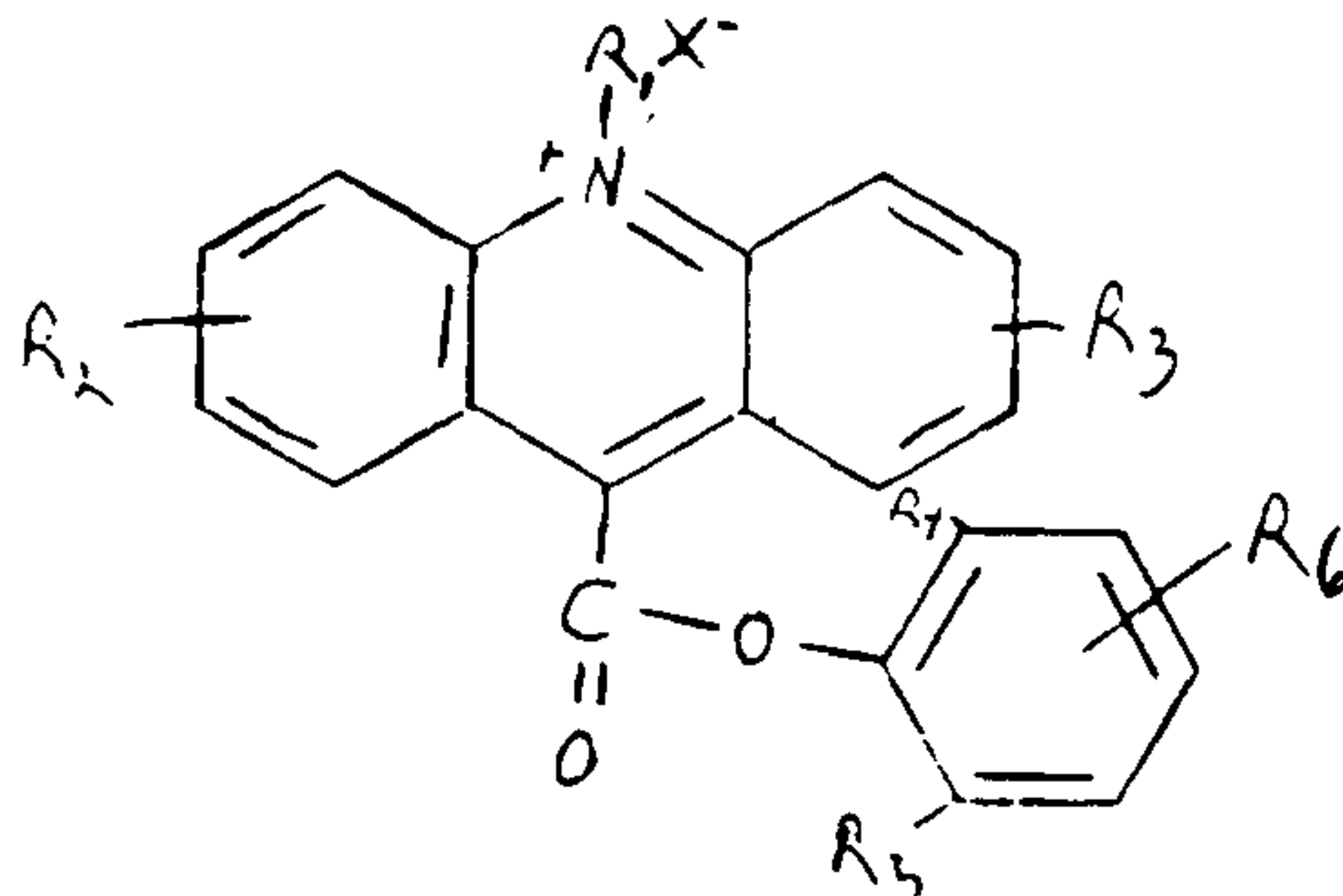
- N_3 or

- NH_2

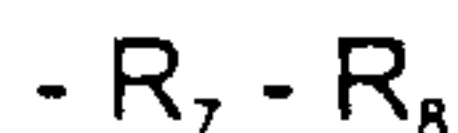
and X^- is an anion.

In accordance with a further aspect of the invention,

A luminescent conjugate for use in luminescent assays comprising a polysubstituted aryl acridinium ester selected from the group having the following structure:

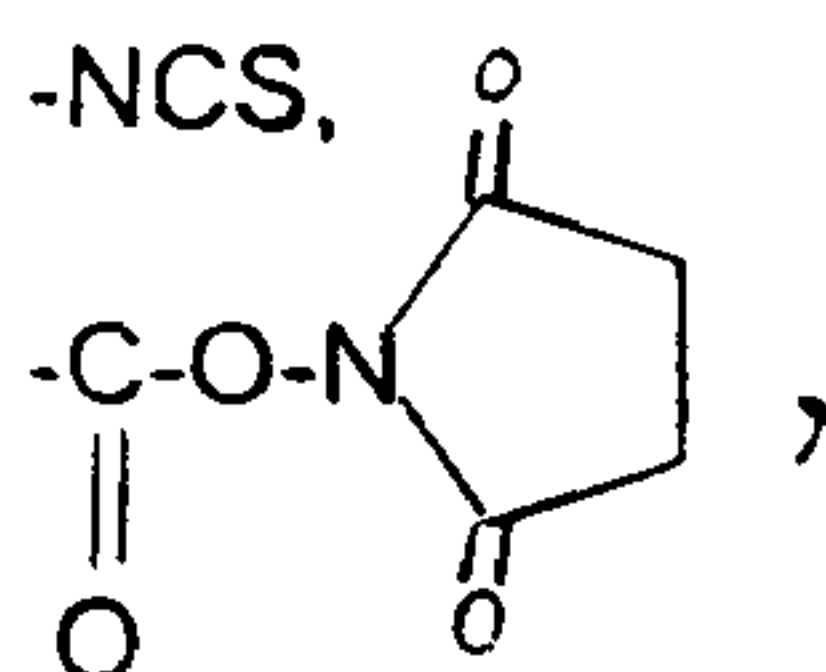
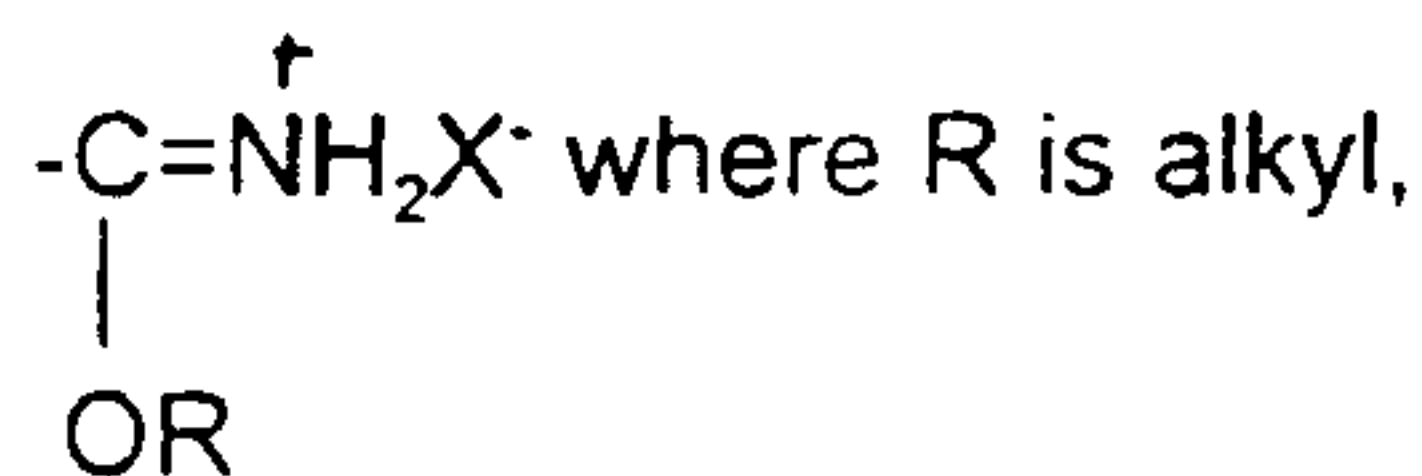


where R_1 is alkyl; R_2 and R_3 are each a hydrogen, carboxyl or amino group; R_4 and R_5 are each an alkyl, aryl or alkoxy group and R_6 is a hydrogen, nitro or halide group or a group of the following formula:



where R_7 is an alkyl, aryl or aralkyl group or a direct bond and R_8 is selected from the following

Co_2R where R is alkyl, aryl or hydrogen,



- N_3 or

- NH_2

and X^- is an anion

wherein the R_6 substituent of the polysubstituted aryl acridinium ester is covalently coupled to a molecule with biological activity which molecule is capable of being covalently coupled to the substituent.

Applicants believe that the remarkable improvements in the performance of the present polysubstituted aryl acridinium esters over the prior unsubstituted ones as labels for specific binding assays arises from the shielding of the aryl ester linkage in the present

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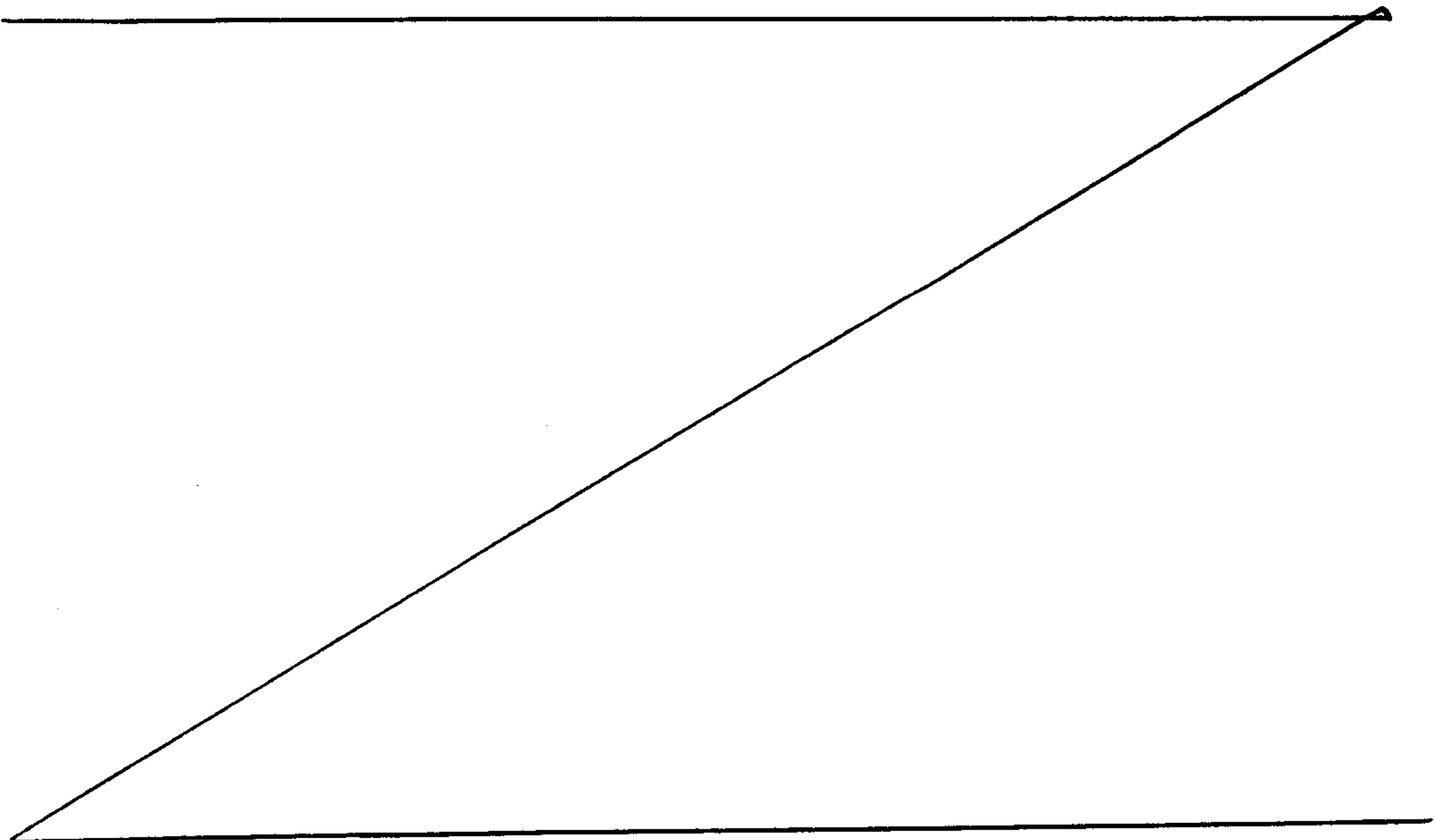
5 compounds. Steric effects, electronic effects, or a combination thereof enable these compounds to be commercially useful in luminescent assays using specific binding phenomena known to the art as antibody/antigen immunological reactions or complementary nucleic acid hybridization reactions.

10 As known in the art, labelling compounds for these assays can be used in many different conventional architectures including being coupled either to the ligand or analyte (such as an antigen), or to the specific binding partner of the ligand or analyte (such as the corresponding antibody).

Modes of the Invention

15 Preferentially, the present polysubstituted aryl acridinium ester compounds should be as described above with the following substituents: R_1 is a methyl group; R_2 , R_3 , R_5 and R_7 are hydrogen; R_4 and R_8 are a methyl group, R_{10} is a N-succinimidyl oxycarbonyl group attached directly to the para-position of the phenoxy ring; and X
20 is CH_3SO_4^- .

The following Examples disclose how to synthesize a preferred compound, the ortho-dimethyl aryl acridinium ester.



Synthesis of Preferred Compounds

Example 1 Preparation of the Potassium Salt of
3,5-Dimethyl-4-Hydroxybenzoic Acid

5

To a solution of 3,5-dimethyl-4-hydroxybenzoic acid (7.8 g, 47 mmole) in 50 ml of methanol was added dropwise 2.5 N NaOH until the pH of the mixture was about 7.0. The reaction mixture was stirred at room temperature for one hour and evaporated to dryness under a vacuum. The residue was triturated with acetone
10 and filtered. The desired potassium salt was collected as a white salt then washed with acetone, and air dried (7.6 g, 80%).

