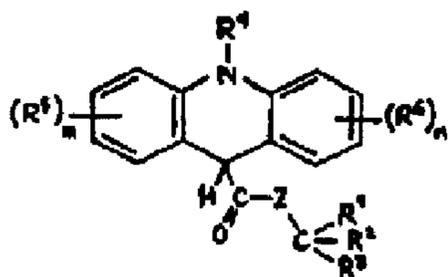
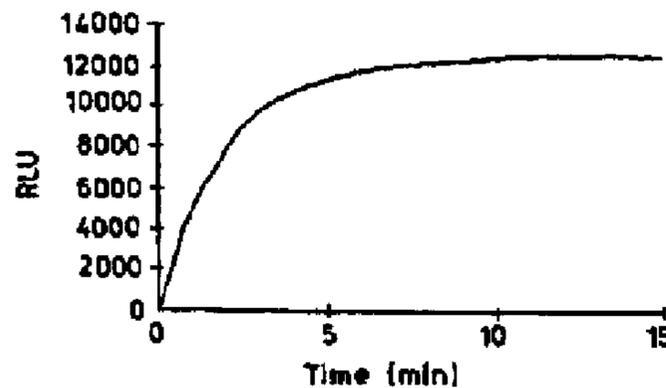
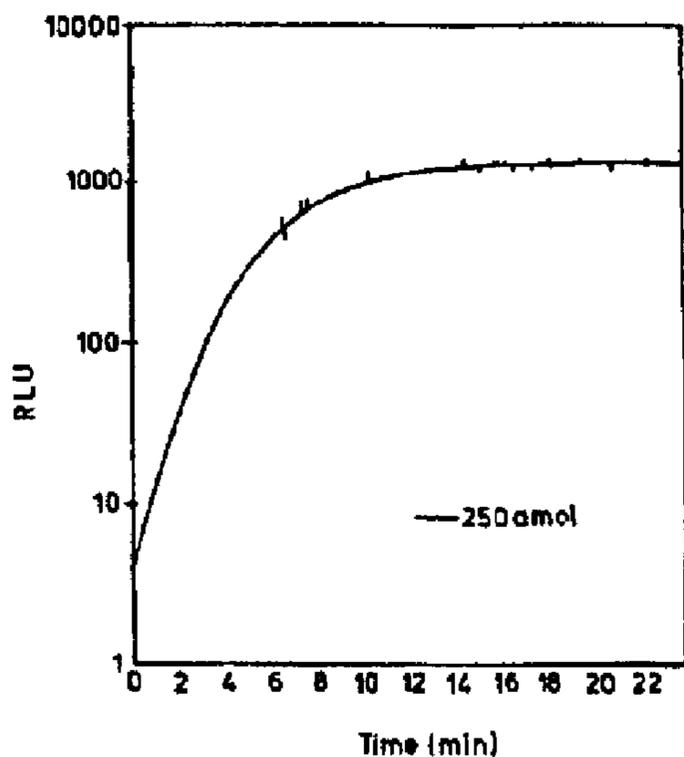




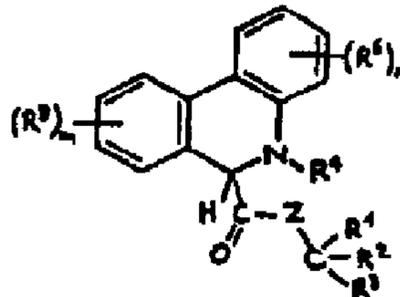
(86) Date de dépôt PCT/PCT Filing Date: 1997/07/15  
 (87) Date publication PCT/PCT Publication Date: 1998/01/22  
 (45) Date de délivrance/Issue Date: 2003/11/25  
 (85) Entrée phase nationale/National Entry: 1999/01/14  
 (86) N° demande PCT/PCT Application No.: NL 1997/000417  
 (87) N° publication PCT/PCT Publication No.: 1998/002421  
 (30) Priorité/Priority: 1996/07/16 (96202022.8) EP

(51) Cl.Int.<sup>6</sup>/Int.Cl.<sup>6</sup> C07D 219/04, C07F 9/64, C07F 9/576,  
C12Q 1/28, C07D 221/18, C07D 221/12, C07D 401/00  
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(54) Titre : ESTERS DIBENZODIHYDROPYRIDINECARBOXYLIQUES ET LEUR UTILISATION DANS LE CADRE  
D'ANALYSES DE CHIMILUMINESCENCE  
 (54) Title: DIBENZODIHYDROPYRIDINECARBOXYLIC ESTERS AND THEIR USE IN CHEMILUMINESCENT ASSAY  
METHODS



(1)



(2)

(57) Abrégé/Abstract:

New dibenzodihydropyridinecarboxylic esters are provided which comply with formula (1) or (2), wherein: R<sup>1</sup> is substituted alkyl or cyano; R<sup>2</sup> and R<sup>3</sup> are independently hydrogen, alkyl, substituted alkyl, aryl or substituted aryl; R<sup>4</sup> is hydrogen, alkyl, substituted alkyl, aryl or substituted aryl; R<sup>5</sup> and R<sup>6</sup> are independently alkyl, alkoxy or another substituent which is relatively

(57) **Abrégé(suite)/Abstract(continued):**

resistant to oxidation; m and n are integers from 0 to 4; wherein, if m or n is at least 2, two groups R<sup>5</sup> or R<sup>6</sup> may be linked together; and Z is oxygen or sulphur. The new compounds are useful as chemiluminogenic reagents for detecting the presence of peroxidase activity at extremely low levels (10<sup>-20</sup> moles).

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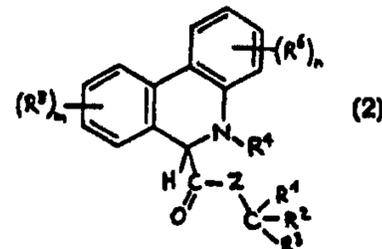
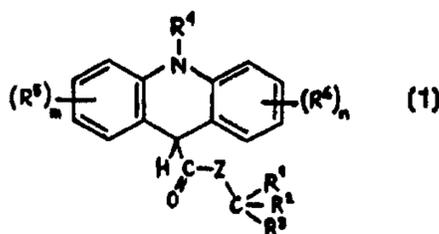
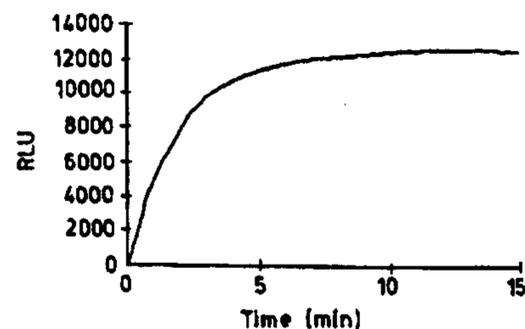
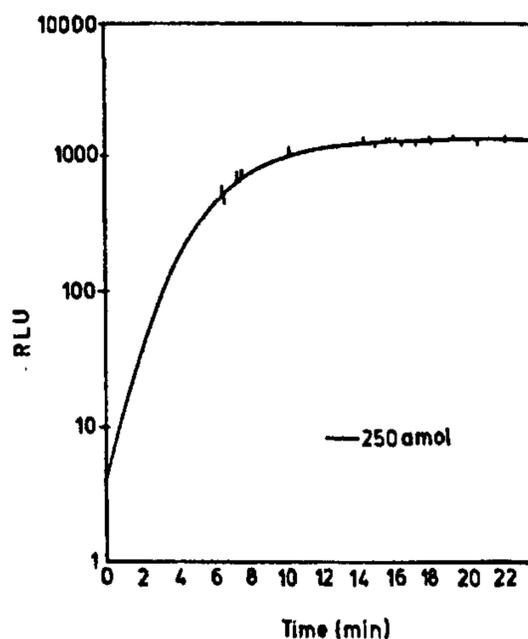
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification <sup>6</sup> : C07D 219/04, G01N 33/58, C07D 221/12</p>	A1	<p>(11) International Publication Number: <b>WO 98/02421</b> (43) International Publication Date: 22 January 1998 (22.01.98)</p>
<p>(21) International Application Number: PCT/NL97/00417 (22) International Filing Date: 15 July 1997 (15.07.97)</p> <p>(30) Priority Data: 96202022.8 16 July 1996 (16.07.96) EP (34) Countries for which the regional or international application was filed: NL et al.</p> <p>(71) Applicant (for all designated States except US): DE STAAT DER NEDERLANDEN, VERTEGENWOORDIGD DOOR DE MINISTER VAN WELZIJN, VOLKSGEZONDHEID EN CULTUURP.O. Box 5406 [NL/NL]; NL-2280 HK Rijswijk (NL).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): JACQUEMIJNS, Marjorie [NL/NL]; Prof. Bronkhorstlaan 28, NL-3451 ES Vleuten (NL). ZOMER, Gijsbert [NL/NL]; Fröbellaan 48, NL-3706 TH Zeist (NL).</p> <p>(74) Agent: DE BRUIJN, Leendert, C.; Nederlandsch Octrooibureau, Scheveningseweg 82, P.O. Box 29720, NL-2502 LS The Hague (NL).</p>		<p>(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published With international search report.</p>

(54) Title: DIBENZODIHYDROPYRIDINECARBOXYLIC ESTERS AND THEIR USE IN CHEMILUMINESCENT ASSAY METHODS

## (57) Abstract

New dibenzodihydropyridinecarboxylic esters are provided which comply with formula (1) or (2), wherein: R<sup>1</sup> is substituted alkyl or cyano; R<sup>2</sup> and R<sup>3</sup> are independently hydrogen, alkyl, substituted alkyl, aryl or substituted aryl; R<sup>4</sup> is hydrogen, alkyl, substituted alkyl, aryl or substituted aryl; R<sup>5</sup> and R<sup>6</sup> are independently alkyl, alkoxy or another substituent which is relatively resistant to oxidation; m and n are integers from 0 to 4; wherein, if m or n is at least 2, two groups R<sup>5</sup> or R<sup>6</sup> may be linked together; and Z is oxygen or sulphur. The new compounds are useful as chemiluminescent reagents for detecting the presence of peroxidase activity at extremely low levels (10<sup>-20</sup> moles).