Example 2 Preparation of the Benzyl Ester of 3,5-Dimethyl-4-Hydroxybenzoic
Acid

15

The potassium salt of 3,5-dimethyl-4-hydroxybenzoic acid in Example 1 (5.6 g, 27 mmole), dibenzo-18-crown-6 (1.0 g, 3 mmole) and 100 ml of DMF/acetonitrile (1:2, v/v) were added to a 250 ml round bottom flask equipped with a reflux condenser, a drying tube, and a stirring bar. The mixture was heated in an oil bath at 80⁰ C for 30 min, before adding benzyl chloride (3.5 ml, 30 mmole). This reaction
20 mixture was heated at 80⁰ C for another 3 hours, cooled and filtered. The filtrate was evaporated to dryness. The residue was taken up with a minimal amount of chloroform and purified on a packed silica gel column (60 A, 230-400 mesh) which was eluted with chloroform/hexane (3:1, v/v). The elution was later changed to 100% chloroform. The fractions having an R_f of 0.6 on E. Merck
25 silica gel TLC plate when developed with toluene/ethyl acetate (4:1, v/v) were pooled, then evaporated to give a white solid, the desired benzyl ester (4.8g, 70%; m.p. 108⁰ C).

Example 3 Preparation of 9-Acridinecarbonyl Chloride

30

A mixture of 9-acridinecarboxylic acid hydrate (7.3 g, 30 mmole), and thionyl chloride (50 ml, 0.685 mole) was heated at reflux in a round bottom flask equipped with a reflux condenser, a drying tube, and a stirring bar. The reaction mixture was heated further for one hour after the dissolution occurred,

35

then cooled and evaporated to remove any excess thionyl chloride. The residue was directly utilized in the subsequent reaction without further purification.

5 Example 4 Preparation of 2',6'-Dimethyl-4'- Benzyloxycarbonylphenyl
Acridine-9-Carboxylate

A solution of the benzyl ester of 3,5-dimethyl-4-hydroxybenzoic acid from Example 2 (6.4 g, 25 mmole) and 4-dimethylaminopyridine (345 mg, 3 mmole) in 50
10 ml of pyridine (over 3 A molecular sieves) was heated at 90⁰ C in a round bottom flask equipped with a drying tube. The solution was cooled and pipetted into a flask containing the dried acridine-9-carbonyl chloride from Example 3 (7.05 g, 29.2 mmole). The reaction mixture was stirred at 100⁰ C for 3 hours, then at room temperature overnight. After the removal of solvent, the crude product was
15 purified on a packed silica gel column which was eluted with chloroform, followed by elution with ethylacetate. The desired product having an R_f of 0.6 in toluene/ethylacetate (4:1, v/v) was further purified by crystallization from ethylacetate/hexane (6.37 g, 50%; m.p. 146-148⁰ C).

20 Example 5 Preparation of 2',6'-Dimethyl-4'-Carboxylphenyl Acridine-9-
Carboxylate

A mixture of 2',6'-dimethyl-4'-benzyloxycarbonylphenyl acridine-9-carboxylate from Example 4 (5.4 g, 11.7 mmole), 100 ml of glacial acetic acid, and 25 ml of
25 48% hydrogen bromide was heated at 100⁰ C for 3 hours and cooled. The reaction mixture was added to 600 ml of water and extracted with 20% methanol in chloroform (3 times with 100 ml). The organic extracts were combined, evaporated, and the residue was washed with hexane on a funnel having a fritted disc. The yellow solid (obtained as a HBr salt) was suspended in chloroform and
30 neutralized with a slight excess of triethylamine before being washed with water, dried over sodium sulfate, and evaporated (3.83 g, 88%). The desired product thus obtained had an R_f of 0.4 in 10% methanol/chloroform.

Example 6 Preparation of 2',6'-Dimethyl-4'-(N-Succinimidylloxycarbonyl)
Phenyl Acridine-9-Carboxylate

A solution of 2',6'-dimethyl-4'-carboxylphenyl acridine-9-carboxylate from
5 Example 5 (3.58 g, 9.64 mmole) in 450 ml of DMF was placed in a one liter round
bottom flask equipped with a drying tube and a stirring bar. The solution was
cooled in an ice bath for 10 minutes, then mixed with a solution of
dicyclohexylcarbodiimide (3.0 g, 14.6 mmole) in 50 ml of DMF. The
reaction mixture was stirred in an ice bath for 30 minutes, mixed with a
10 solution of N-hydroxysuccinimide (1.7 g, 14.5 mmole) in 50 ml of DMF,
stirred at room temperature overnight, and evaporated to dryness. The
residue was extracted with 150 ml of methylene chloride, then filtered.
The filtrate was concentrated to a wet solid, triturated with 20 ml of
ethylacetate, filtered, and dried in a vacuum desiccator to obtain the
15 crude product (3.5 g). Further purification of the product was carried
out using conventional flash chromatography technique. The silica gel (J.
T. Baker, #7024-1) column was packed and eluted with
chloroform/ethylacetate (4:1, v/v). The fractions having an R_f of 0.28 on
silica gel TLC plate when developed with ethyl ether/ethylacetate (4:1, v/v)
20 were pooled and evaporated to give a white solid (95% pure by HPLC). Repeated
flash chromatography purification using a packed silica gel column packed and
eluted with hexane/acetone (2:1) yielded the pure desired product (34% yield,
m.p. 276-277⁰ C).

25

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Example 7 Preparation of 2',6'-Dimethyl-4'-(N-Succinimidylloxycarbonyl)
Phenyl 10-Methyl-Acridinium-9-Carboxylate Methosulfate

To a solution of 2',6'-dimethyl-4'-(N-succinimidylloxycarbonyl) phenyl
5 acridine-9-carboxylate from Example 6 (91.5 mg, 0.195 mmole) in 3.8 ml of
methylene chloride is added 2.0 ml of redistilled dimethyl sulfate. The
resulting solution is refluxed for four days under anhydrous condition. To the
yellow homogeneous solution at room temperature is added 15 ml of diethyl ether
which precipitates the desired product. Purification is by means of
10 recrystallization from methylene chloride and diethyl ether mixture to give a
yellow solid, (92.7 mg, 80%) m.p. 228-230⁰ C.

Example 8 Preparation of 2',6'-Dimethyl-4'-Benzyloxycarbonylphenyl 10-
Methyl-Acridinium-9-Carboxylate Methosulfate

15 A solution of 2',6'-dimethyl-4'-benzyloxycarbonylphenyl acridine-9-carboxylate
from Example 4 (46 ml, 0.1 mmole) in 1 ml of chloroform and 0.3 ml of dimethyl
sulfate (3.17 mmole) were placed in a 25 ml round bottom flask equipped with a
Vigreux distillation column and a stirring bar. The solution was heated at
20 105⁰ C for 10 minutes, and cooled. The reaction mixture was diluted with 2
ml of methylene chloride, then anhydrous ethyl ether was added to obtain
maximal precipitation of the desired product. The mixture was filtered,
washed with ethyl ether, and air dried to give a yellow solid (46 mg,
78%).

25

It should be noted that during synthesis the R₁₀ moiety can be either as
defined above, or, more often, it can be a precursor which later requires
transformation into the defined moiety. For example, the precursor may comprise
protective groups linked to the defined moiety which are removed to yield the
30 defined moiety. Such synthesis techniques are known to the art.

Preparation of Present Conjugates

Depending on which R_{10} coupling moiety is selected, the ester label can be reacted directly with the specific binding partner or the ligand, either
5 in an aqueous or an organic media. Again such coupling techniques are well known to the art.

Light Emitting Efficiency

10 Interestingly, whereas alone the preferred ortho-dimethyl substituted aryl acridinium compound has one third the light emitting efficiency of the prior unsubstituted compound, when it is coupled to an antibody to form a conjugate, the light emitting efficiency of the present conjugate is tripled, and thus, unexpectedly becomes as efficient
15 as the prior art conjugates.

It is believed that the present conjugate has a higher specific activity than past ones because the substituted acridinium ester has a much greater stability in the alkaline buffer solutions necessary for coupling
20 than the unsubstituted compound.

More particularly, the following assay was performed on both the present ortho-dimethyl ester and the prior unsubstituted compound.

25 A conjugate was prepared by coupling an anti-TSH antibody to the polysubstituted acridinium ester of Example 7 according to a conventional protein-coupling procedure, however any number of such known methods would be suitable.

30

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-9-

The final antibody concentration was 0.8 ng/ml. Ten microliters of a 1 in 1200 aqueous dilution of the conjugate were flashed by injecting 0.3 ml of a first solution containing 0.1N HNO₃, 0.1% H₂O₂, and 0.2% of ArquadTM surfactant, followed by a second solution of 0.3 ml of 0.1 N NaOH. Light emission was integrated for two seconds on a Berthold luminometer.

The above process was duplicated except for the substitution of the prior unsubstituted acridinium ester compound.

The light emitting efficiencies of a conjugation made with the present ortho-dimethyl ester were comparable or better than similar conjugates made with the prior unsubstituted ester:

Table I

Conjugate	Total Counts
Unsubstituted Ester	3,408,030
Unsubstituted Ester	3,110,990
Ortho-dimethyl Ester	3,913,690
Ortho-dimethyl Ester	3,467,030

Conjugate Stability

The above prior unsubstituted ester conjugate and the ortho-dimethyl ester conjugate were tested also for stability. The retention of luminescent activity was tested under various pHs (using citrate-phosphate buffers) and temperatures. Five milliliter aliquots of each of the above conjugates were placed into two sets of three different buffers (pH 5.0, 7.4, and 8.0), each containing 0.1% BSA. One set was kept at 4-8°C as a control, while the other was subjected to 37°C for seven days. At three days and seven days, 25 microliters of each buffered sample were flashed as described above. The results were as follows:

Table 2

Relative Stability of Heat-Stressed
Versus Non-Heat-Stressed Conjugates

	pH	3 days at 37°C	7 days at 37°C
20	5.0	77% / 91%*	62% / 77%
	7.4	66% / 101%	46% / 92%
	8.0	45% / 102%	13% / 106%

*The relative activity of the prior unsubstituted ester is expressed first, followed by that of the present ortho-dimethyl ester second. Thus, in the noted case the present ortho-dimethyl ester has 91% relative activity, while the prior unsubstituted ester has 77% relative activity. All activities are related back to their 2-8°C stability.

13 4 1 4 9 8

-11-

Signal-to-Noise Comparison

The above prior unsubstituted ester conjugate and the present ortho-dimethyl ester also were tested for signal-to-noise (S/N) ratios in an immunoassay. The assay worked as follows:

100 microliters of either of the above conjugates is incubated for two hours at room temperature with 200ul of a TSH standard (Ciba Corning Diagnostics Corp., Medfield, MA). Incubations were done separately with four standards containing either 0, 0.4, 1.0, or 100uIU/ml of TSH. A second incubation was then performed by adding 500u microliters of sheep anti-TSH, MAGIC^R magnetic particle conjugate (also available from Ciba Corning Diagnostics Corp.) to the above mixture, then waiting for 30 minutes at room temperature. A wash was done first by magnetically separating the particles from the solution, decanting the solution, then adding 500 microliters of water, followed by another magnetic separation. The washed particles were resuspended in 100 microliters of a solution containing 0.1N HNO₃ and 0.1% of H₂O₂. Flashing and counting were done according to the above-described procedures. The results were tabulated using ratios of the counts with a TSH standard containing TSH versus the zero TSH standard.

Table 3

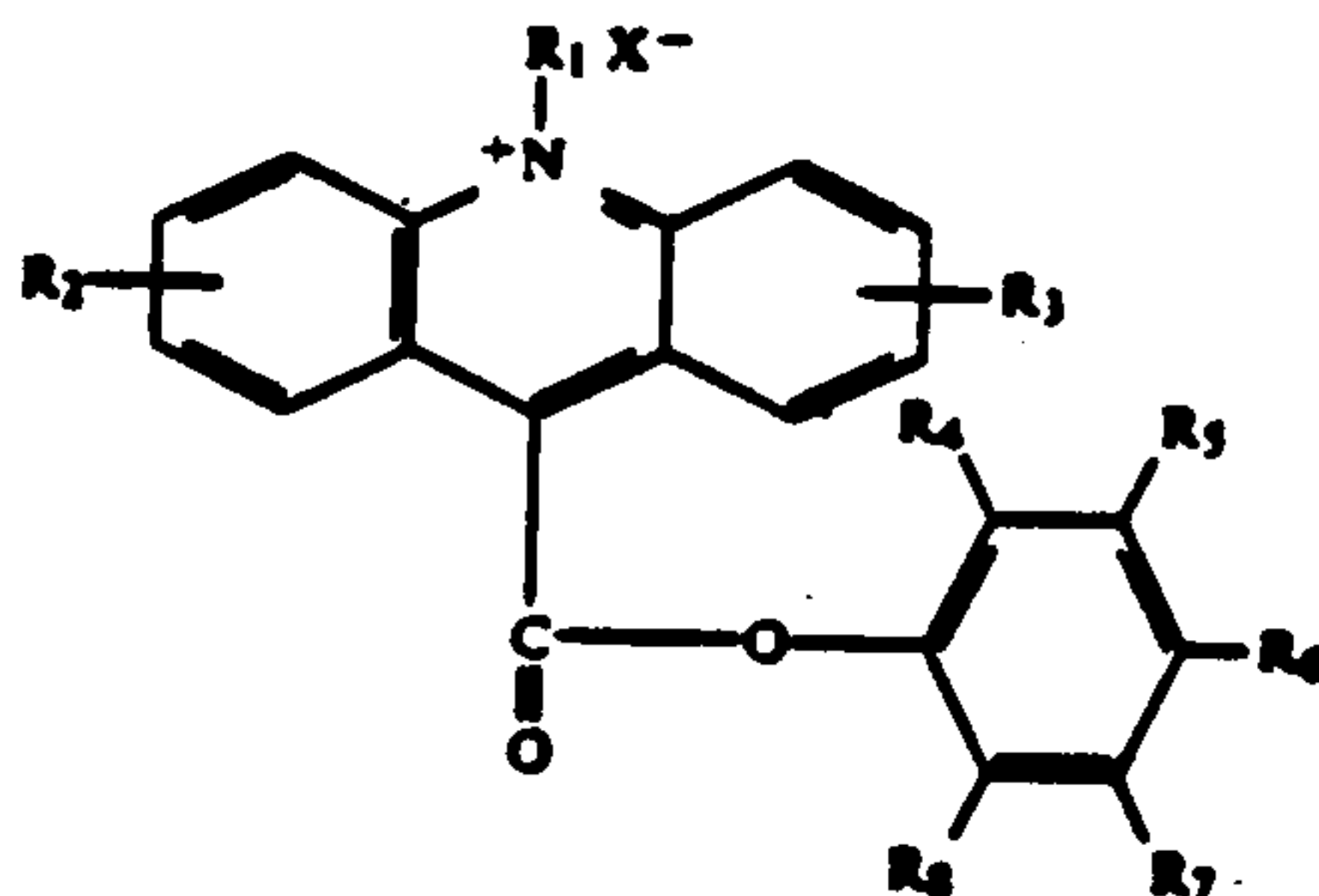
Conjugate	Standard		
	0.4uIU/ml	1.0uIU/ml	100uIU/ml
Unsubstituted Ester	1.4	1.9	20.7
Ortho-dimethyl Ester	1.9	2.3	44.5

The comparison of results indicated the present conjugate had a mean increase in its signal-to-noise ratio of 57% over prior conjugates.