## **Dibenzodihydropyridinecarboxylic esters and their use in chemiluminescent assay methods**

The invention relates to novel dibenzodihydropyridinecarboxylic esters, in particular acridanecarboxylic esters, and to their use as a chemiluminescent substrate in peroxidase assay methods.

Aromatic acridane esters have been known for more than 20 years (McCapra, *Acc. Chem. Res.*, 1976; 9: 201). The chemiluminescence of acridane esters proceeds in dipolar aprotic solvents upon addition of strong base. The mechanism is reported to involve a reaction with oxygen of the corresponding carbanion formed by the addition of the base. EP-A-625510 discloses certain aromatic acridane esters, in particular p-hydroxyphenyl 10-methylacridan-9-carboxylate, which can be used as a part of a signal reagent for the enzyme horseradish peroxidase (HRP). The signal reagent also contained an enhancer e.g. para-iodophenol, a chelating agent (EDTA), a nonionic surfactant, and hydrogen peroxide. According to EP-A-625510, HRP can be detected with great sensitivity at an optimal pH range of 8-9. More potent chemiluminescent polyfluorophenyl esters of acridane-9-carboxylic acid are disclosed in WO 95/23971 and phenyl thioesters are described in WO 95/28495.

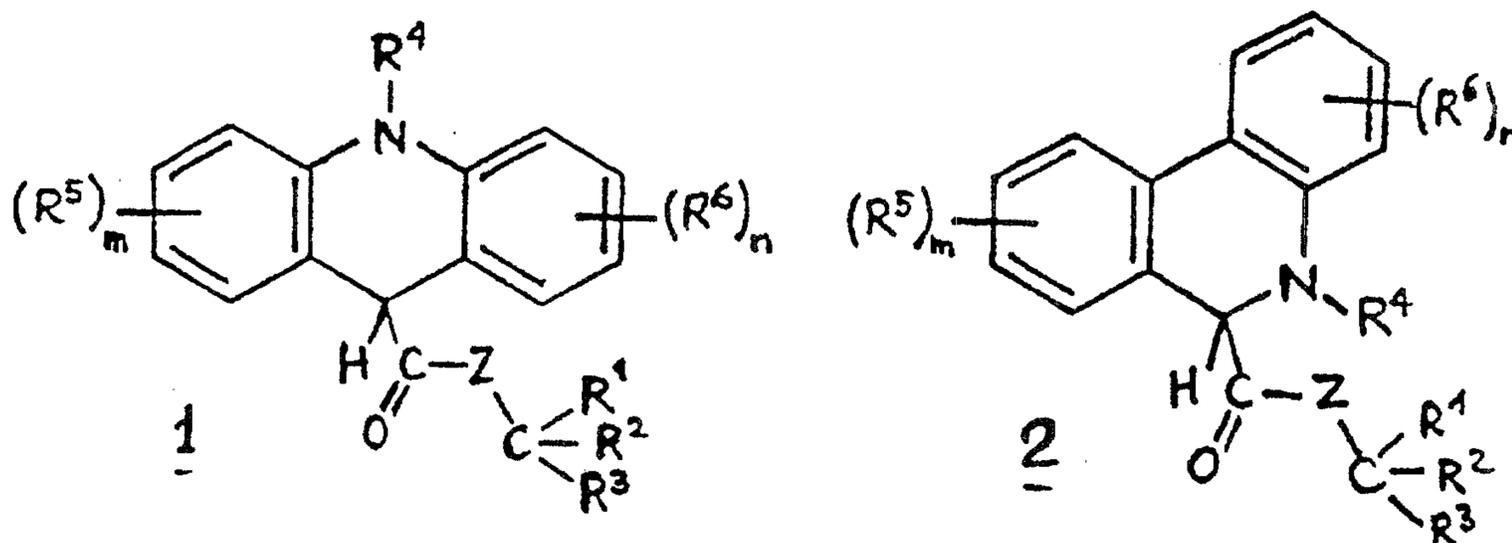
The optimum pH for HRP is around pH 5. Therefore, it seems unsatisfactory that the optimum pH for chemiluminescence detection of HRP is at pH 8-9. This pH is presumably a compromise between the best pH for the enzyme and the optimal pH for intermediate acridinium ester chemiluminescence, which occurs at basic pH's (Akhavan-Tafti et al. in: *Bioluminescence and Chemiluminescence. Fundamentals and Applied Aspects. Proceedings of the 8th International Symposium on Bioluminescence and Chemiluminescence*. Cambridge, September 1994. Ed. Campbell, Kricka, Stanley. John Wiley and Sons 1994: 199).

The present invention aims at an enzyme assay and at reagents to be used therein, which allow a higher sensitivity than the assay and reagents of the prior art. The present invention is based on the surprising discovery that certain aliphatic acridane esters and phenanthridane esters are capable of very intense and prolonged chemiluminescence upon reaction with hydrogen peroxide and certain enhancers. This reaction proceeds over a wide pH range (optimum pH range 6-8), and without the necessity of

adding chelating compounds (EDTA) or surfactants.

Moreover, these aliphatic esters produce a very low background luminescence, i.e. without enzyme.

Thus the invention is concerned with dibenzodihydro-  
5 pyridinecarboxylic ester having formula 1 or 2:



wherein:

$R^1$  is substituted alkyl or cyano;

$R^2$  and  $R^3$  are independently selected from hydrogen, alkyl, substituted alkyl, aryl, heteroaryl and substituted aryl and heteroaryl;

$R^4$  is selected from hydrogen, alkyl, substituted alkyl, aryl, heteroaryl and substituted aryl and heteroaryl;

$R^5$  and  $R^6$  are independently selected from alkyl, alkoxy, halogen, and other substituents which are relatively resistant to oxidation;

$m$  and  $n$  are integers from 0 to 4; wherein, if  $m$  or  $n$  is at least 2, two groups  $R^5$  or  $R^6$  may be bound together; and  $Z$  is oxygen or sulphur.

The dibenzodihydropyridinecarboxylic esters according to the invention can be acridane-9-carboxylic esters having formula 1, or phenanthridane-6-carboxylic esters having formula 2.

A key feature of the compounds according to the invention is that the  $\alpha$ -carbon atom of the ester alkyl group is an aliphatic carbon atom. It may be substituted with various aliphatic or other groups, designated in the above formulae 1 and 2 by  $R^1$ ,  $R^2$  and  $R^3$ .

In the specification of the compounds according to the present invention, "alkyl" means straight or branched, saturated or unsaturated C<sub>1</sub>-C<sub>24</sub>, preferably C<sub>1</sub>-C<sub>12</sub>, most preferably C<sub>1</sub>-C<sub>4</sub> alkyl, such as methyl, ethyl, propyl, isopropyl, butyl and decyl. "Substituted alkyl" means alkyl as defined above containing one or more substituents such as halogen, hydroxy, alkoxy, alkanoyl, carboxyl, alkoxy-carbonyl, alkanoyloxy, aryl, aryloxy, aroyl, aroyloxy, cyano, nitro and the like, especially halogen, hydroxy, alkoxy and aryl; the substituents themselves may also be substituted, e.g. with halogen, alkyl or alkoxy. "Aryl" means phenyl, naphthyl and higher carbocyclic aryl, and "heteroaryl" means such groups as furyl, thienyl, pyrrolyl, imidazolyl, thiazolyl, oxazolyl, pyridyl, pyrimidyl, quinolyl and the like. "Substituted aryl or heteroaryl" means aryl or heteroaryl as defined above containing one or more substituents as exemplified above for substituted alkyl; examples include chlorophenyl, trifluoromethyl-phenyl, methoxyphenyl, methylenedioxyphenyl and hydroxy-pyridyl. In "alkoxy", the alkyl part may be as defined above; examples include methoxy, ethoxy, t-butoxy, 2-chloroethoxy, 2-methoxyethoxy, hexafluoroisopropoxy, benzyloxy, 2-phenylethoxy and the like.

Substituents which are relatively resistant to oxidation are understood to comprise substituents that do not interfere with the enzymatic oxidation process, such as halogen, alkyl, haloalkyl, hydroxyalkyl, alkoxyalkyl, alkoxy, carboxyl, alkoxy-carbonyl, alkanoyl, alkanoyloxy, cyano, nitro, carbamoyl, acylamino, sulpho, phospho, penyl, pyridyl and other aryl groups, and the like.

Preferably, at least one of R<sup>1</sup>, R<sup>2</sup> and R<sup>3</sup> is a group containing electron-withdrawing substituents such as halogen, nitro, sulpho, cyano, acyl, sulphonyl, carboxyl. A suitable group with electron-withdrawing substituents is haloalkyl, such as mono-, di- and trichloromethyl, mono-, di- and trifluoromethyl, tri- or pentafluoroethyl, heptafluoropropyl and higher homologues. Especially preferred are trifluoromethyl groups for R<sup>1</sup> and optionally also for

## 3a

$R^2$ .  $R^3$  may then be e.g. hydrogen, lower alkyl, haloalkyl, phenyl or halophenyl.  $R^1$  may also be cyano, in which case  $R^2$  may be e.g. hydrogen, methyl, phenyl or trifluoromethyl and  $R^3$  e.g. hydrogen.

5       The nature of the substituent represented by  $R^4$  is not very critical. For reasons of convenience,  $R^4$  may be lower alkyl, especially methyl, but it may also be e.g. hydrogen, substituted alkyl (examples: carboxymethyl, hydroxyethyl, benzyl), phenyl or substituted phenyl.