It should be apparent to one having ordinary skill in the art that many variations are possible without departing from the spirit and scope of the invention.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A luminescent conjugate for use in luminescent
assays comprising a polysubstituted aryl acridinium ester
of the formula:

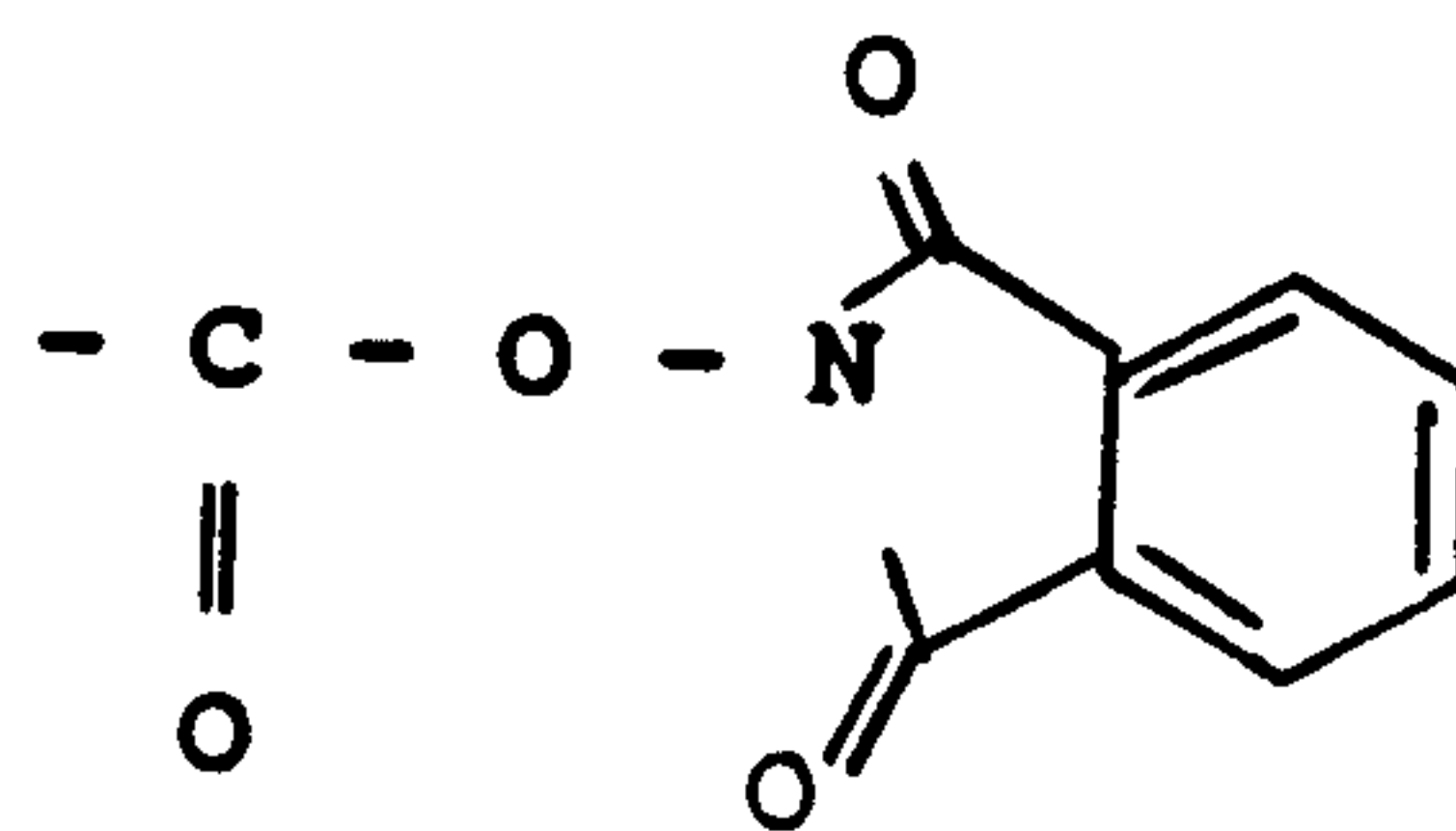
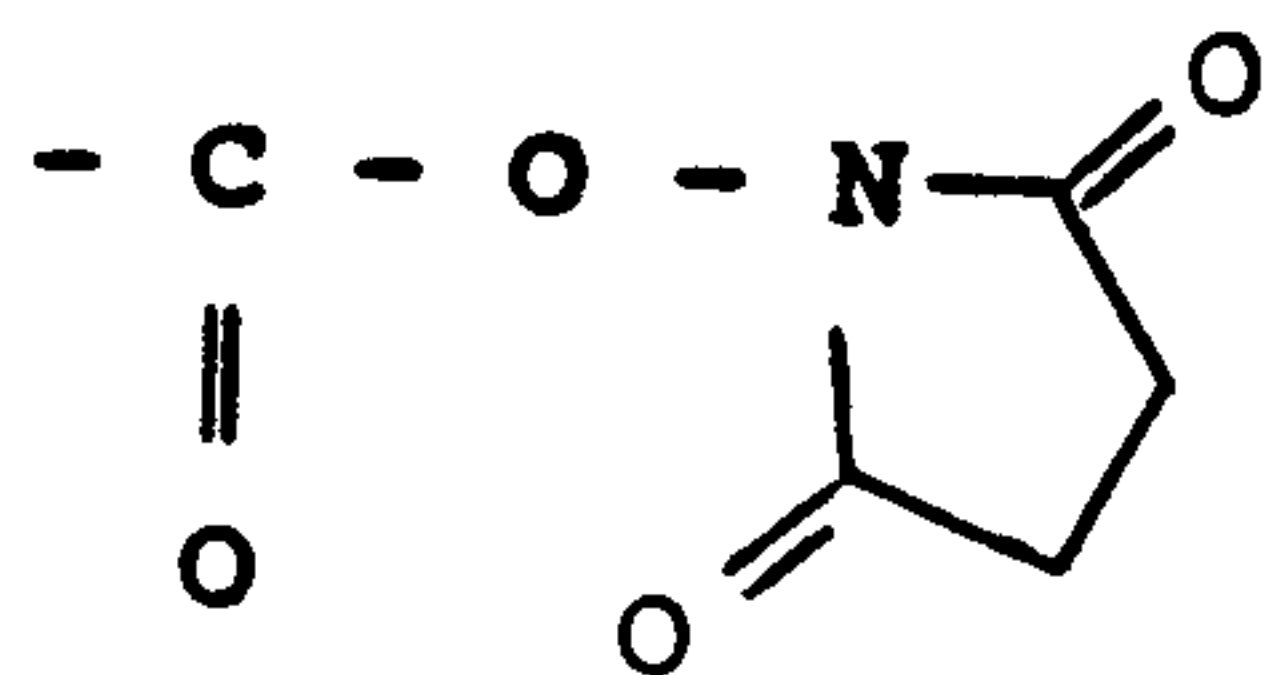


where R_1 is an alkyl, alkenyl, alkynyl or aryl group; R_2 ,
 R_3 , R_5 or R_7 is a hydrogen, amino, carboxyl, hydroxyl,
alkoxyl, nitro, or halide group; R_4 or R_8 is an alkyl,
alkenyl, alkylanyl, aryl, alkoxyl, amino, amido,
sulfonamido, or sulfide group; and R_6 represents hydrogen
amino, carboxyl, hydroxyl, alkoxyl, nitro or halide group
or the following substituent:

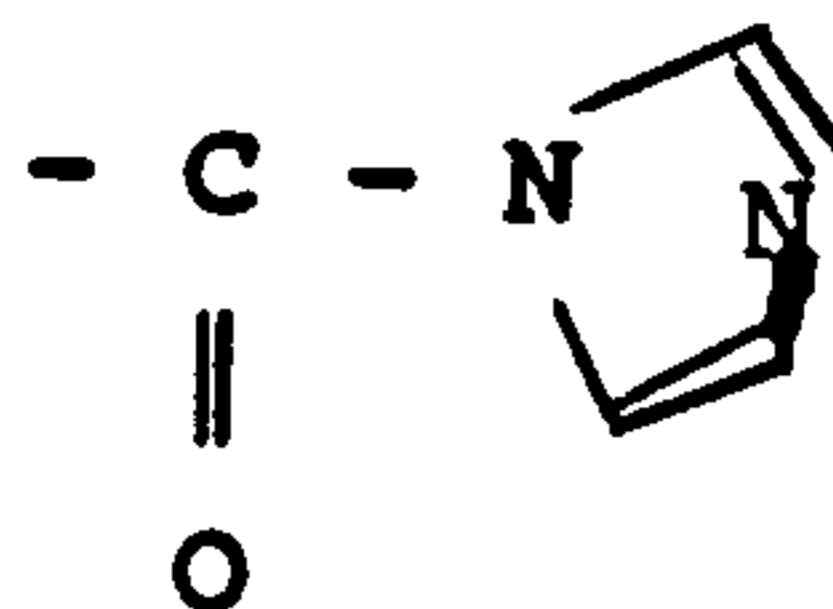
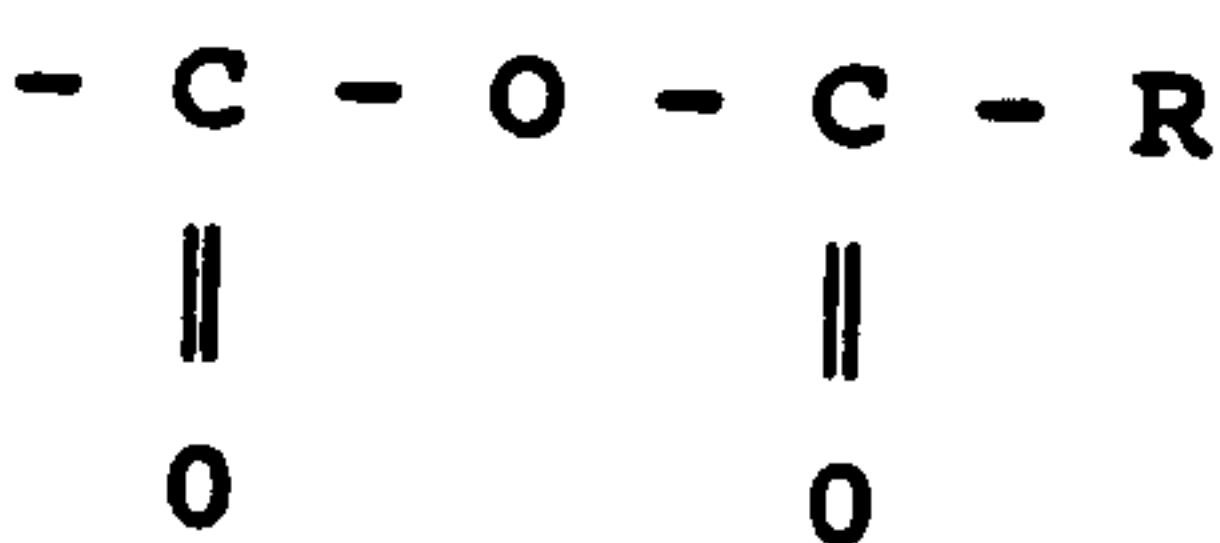
$R_6 = -R_9 - R_{10}$

where R_9 is not required but optionally can be an alkyl,
aryl, or aralkyl group, and R_{10} is selected from the
following:

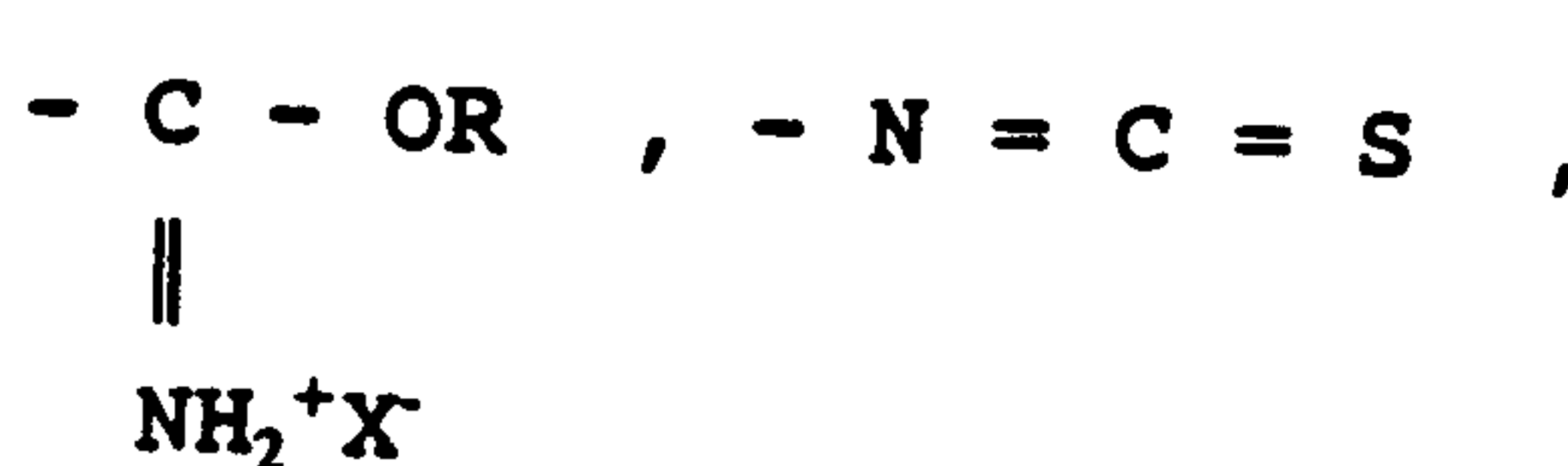
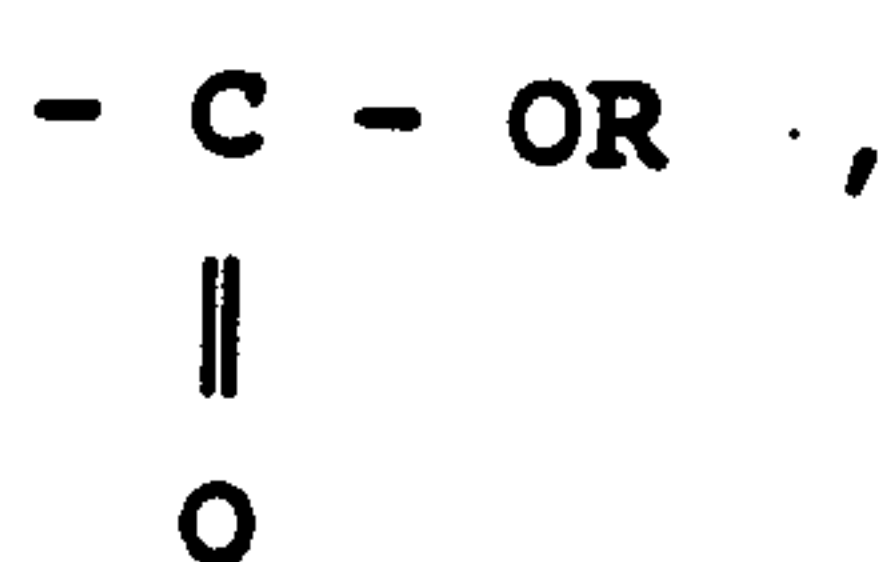
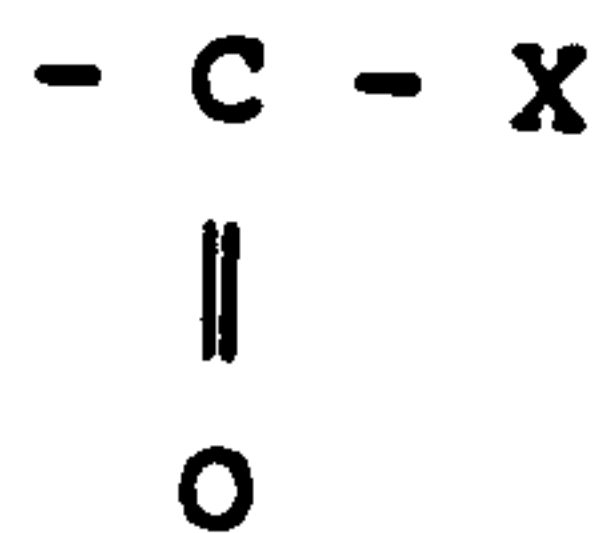
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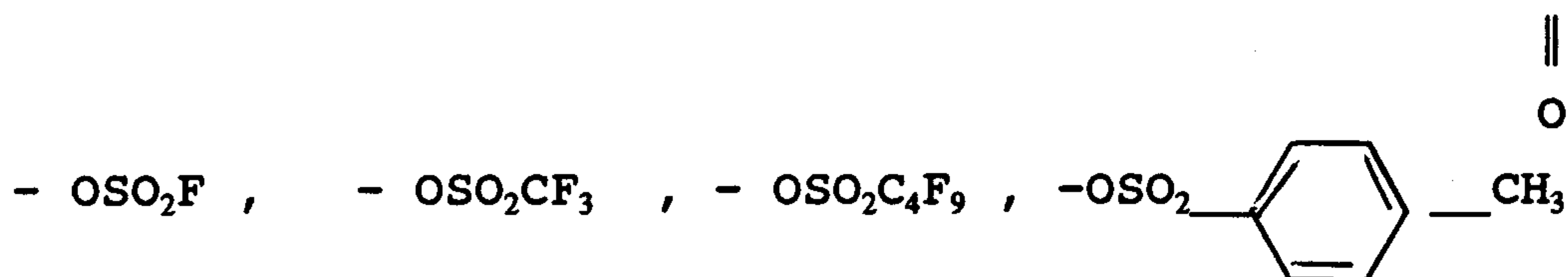
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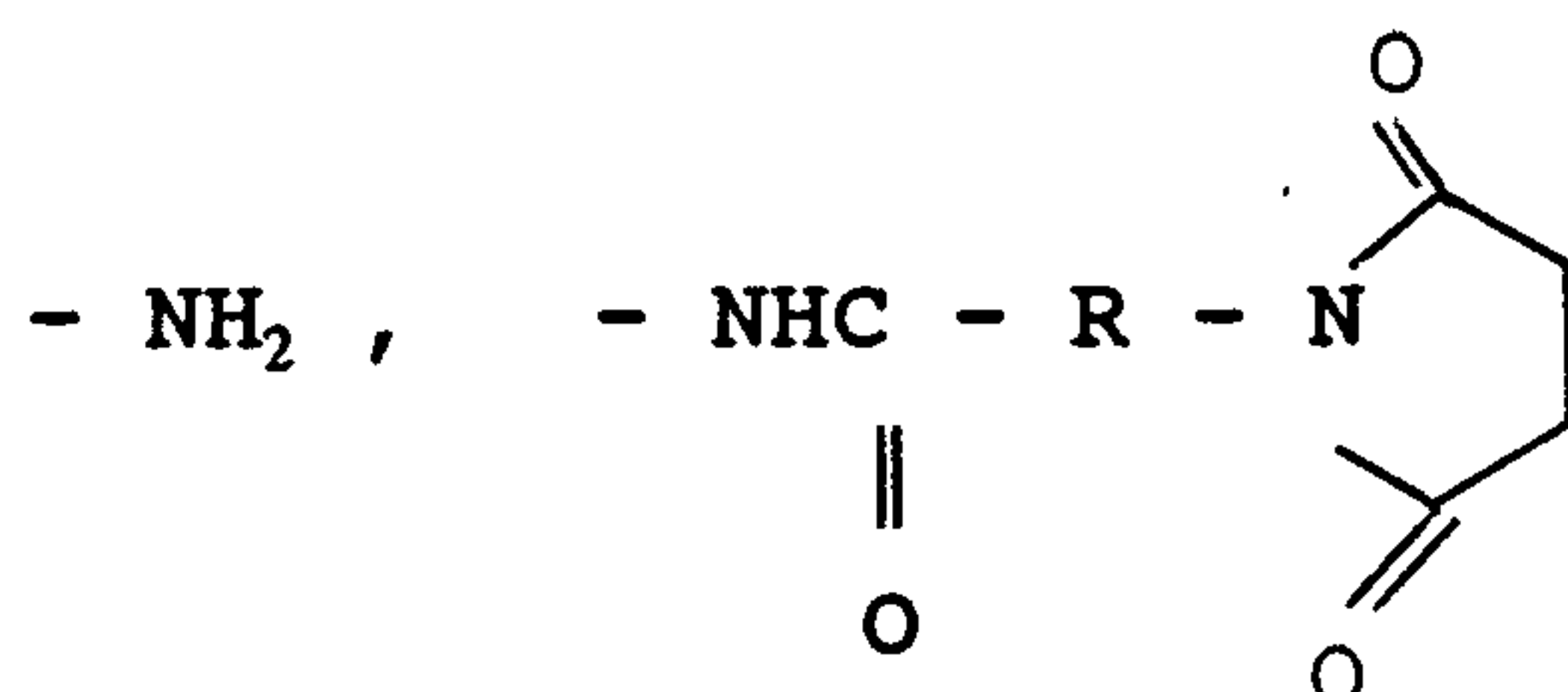
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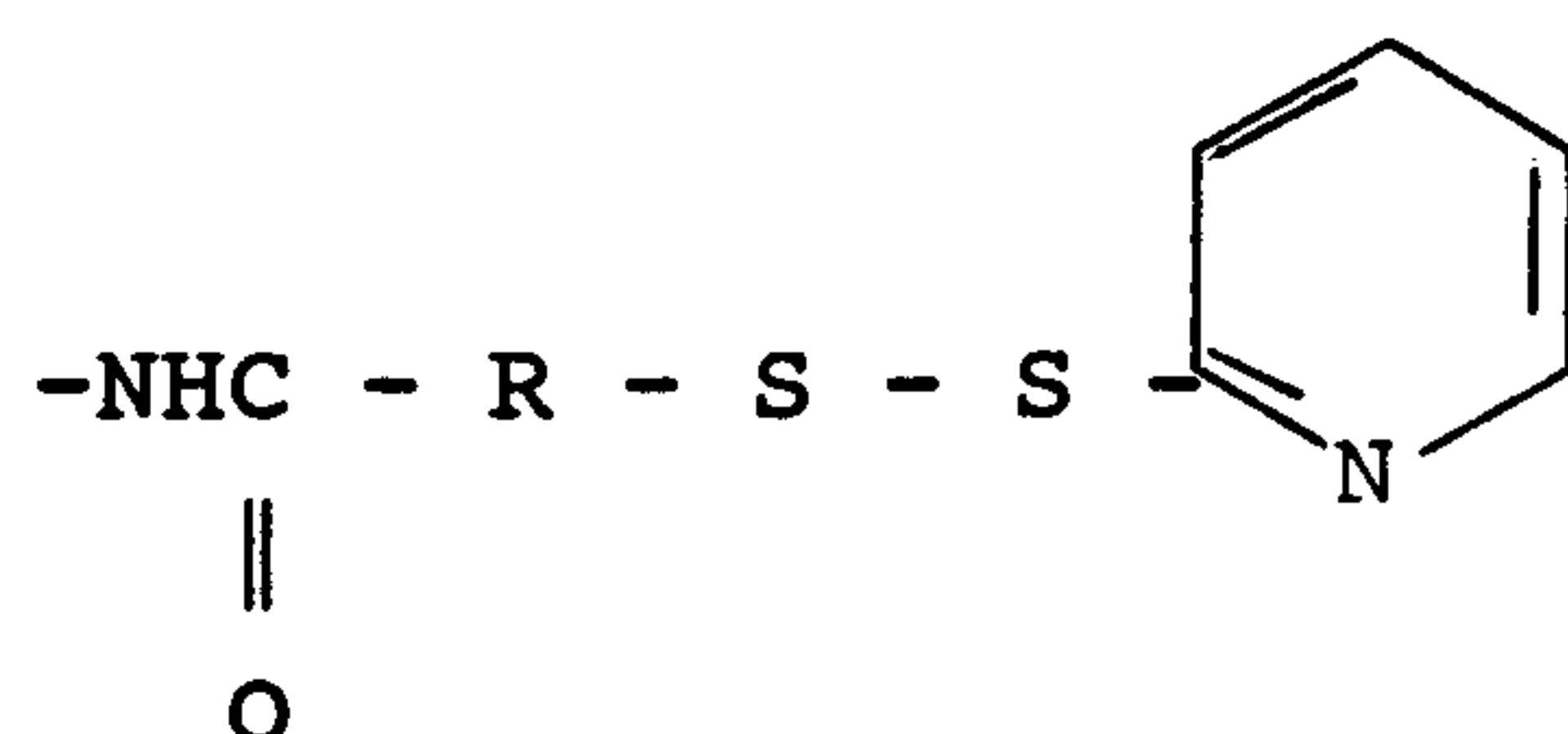
- N = C = O , - N₂⁺X⁻ , a halide , -N₃ , - C - OH



5

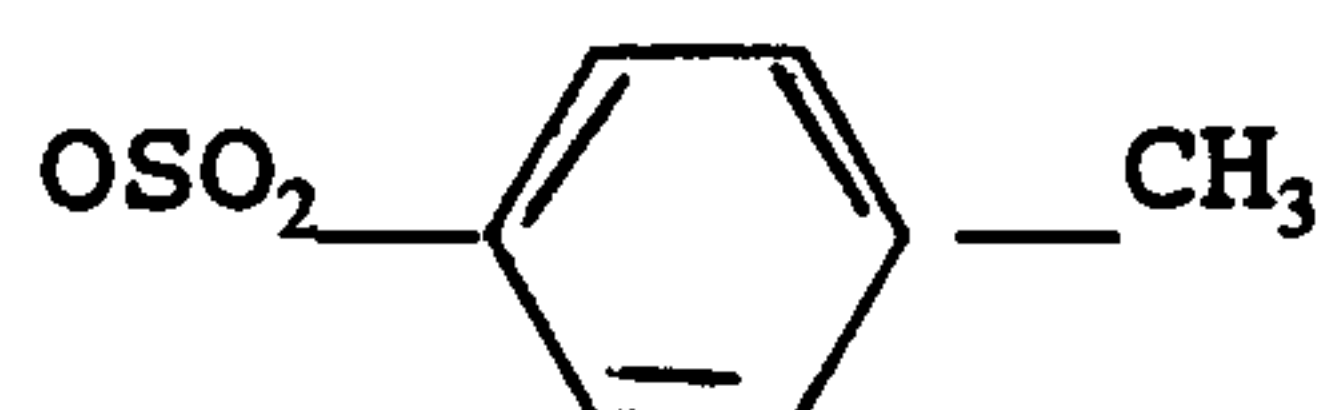


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X is CH₃SO₄⁻ , OSO₂F⁻ , a halide , OSO₂CF₃⁻ , OSO₂C₄F₉⁻ ,



20

R is alkyl, aryl, aralkyl; and finally R₅, R₆, and R₇ substituent positions on the phenoxy ring are interchangeable, wherein the R₆ substituent of the polysubstituted aryl acridinium ester is covalently coupled to a molecule with biological activity which molecule is capable of being covalently coupled to the R₆ substituent.

25

2. A luminescent conjugate as recited in claim 1 wherein R₁ is a methyl group; R₂, R₃, R₅ and R₇ are hydrogen; R₄ and R₈ are a methyl group; R₁₀ is a N-succinimidyl oxycarbonyl group attached directly to the para-position of the phenoxy ring; and X is CH₃SO₄⁻

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3. A luminescent conjugate as recited in claim 1, wherein the molecule with biological activity is a molecule with immunological activity.

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4. A luminescent conjugate as recited in claim 3 wherein the molecule with immunological activity is an antibody.