10       The substituents  $R^5$  and  $R^6$  are preferably attached to the m- and/or p-positions with respect to the ring nitrogen, most preferably in the p-positions. Combinations of substituents  $R^5$  and  $R^6$  (m or n being a number of 2 or higher) that may be bound.....

together include C<sub>1</sub>-C<sub>6</sub> alkylene and other bivalent groups that, together with the benzo groups to which they are attached, form an additional ring such as cyclopenta ((R<sup>5</sup>)<sub>2</sub> = (CH<sub>2</sub>)<sub>3</sub>), cyclohexa ((R<sup>5</sup>)<sub>2</sub> = (CH<sub>2</sub>)<sub>4</sub>), furo ((R<sup>5</sup>)<sub>2</sub> = CH<sub>2</sub>-O-CH<sub>2</sub>), dioxolo ((R<sup>5</sup>)<sub>2</sub> = O-CH<sub>2</sub>-O), benzo ((R<sup>5</sup>)<sub>2</sub> = CH=CH-CH=CH), pyrido ((R<sup>5</sup>)<sub>2</sub> = N=CH-CH=CH or CH=N-CH=CH), and the like. Preferred groups R<sup>5</sup> and/or R<sup>6</sup> are chloro, fluoro, bromo, methoxy and methylenedioxy, most preferred group R<sup>5</sup> is chloro.

The dibenzodihydropyridinecarboxylic esters according to the invention can be prepared by methods known per se. A general synthesis scheme for the acridane esters, which is further illustrated in the examples, can be as follows:

A suitably substituted aniline (I) having the formula (R<sup>5</sup>)<sub>m</sub>C<sub>6</sub>H<sub>(5-m)</sub>NH<sub>2</sub> is converted to the acetanilide (II), which is reacted with a suitably substituted phenyl bromide (III) having the formula (R<sup>6</sup>)<sub>n</sub>C<sub>6</sub>H<sub>(5-n)</sub>Br in the presence of copper iodide to produce the diphenylamine (IV) having the formula (R<sup>5</sup>)<sub>m</sub>C<sub>6</sub>H<sub>(5-m)</sub>-NH-C<sub>6</sub>H<sub>(n-5)</sub>(R<sup>6</sup>)<sub>n</sub>. This diphenylamine (IV) is converted to the acridine-9-carboxylic acid (V) by reaction with oxalic chloride in the presence of a Lewis acid. After esterification with the appropriate alcohol R<sup>1</sup>R<sup>2</sup>R<sup>3</sup>COH or thiol R<sup>1</sup>R<sup>2</sup>R<sup>3</sup>CSH and N-alkylation, the 9-alkoxycarbonyl- or 9-alkylthiocarbonyl-acridinium compound with formula 3 is obtained, which can then be reduced to produce the desired acridane ester having formula 1.

The synthesis of the phenanthridane esters proceeds in an analogous way starting with optionally substituted 2-aminobiphenyl. Acetylation and ring closure yields (substituted) 5-methylphenanthridine. The 6-carboxy group is introduced by reaction of (substituted) 5-methylphenanthridine with formaldehyde, followed by oxidation to give (substituted) 6-carboxy-5-methylphenanthridine. Esterification with the appropriate alcohol or thiol, followed by N-alkylation yields the 6-alkoxycarbonyl- or 6-alkylthiocarbonyl-phenanthridinium compound with formula 4.

The invention furthermore relates to a method for assaying a peroxidase, wherein a dibenzodihydropyridinecarboxylic ester as described above is used as a chemiluminescent substrate. The method can be used for assaying the peroxidase enzyme itself, or for assaying an analyte labelled with the peroxidase. The method is extremely sensitive in that peroxidase levels as low as 10<sup>-20</sup> moles can be reliably detected.

In the method of the invention, the dibenzodihydropyridinecarboxylic ester according to the invention is contacted with a sample containing the peroxidase or the