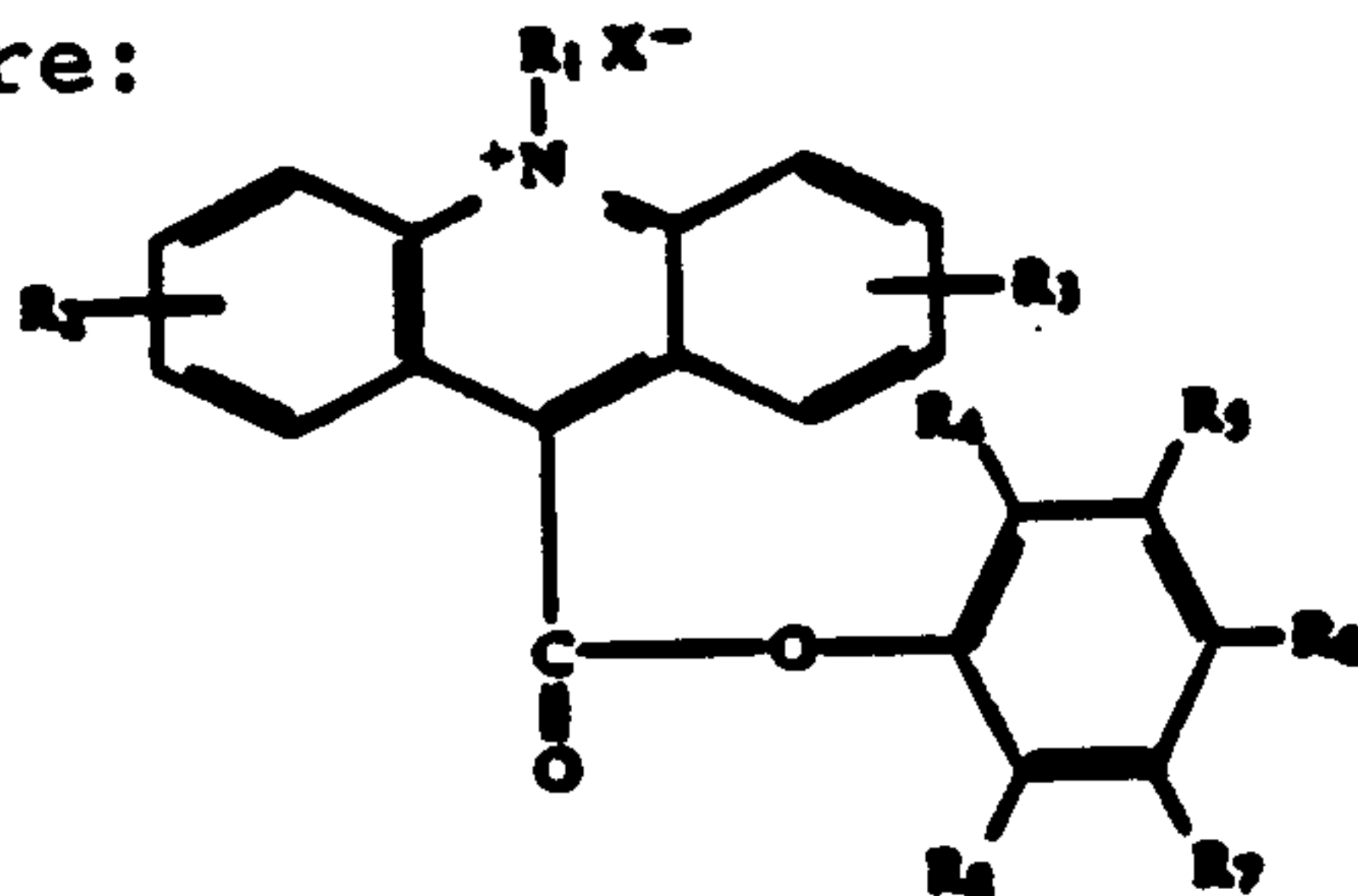
5

5. A luminescent conjugate as recited in claim 3 wherein the molecule with immunological activity is an antigen.

10 6. A luminescent conjugate as recited in claim 1 wherein the molecule with biological activity is a nucleic acid.

15 7. A luminescent conjugate as recited in claim 1 wherein the molecule with biological activity comprises nucleic acids.

8. A luminescent compound comprising a polysubstituted aryl acridinium ester selected from the group having the following structure:



25

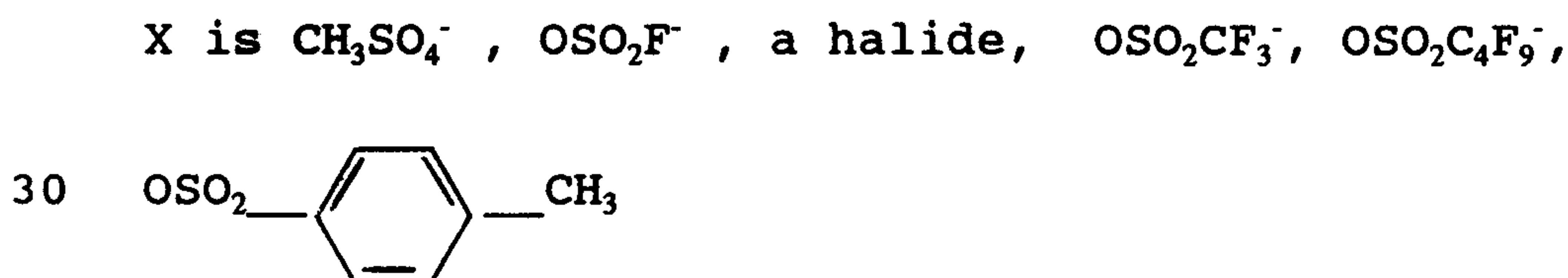
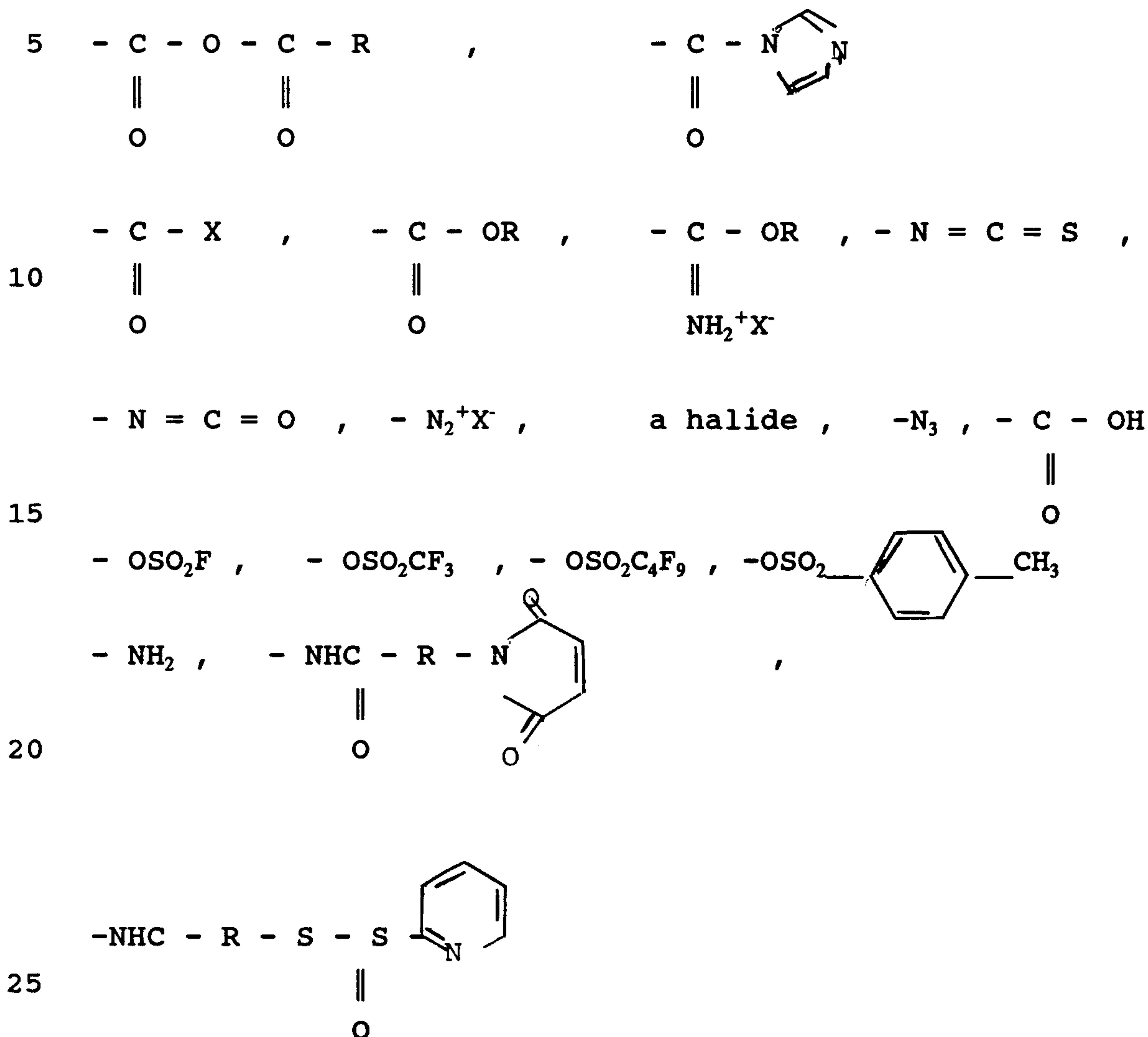
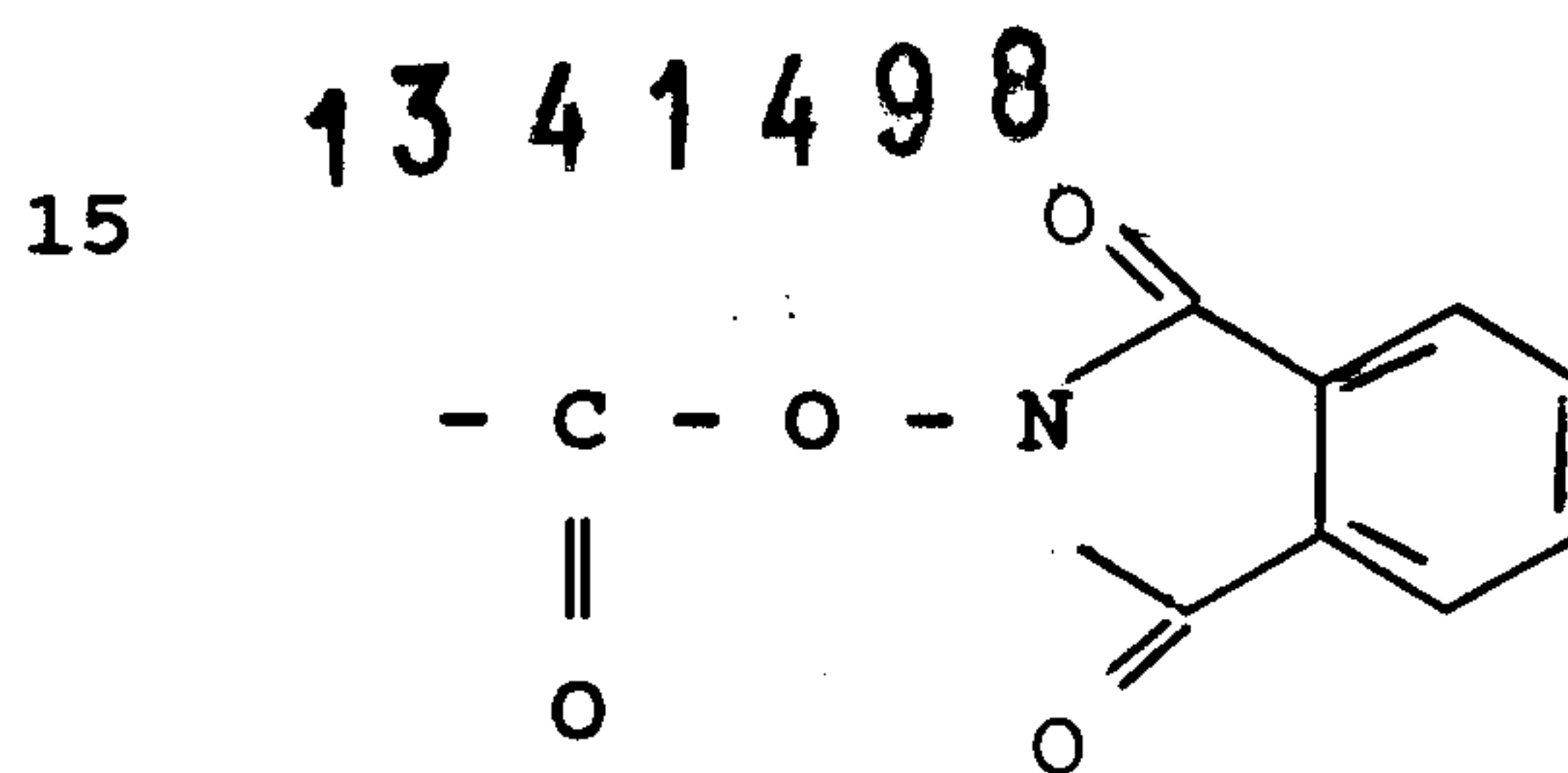
where R₁ is an alkyl, alkenyl, alkynyl or aryl group; R₂, R₃, R₅ or R₇ is a hydrogen, amino, carboxyl, hydroxyl, alkoxyl, nitro, or halide group; R₄ or R₈ is an alkyl group; R₆ represents hydrogen, amino, carboxyl, hydroxyl, alkoxyl, nitro or halide group or the following substituent:

30



where R₉ is not required but if present is an alkyl, aryl, or aralkyl group, and R₁₀ is selected from the following:

35



R is alkyl, aryl, aralkyl; and finally R₅, R₆, and R₇ substituent positions on the phenoxy ring are interchangeable.