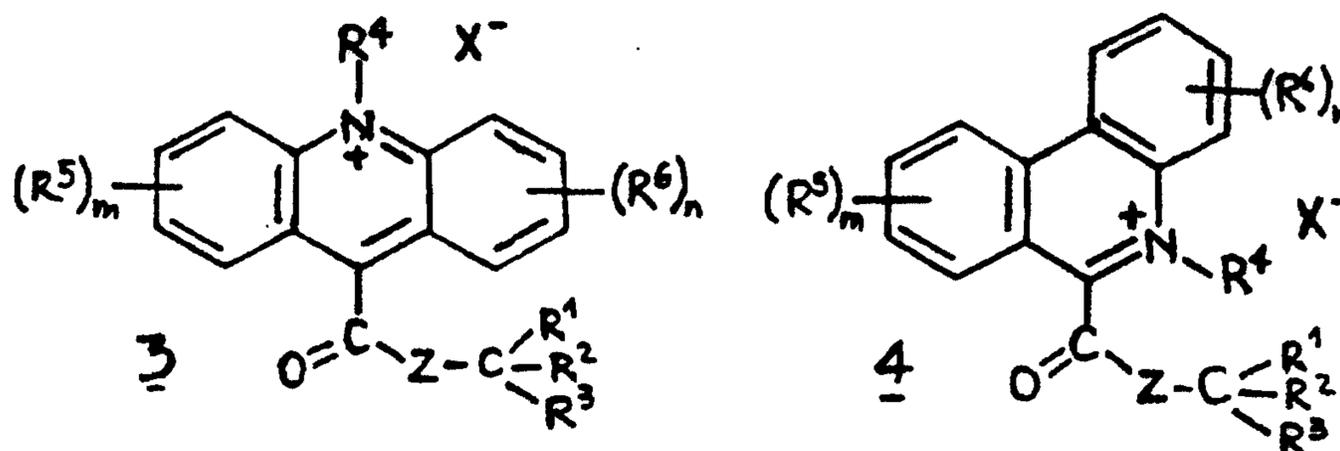
analyte labelled with the peroxidase, and with a peroxide and optionally an enhancer so as to generate light. The light is most probably generated by an excited state of the dihydropyridone resulting from the reaction with peroxide. The preferred peroxidase is horseradish peroxidase (HRP). The preferred peroxide is hydrogen peroxide; other suitable peroxides include urea peroxide, perborates and the like. The preferred enhancer is p-phenylphenol, but other phenolic enhancers such as p-iodophenol, p-thiazolylphenol, p-hydroxycinnamic acid, other substituted phenols and optionally substituted naphthols, hydroxy-benzothiazoles, hydroxy-benzoxazoles and hydroxy-fluorenones, can also be used. The pH to be used in the method of the invention can vary over a wide range, for example from 4 to 10. The preferred pH range is from 5.5 to 8, in particular from 6 to 7.5. The method is preferably performed in a buffered aqueous system. The dibenzodihydropyridinecarboxylic ester may be used in a concentration of  $10^{-7}$  to  $10^{-4}$  molar, the enhancer in a similar or somewhat higher concentration (e.g.  $10^{-7}$  to  $10^{-3}$  molar). The peroxide may be added in a concentration of e.g.  $10^{-5}$  to  $10^{-2}$ . The enzyme may be present in the attomol ( $10^{-18}$  mole) range or even lower. The presence of surfactants such as poly(ethylene oxides) or chelating agents such as EDTA is not necessary, and is therefore preferably dispensed with.

The method of the invention can also be used to assay peroxides such as hydrogen peroxide which may be generated by the action of dehydrogenase or oxidase enzymes in solution, or to assay molecular oxygen in a solution in an aprotic solvent such as acetonitrile, nitromethane, dimethyl sulphoxide and the like.

The method of the invention can further be used for assaying hydrolytic enzymes such as phosphatases and glycosidases by using a phosphorylated or glycosylated phenol which, after hydrolytic cleavage of the phosphate or glycosyl group, serves as an enhancer in a peroxidase reaction.

The invention also relates to a kit for assaying peroxidase activity comprising a dibenzodihydropyridinecarboxylic ester as described above and optionally a peroxide, a phenolic enhancer and/or further constituents for performing the assay as described above. The kit may further contain an analyte, such as an antibody, labelled with a peroxidase enzyme such as HRP. If used for peroxide detection, the kit may also contain a peroxidase as such.

The invention also relates to novel dibenzopyridinium (acridinium and phenanthridinium) compounds having formula 3 or 4:



wherein  $\text{R}^1$  is haloalkyl or cyano and  $\text{R}^2$  to  $\text{R}^6$  and  $\text{Z}$  are as defined above for compounds 1 and 2. The counterion  $\text{X}^-$  may be any suitable, relatively inert ion, such as halide, sulphate,  $\text{R}^7$ -sulphonate wherein  $\text{R}^7$  is e.g. alkyl, haloalkyl or aryl, tetrafluoroborate etc. Preferably  $\text{R}^1$  is trifluoromethyl or cyano,  $\text{R}^2$  is trifluoromethyl and  $\text{R}^3$  is hydrogen, trifluoromethyl, phenyl or substituted phenyl.  $\text{R}^5$  and/or  $\text{R}^6$  are preferably halogen or methoxy, and  $m$  and  $n$  are preferably 0, 1 or 2, most preferably at least one of them is 1.

The dihydropyridinium salts can be used as a direct label for detecting the presence of analytes such as antibodies, antigens, DNA-probes and other biologically interesting substances as described in EP-A-324202. Suitable compounds 3 and 4 have substituents containing a moiety capable of chemically reacting with (bio)molecules. Examples thereof are carboxy, aldehyde, alcohol, amino or thiol functions that can be present in any one of  $\text{R}^1$  to  $\text{R}^6$ , especially in  $\text{R}^4$ , such as when  $\text{R}^4$  is carboxymethyl.

## EXAMPLES

### Synthesis of the acridan derivatives GZ-1/GZ-12.

The compounds given in table I were synthesised.

The general synthesis procedure was as follows:

#### *Acetanilide (II):*

The particular aromatic amine I (20 g), acetic acid anhydride (20 ml), acetic acid (glacial, 20 ml) and zinc powder (0.1 g) were mixed and refluxed. After 30 min. the solution was poured into 500 ml ice water and the precipitate was filtered and dried to obtain the acetanilide.

*Diphenyl amine (IV):*

The acetanilide II (13.5 g), the particular aromatic bromide III (25 g), potassium carbonate (13.2 g) and copper iodide (1.9 g) were heated (190 °C) and stirred overnight. After cooling to room temperature toluene was added and the precipitate filtered. The solution was concentrated and the excess of bromide removed by distillation under reduced pressure. The residue was dissolved in ethanol (200 ml), potassium hydroxide (10.3 g) was added and the mixture refluxed overnight. Ethanol was evaporated, the residue dissolved in dichloromethane, and washed with brine. The organic layer was dried over MgSO<sub>4</sub> and concentrated to obtain the crude diphenyl amine.

**Table 1**

	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>	R <sup>5</sup> , R <sup>6</sup>	Z
GZ-1	CF <sub>3</sub>	CF <sub>3</sub>	H	CH <sub>3</sub>	-	O
GZ-2	CF <sub>3</sub>	CF <sub>3</sub>	H	CH <sub>3</sub>	2-OCH <sub>3</sub> , 7-OCH <sub>3</sub>	O
GZ-3	CF <sub>3</sub>	CF <sub>3</sub>	H	CH <sub>3</sub>	2-OCH <sub>3</sub> , 3-OCH <sub>3</sub>	O
GZ-4	CF <sub>3</sub>	CF <sub>3</sub>	H	CH <sub>3</sub>	2-F	O
GZ-5	CF <sub>3</sub>	CF <sub>3</sub>	H	CH <sub>3</sub>	2-Cl	O
GZ-6	CF <sub>3</sub>	CF <sub>3</sub>	H	CH <sub>3</sub>	2-OCH <sub>3</sub> , 6-OCH <sub>3</sub> , 3-OCH <sub>3</sub> , 7-OCH <sub>3</sub>	O
GZ-7	CF <sub>3</sub>	CF <sub>3</sub>	H	CH <sub>3</sub>	2-Br, 7-Br	O
GZ-8	CF <sub>3</sub>	CF <sub>3</sub>	C <sub>6</sub> H <sub>5</sub>	CH <sub>3</sub>	2-Cl	O
GZ-9	CF <sub>3</sub>	CF <sub>3</sub>	C <sub>6</sub> H <sub>5</sub>	CH <sub>3</sub>	2-OCH <sub>3</sub> , 3-OCH <sub>3</sub>	O
GZ-10	CF <sub>3</sub>	CF <sub>3</sub>	H	CH <sub>3</sub>	3-OCH <sub>3</sub>	O
GZ-11	CF <sub>3</sub>	CF <sub>3</sub>	CF <sub>3</sub>	CH <sub>3</sub>	2-Cl	O
GZ-12	CF <sub>3</sub>	H	H	CH <sub>3</sub>	-	S

*Acridine-9-carboxylic acid (GW):*

To a solution of oxalic chloride (5 g) in dichloromethane, a solution of the diphenyl amine IV (5 g) in dichloromethane was added dropwise and refluxed for 30 min.. The solution was concentrated (50 %) and aluminium trichloride (8 g) added in portions. The mixture was refluxed for 45 min. and the solvent evaporated. To this residue hydrochloric acid in ice water (1 molar) was added and the red coloured precipitate filtered. The precipitate was dissolved in potassium hydroxide (10 % in water), refluxed overnight and poured into hydrochloric acid in ice water (5 molar). The yellow acridine-9-carboxylic acid (GW) was filtered, washed with water and dried.

*Acridine-9-carboxylic ester (GX):*

The acridine-9-carboxylic acid GW (1 g) was mixed with thionyl chloride (20 ml) and refluxed until a clear solution was obtained. The solution was concentrated and the excess thionyl chloride coevaporated with toluene. To the residue pyridine (20 ml), dimethylaminopyridine (1 g) and an excess of the particular alcohol (or thiol)  $R^1R^2R^3COH$  (-SH) were added. The mixture was stirred overnight at room temperature, poured into hydrochloric acid in ice water (1 molar) and extracted with dichloromethane. The organic layer was washed with water, dried over  $MgSO_4$  and concentrated. The crude product was purified by flash-chromatography (66% hexanes/ethyl acetate).

*10-Methylacridinium-9-carboxylic ester trifluoromethanesulfonate (GY):*

The acridine-9-carboxylic ester GX (100 mg) was dissolved in dichloromethane (5 ml). To this solution an excess of methyl trifluoromethanesulfonate was added. The mixture was stirred overnight at room temperature. Diethyl ether was added and the obtained precipitate was filtered, washed with diethyl ether and dried.

*10-Methylacridane-9-carboxylate (GZ):*

The 10-methylacridinium-9-carboxylic ester trifluoromethanesulfonate GY (100 mg) was dissolved in dichloromethane (100 ml). To this solution perchloric acid (20 drops) and zinc powder (6 g) were added. The mixture was placed in an ultrasone bath for three hours and filtered. The solution was washed with water (1x) and hydrochloric acid (2x, 0.1 molar), dried over  $MgSO_4$  and concentrated.

**Example 1. Synthesis of GZ-1.***Hexafluoroisopropyl acridine-9-carboxylate (GX-1)*

$^1H$  NMR (acetone- $d_6$ )  $\delta$  7.2 (m, 1H), 7.8-8.3 (m, 8H).