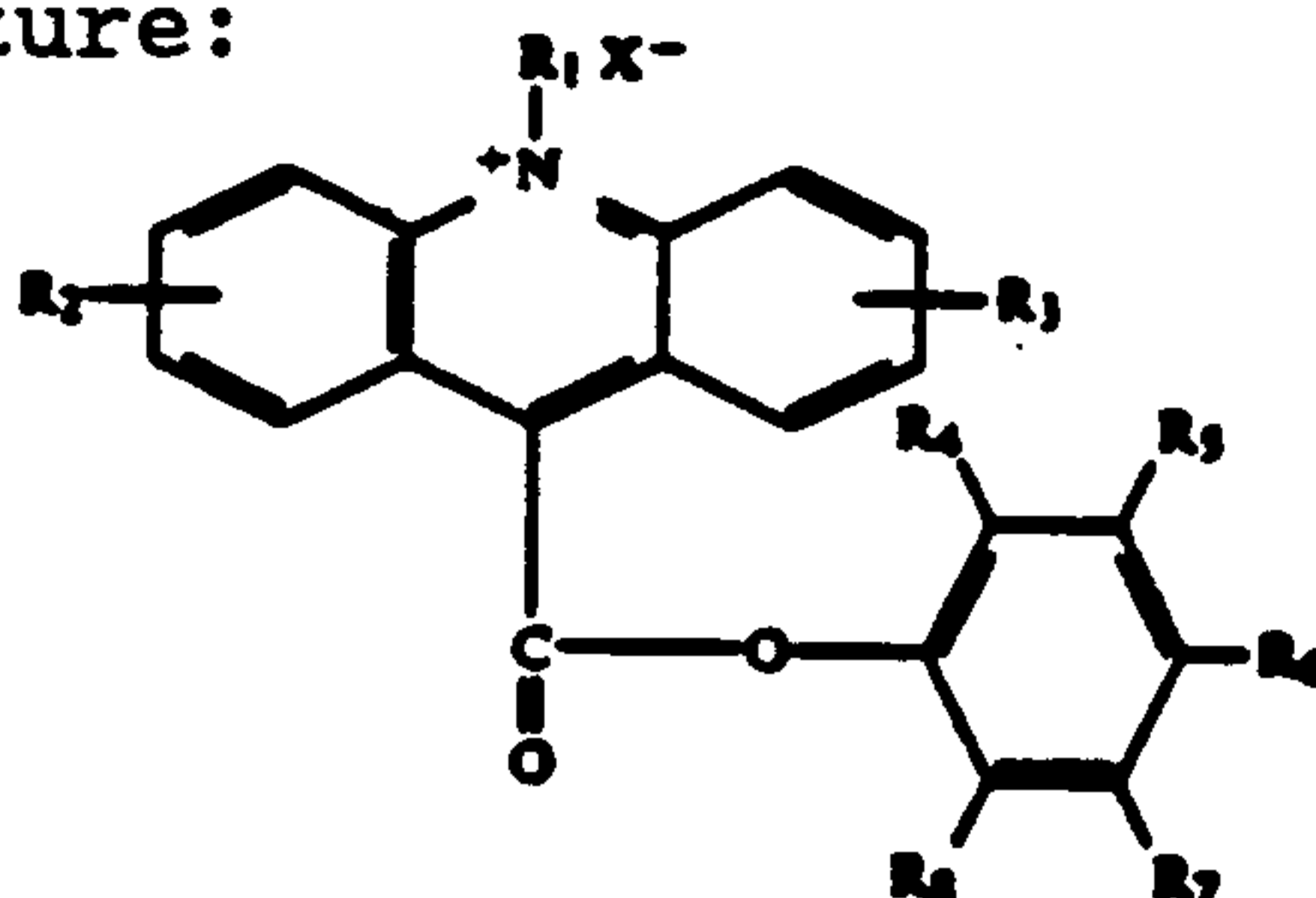
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9. A luminescent compound according to claim 1 wherein R₁ is a methyl group; R₂, R₃, R₅ and R₇ are hydrogen; R₄ and

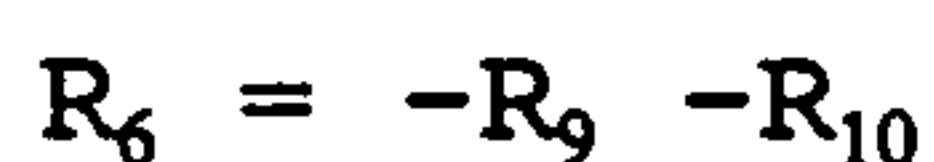
R_8 are a methyl group; R_{10} is a N-succinimidyl oxycarbonyl group attached directly to the para-position of the phenoxy ring; and X is CH_3SO_4 .

- 5 10. A luminescent compound comprising a polysubstituted aryl acridinium ester selected from the group having the following structure:

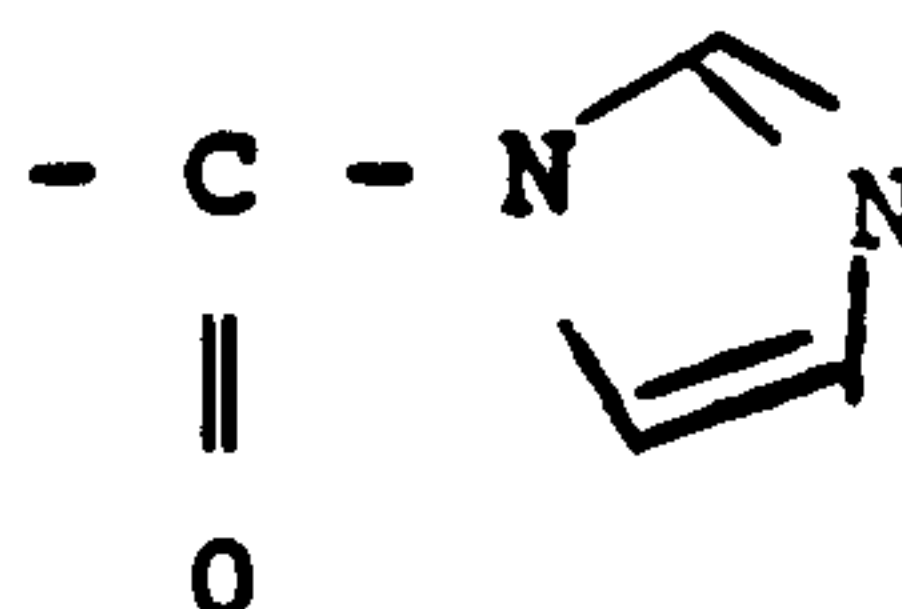
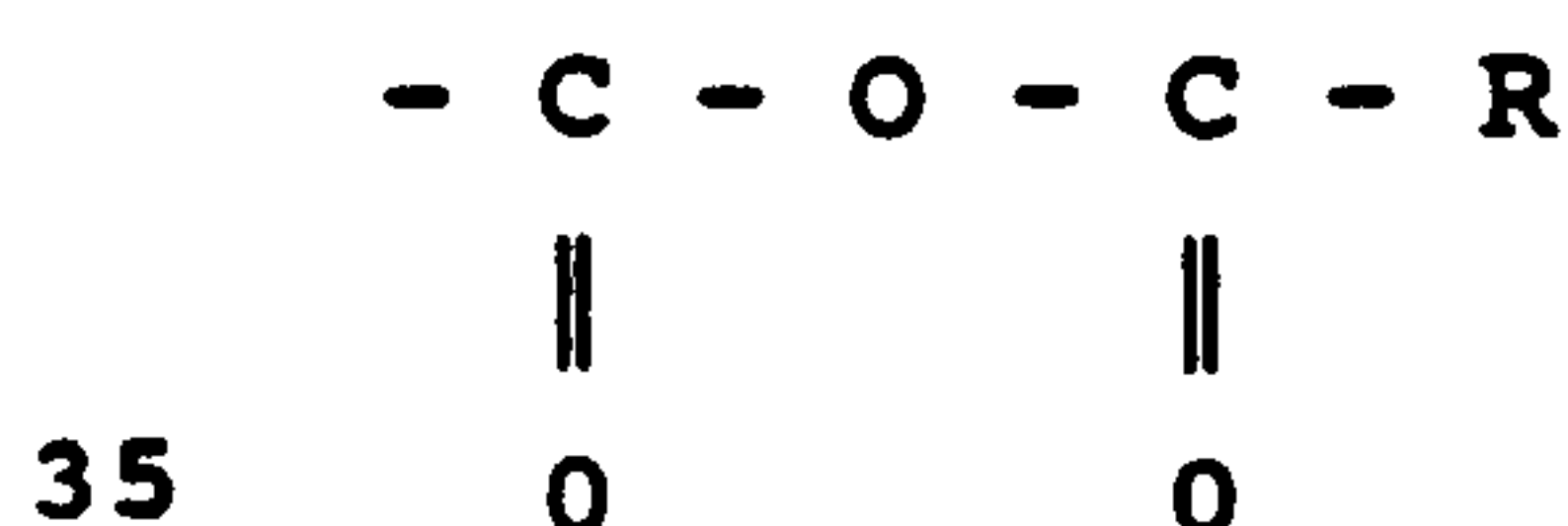
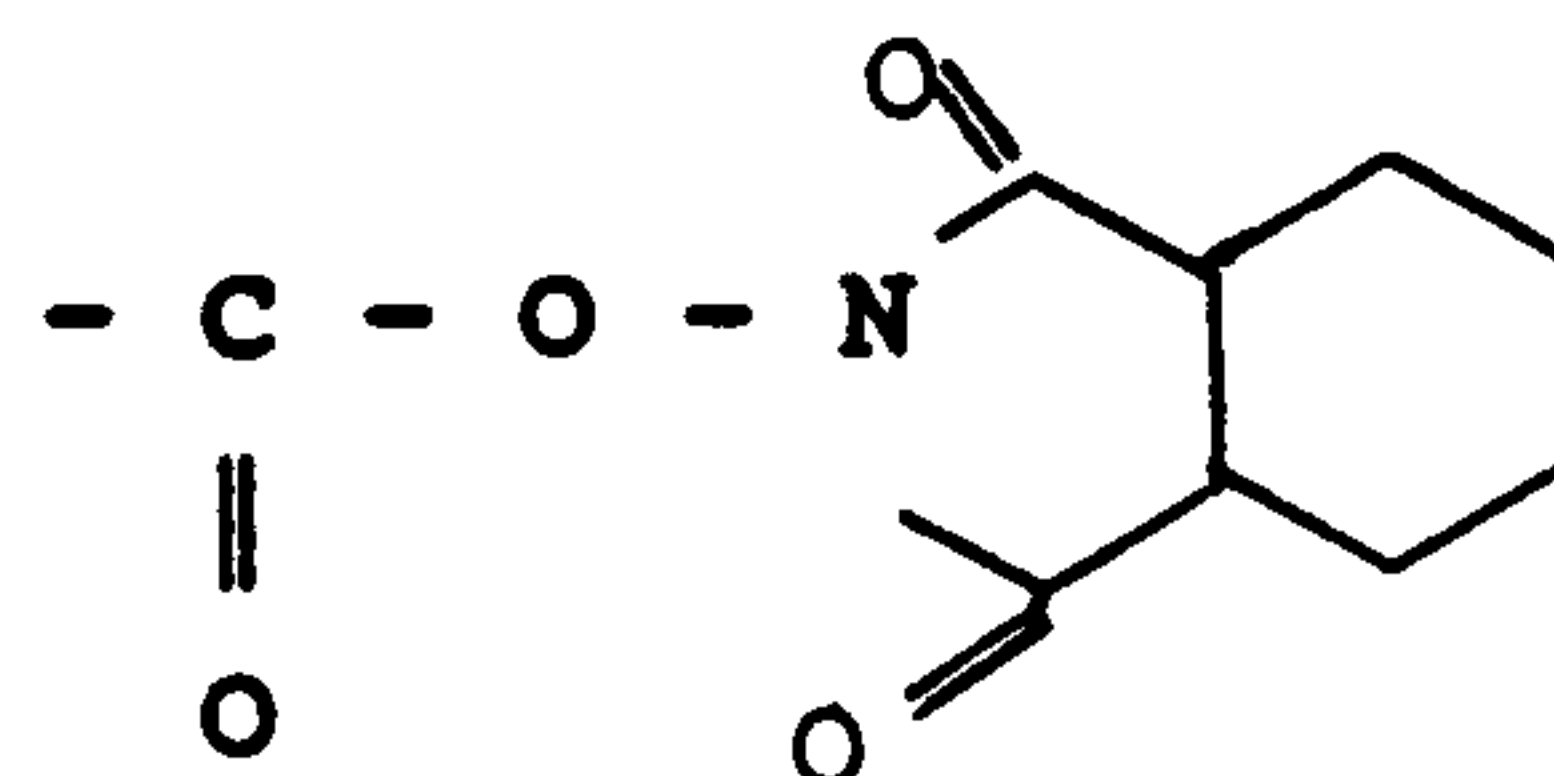
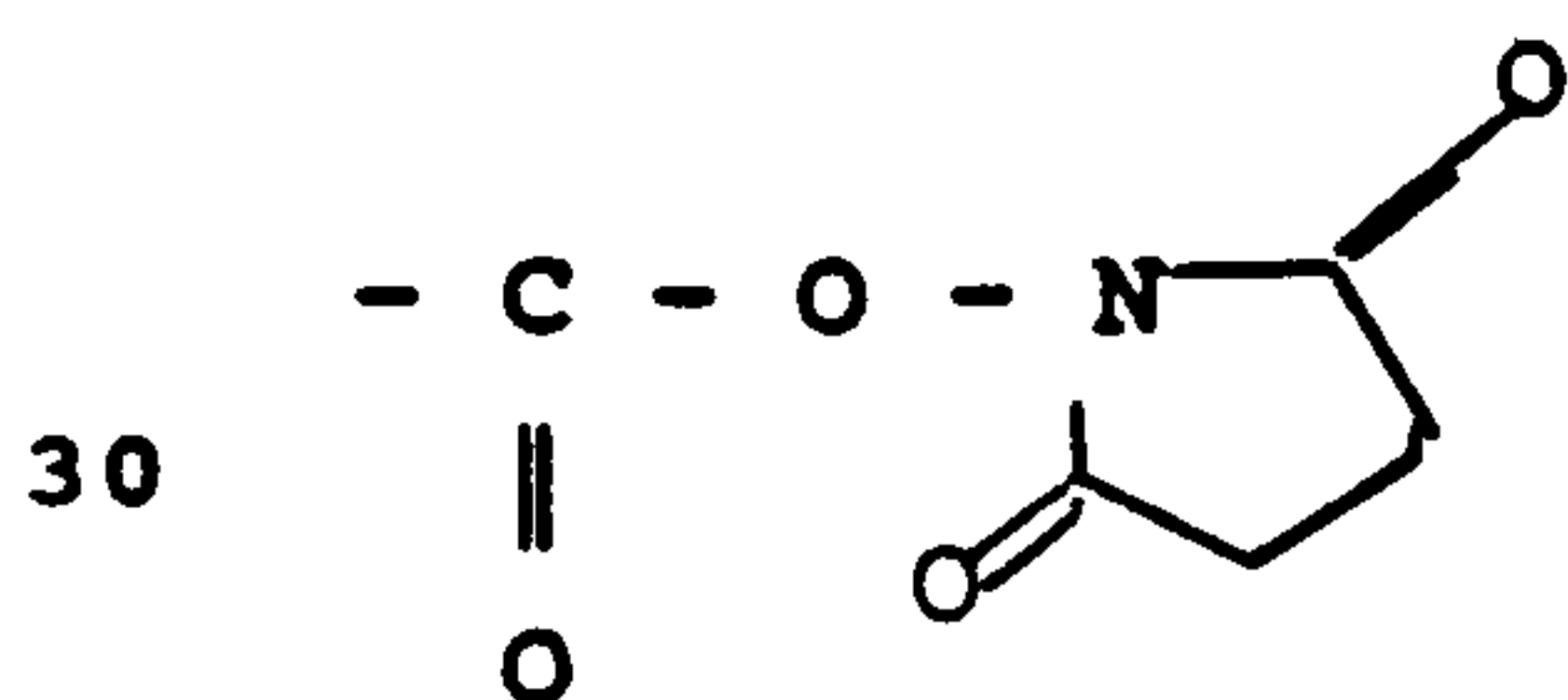
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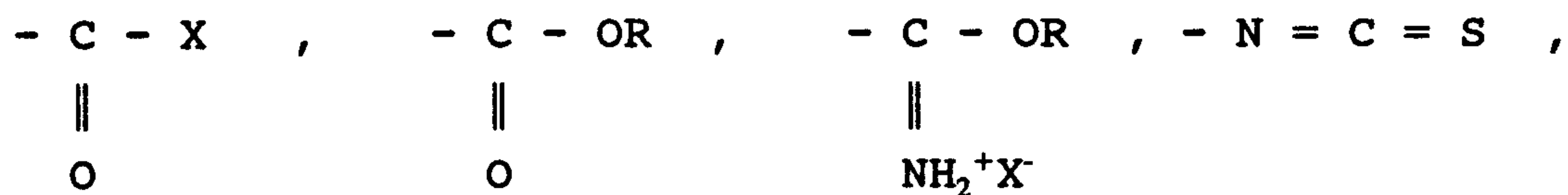


- 15 where R_1 is an alkyl, alkenyl, alkynyl or aryl group; R_2 , R_3 , R_5 or R_7 is a hydrogen, amino, carboxyl, hydroxyl, alkoxy, nitro, or halide group, R_4 or R_8 is an alkyl, alkenyl, alkynyl, aryl, alkoxy, amino, amido, sulfonamido, or sulfide group; and R_6 represents hydrogen, amino, carboxyl, hydroxyl, alkoxy, nitro or halide group
20 or the following substituent:



- 25 where R_9 is not required but optionally can be an alkyl, aryl, or aralkyl group, and R_{10} is selected from the following:

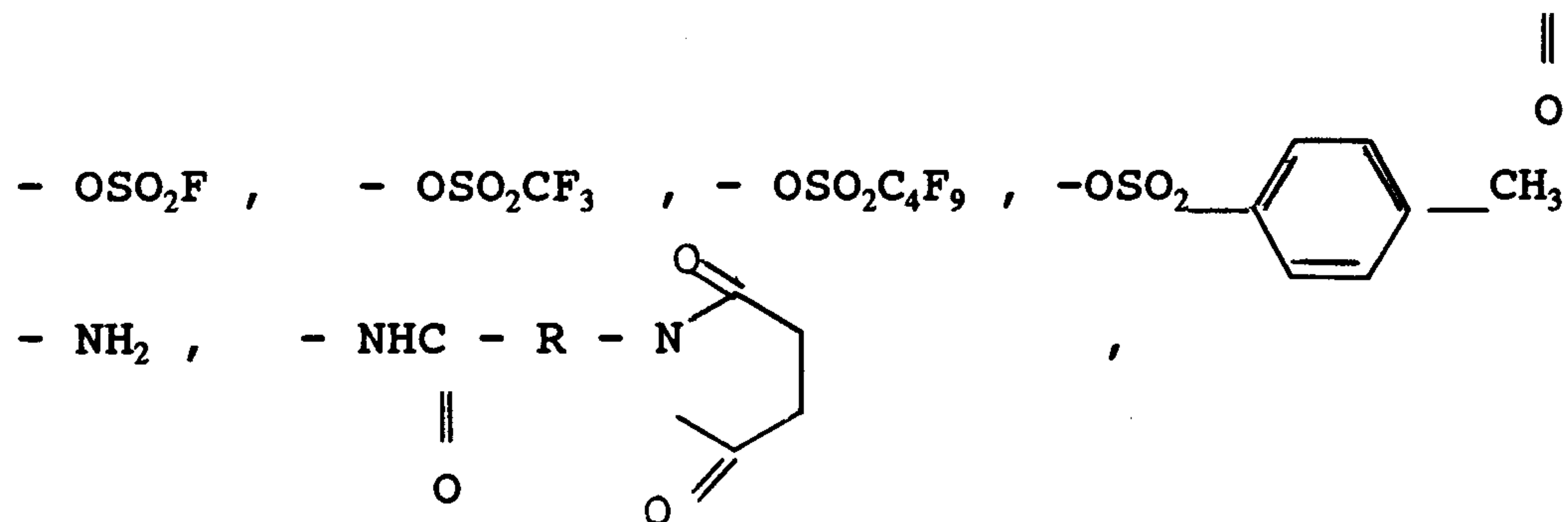




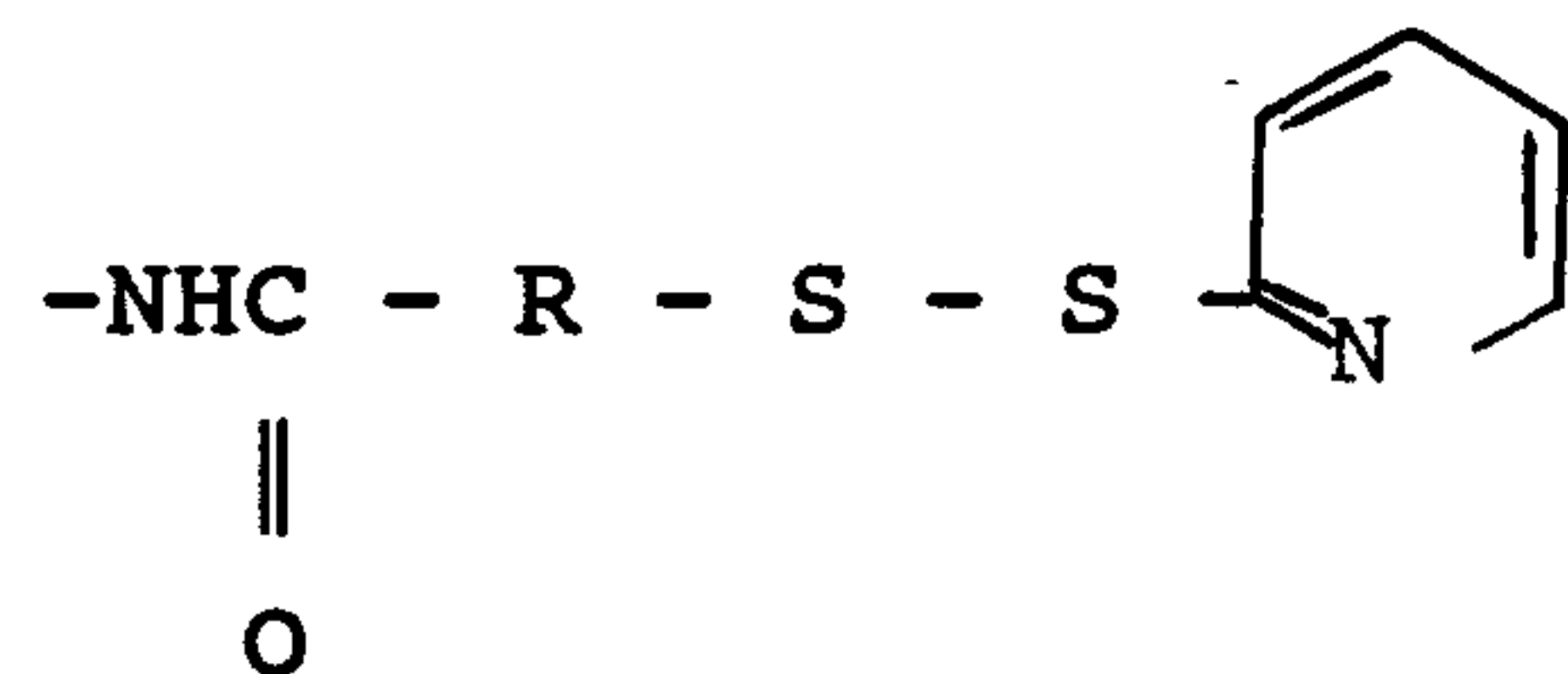
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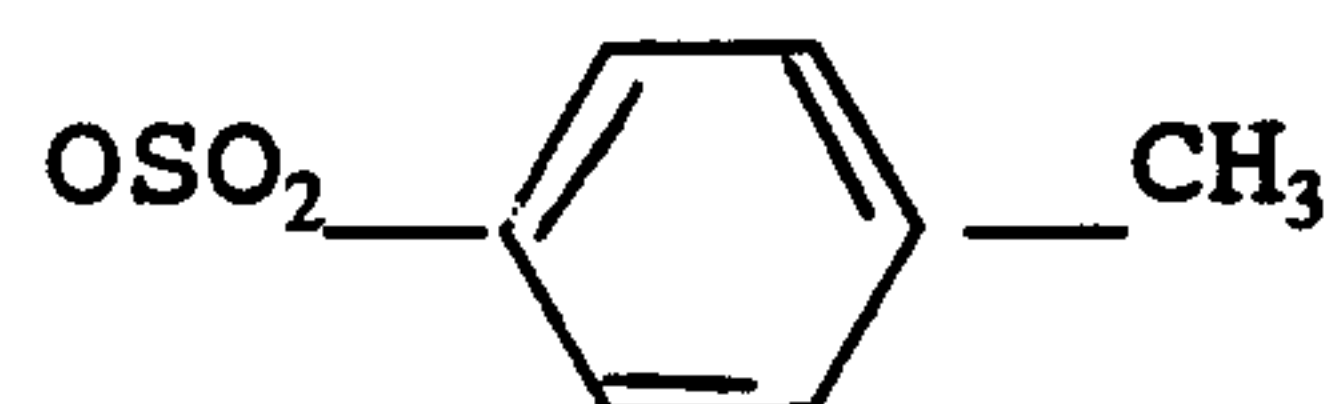
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X is $CH_3SO_4^-$, OSO_2F^- , a halide, $OSO_2CF_3^-$, $OSO_2C_4F_9^-$,

25



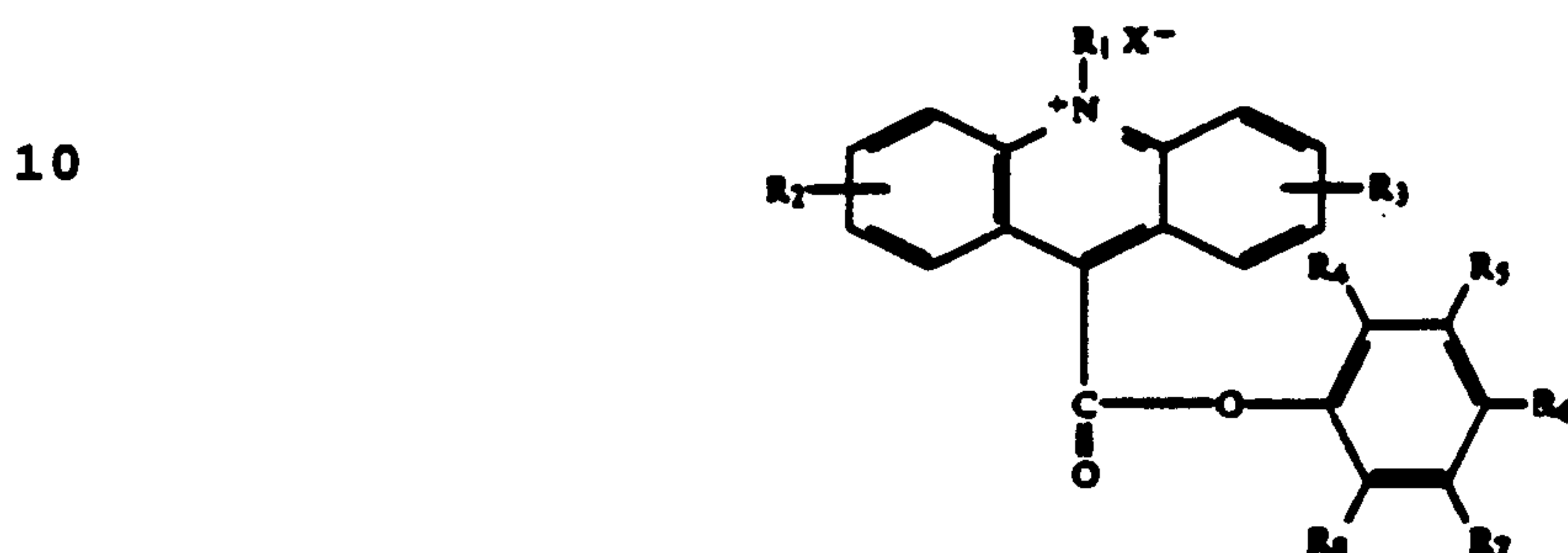
R is alkyl, aryl, aralkyl; and finally R_5 , R_6 , and R_7 substituent positions on the phenoxy ring are interchangeable.

30

11. A luminescent compound according to claim 10 wherein R_1 is a methyl group; R_2 , R_3 , R_5 and R_7 are hydrogen; R_4 and R_8 are a methyl group; R_{10} is a N-succinimidyl oxycarbonyl group attached directly to the para-position of the phenoxy ring; and X is $CH_3SO_4^-$

35

- 5 12. A luminescent compound comprising a polysubstituted aryl acridinium ester selected from the group having the following structure:



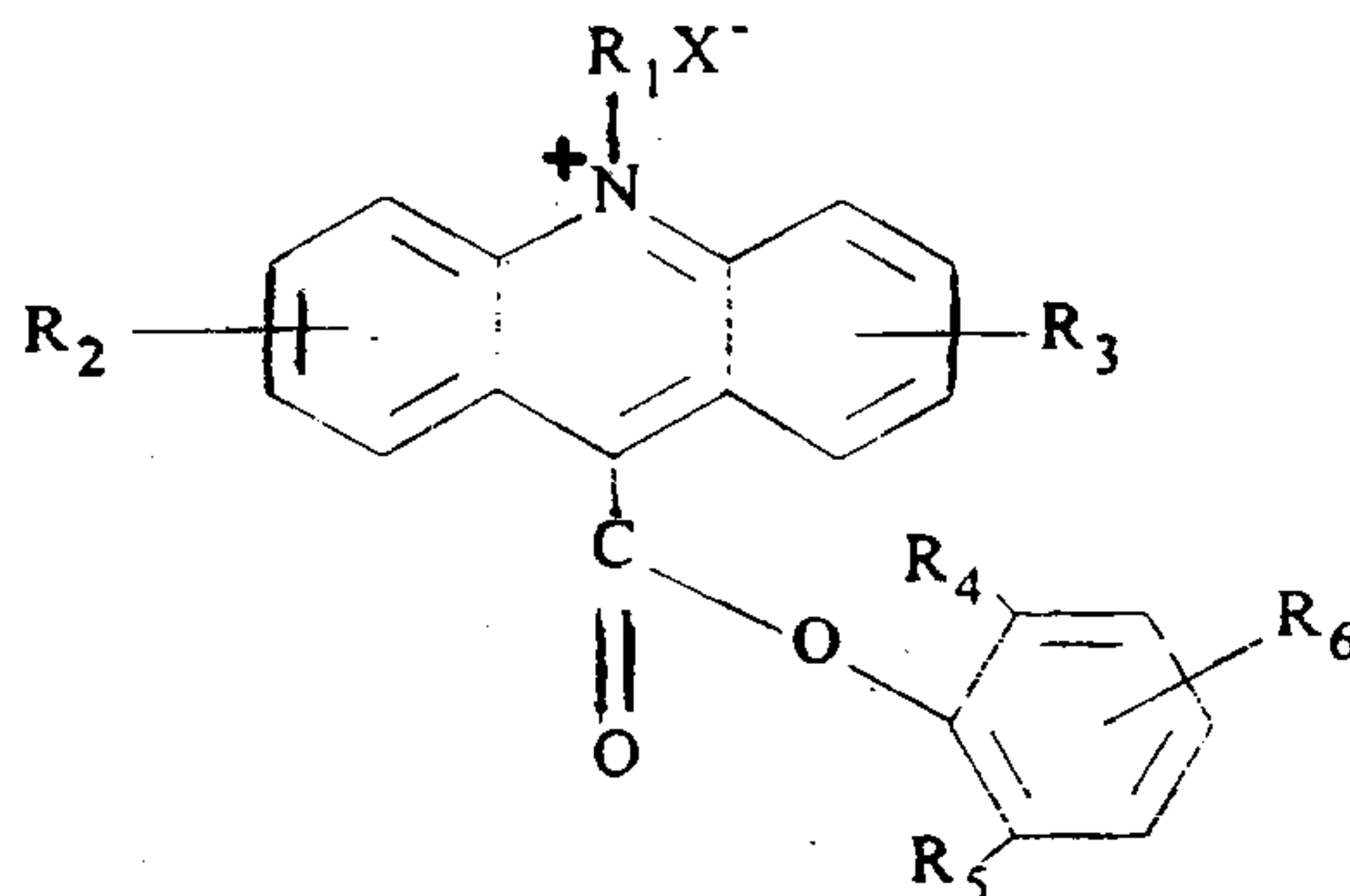
- where R_1 is an alkyl, alkenyl, alkynyl or aryl group; R_2 , R_3 , R_5 , R_6 or R_7 is a hydrogen, amino, carboxyl, hydroxyl, alkoxyl, nitro, or halide group; R_4 or R_8 is an alkyl, alkenyl, alkylanyl, aryl, alkoxyl, amino, amido, sulfonamido, or sulfide group; and finally R_5 , R_6 , and R_7 substituent positions on the phenoxy ring are interchangeable.
- 20

13. A luminescent compound according to claim 12 wherein R_1 is a methyl group; R_2 , R_3 , R_5 , R_6 and R_7 are hydrogen; R_4 and R_8 are a methyl group; and X is CH_3SO_4 .
- 25

14. A luminescent conjugate comprising the compound of claim 1 wherein the R_6 substituent is covalently coupled to a ligand or a specific binding partner thereto.
- 30

15. A luminescent conjugate comprising the compound of claim 11 wherein the R_6 substituent is covalently coupled to a ligand or a specific binding partner thereto.
- 35

16. A luminescent compound comprising a polysubstituted aryl acridinium ester selected from the group having the following structure:

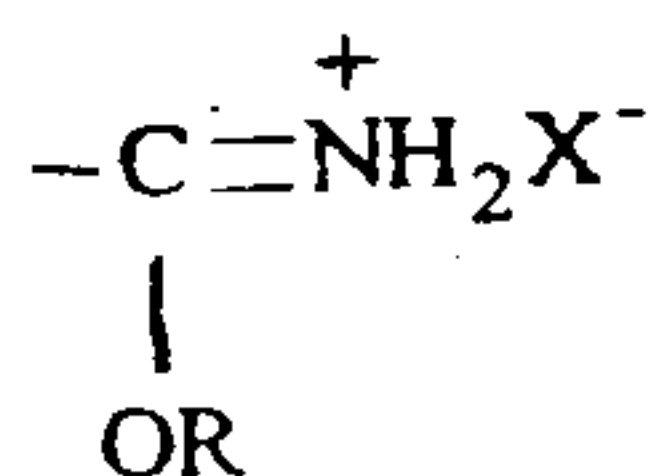


where R_1 is alkyl; R_2 and R_3 are each a hydrogen, carboxyl or amino group; R_4 and R_5 are each an alkyl, aryl or alkoxy group and R_6 is a hydrogen, nitro or halide group or a group of the following formula:



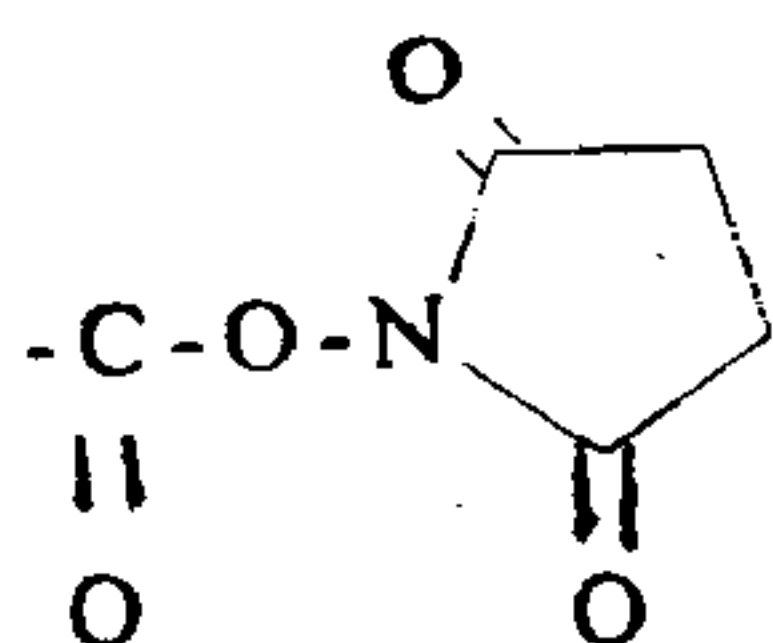
where R_7 is an alkyl, aryl or aralkyl group or a direct bond and R_8 is selected from the following:

CO_2R where R is alkyl, aryl or hydrogen,



where R is alkyl or

$-\text{NCS}$,

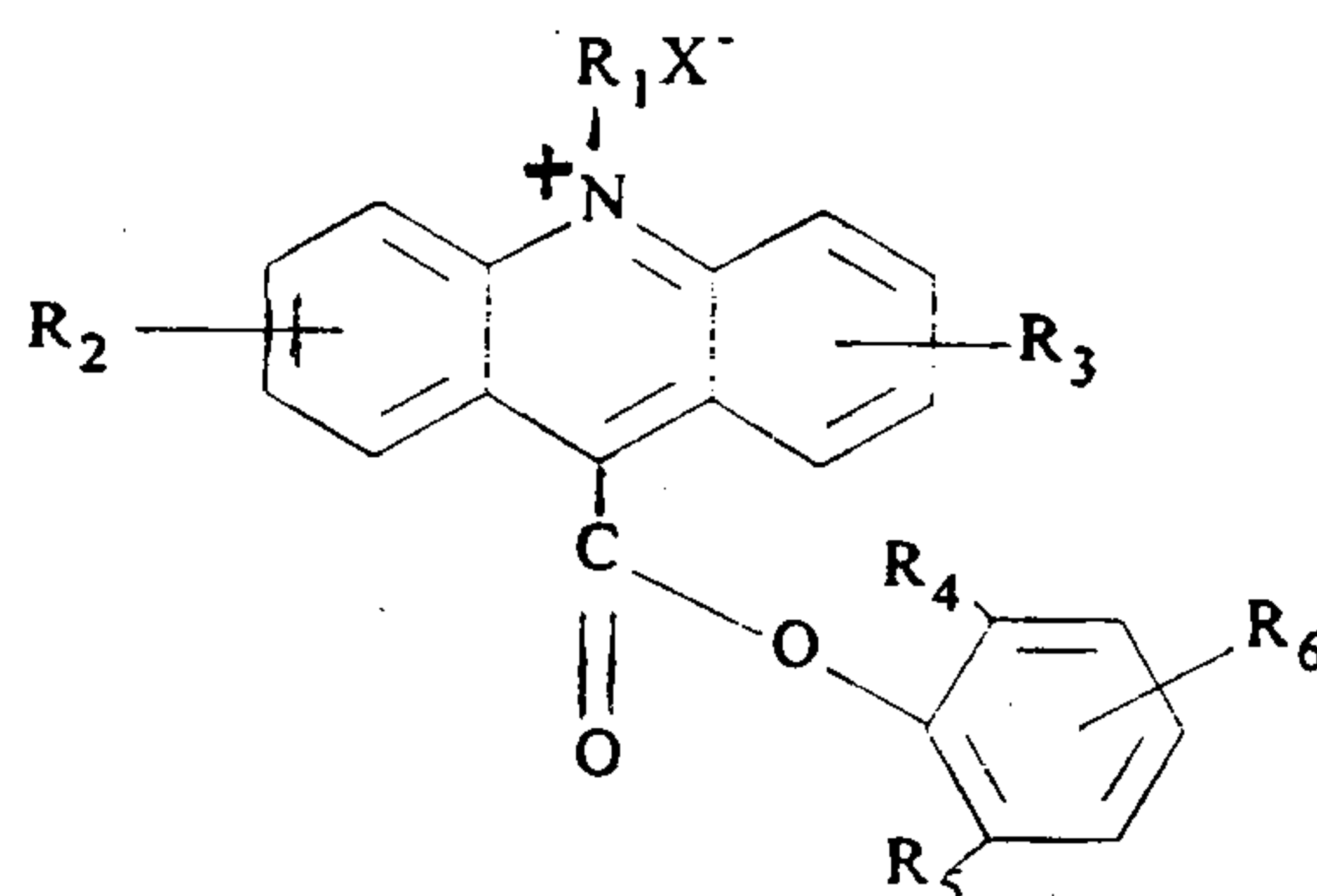


$-\text{N}_3$ or

$-\text{NH}_2$

and X is an anion.

17. A luminescent compound comprising a polysubstituted aryl acridinium ester selected from the group having the following structure:

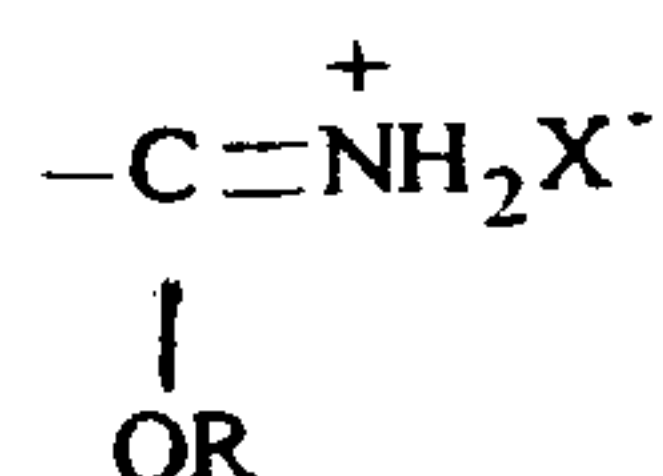


where R_1 is alkyl; R_2 and R_3 are each a hydrogen, carboxyl or amino group; R_4 and R_5 are each an alkyl, aryl or alkoxy group and R_6 is a hydrogen, nitro or halide group or a group of the following formula:



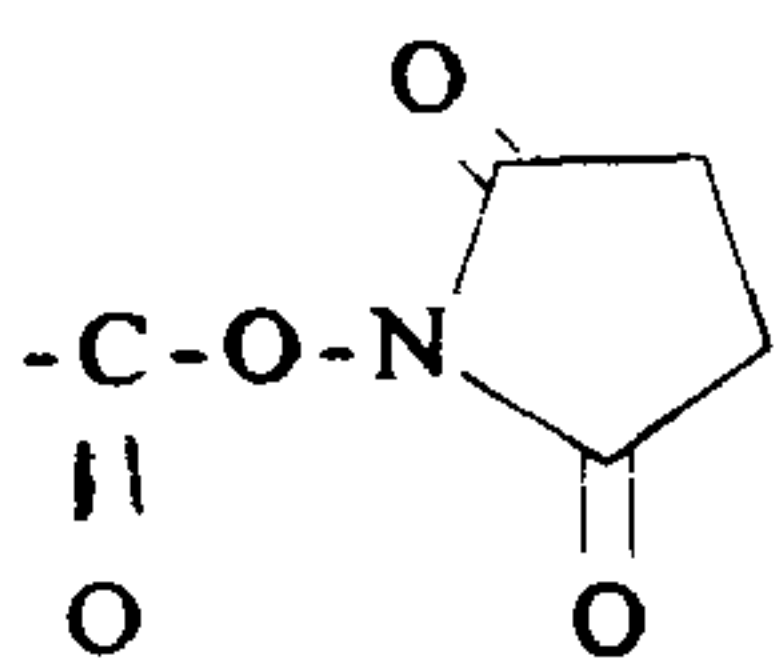
where R_7 is an alkyl, aryl or aralkyl group or a direct bond and R_8 is selected from the following:

CO_2R where R is alkyl, aryl or hydrogen,



where R is alkyl or

$-\text{NCS}$,



$-\text{N}_3$ or

$-\text{NH}_2$

and X is an anion

wherein the R_6 substituent of the polysubstituted aryl acridinium ester is covalently coupled to a molecule with biological activity which molecule is capable of being covalently coupled to the substituent.