*9-Hexafluoroisopropoxycarbonyl-10-methylacridinium trifluoromethanesulfonate (GY-1)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 5.3 (s, 3H), 7.4 (m, 1H), 8.2-9.2 (m, 8H).

<sup>13</sup>C NMR (acetone-d<sub>6</sub>) δ 38.3, 41.4, 70.4, 120.5, 121.6, 124.2, 125.3, 127.8, 131.6, 141.2, 144.1, 146.4, 149.4, 163.4, 165.2.

*Hexafluoroisopropyl 10-methylacridane-9-carboxylate (GZ-1)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 3.5 (s, 3H), 5.4 (s, 1H) 6.2 (m, 1H), 6.9-7.4 (m, 8H).

**Example 2. Synthesis of GZ-2.**

*2,7-Dimethoxyacridine-9-carboxylic acid (GW-2)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 4.0 (s, 6H), 7.1-8.2 (m, 6H)

*Hexafluoroisopropyl 2,7-dimethoxyacridine-9-carboxylate (GX-2)*

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 4.0 (s, 6H), 6.4 (m, 1H), 7.0-8.1 (m, 6H).

*9-Hexafluoroisopropoxycarbonyl-2,7-dimethoxy-10-methylacridinium trifluoromethanesulfonate (GY-2)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 4.1 (s, 6H), 5.2 (s, 3H), 7.2 (d, 2H-acr), 7.3 (m, 1H), 8.1 (dd, 2H-acr), 9.0 (d, 2H-acr).

*Hexafluoroisopropyl 2,7-dimethoxy-10-methylacridane-9-carboxylate (GZ-2)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 3.4 (s, 3H), 3.7 (s, 6H), 5.3 (s, 1H), 6.2 (m, 1H), 6.8-7.0 (m, 6H).

**Example 3. Synthesis of GZ-3.**

*2,3-Dimethoxyacridine-9-carboxylic acid (GW-3)*

<sup>1</sup>H NMR (DMSO-d<sub>6</sub>) δ 3.9 (s, 3H), 4.0 (s, 3H), 7.2-8.1 (m, 6 H).

*Hexafluoroisopropyl-2,3-dimethoxyacridine-9-carboxylate (GX-3)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 4.0 (s, 3H), 4.1 (s, 3H), 7.1 (s, 1H-acr), 7.2 (m, 1H), 7.5-8.2 (m, 5H-acr).

*9-Hexafluoroisopropoxycarbonyl-2,3-dimethoxy-10-methylacridinium trifluoromethanesulfonate (GY-3)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 4.2 (s, 3H), 4.4 (s, 3H), 5.1 (s, 3H), 7.3 (s, 1H-acr), 7.4 (m, 1H), 8.1-8.9 (m, 5H-acr).

*Hexafluoroisopropyl 2,3-dimethoxy-10-methylacridane-9-carboxylate (GZ-3)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 3.5 (s, 3H), 3.8 (s, 3H), 3.9 (s, 3H), 5.3 (s, 1H), 6.2 (m, 1H), 6.8-7.3 (m, 6H).

**Example 4. Synthesis of GZ-4.**

*2-Fluoroacridine-9-carboxylic acid (GW-4)*

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  7.7-8.4 (m, 7H).

*Hexafluoroisopropyl 2-fluoroacridine-9-carboxylate (GX-4)*

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  6.3 (m, 1H), 7.5-8.4 (m, 7H).

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  67.5 (m), 106.5 (d), 118.3, 122.4 (d), 122.5 (d), 122.9, 123.7, 128.9, 130.4 (d), 133.3 (d), 146.0, 148.0 (d), 159.4, 163.1, 164.2.

*9-Hexafluoroisopropoxycarbonyl-2-fluoro-10-methylacridinium trifluoromethane-sulfonate (GY-4)*

$^1\text{H}$  NMR (acetone- $\text{d}_6$ )  $\delta$  5.3 (s, 3H), 7.3 (m, 1H), 7.9-9.3 (m, 7H).

*Hexafluoroisopropyl 2-fluoro-10-methylacridane-9-carboxylate (GZ-4)*

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.4 (s, 3H), 5.1 (s, 1H), 5.5 (m, 1H), 6.9-7.4 (m, 7H).

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  33.4 (d), 48.8 (d), 76.3 (m), 112.9, 113.8 (d), 115.4 (2xd), 118.0, 119.9, 120.0, 121.0 (q), 122.2, 128.9 (d), 138.8, 142.2, 155.8, 159.3, 168.0.

**Example 5. Synthesis of GZ-5.**

*2-Chloroacridine-9-carboxylic acid (GW-5)*

$^1\text{H}$  NMR ( $\text{DMSO-}d_6$ )  $\delta$  7.7-8.3 (m, 7H).

*Hexafluoroisopropyl 2-chloroacridine-9-carboxylate (GX-5)*

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  6.3 (m, 1H), 7.7-8.3 (m, 7H).

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  67.6 (m), 118.3, 122.6, 124.0, 128.9, 130.5, 130.8, 131.6, 131.9, 146.8, 148.6, 164.1.

*9-Hexafluoroisopropoxycarbonyl-2-chloro-10-methylacridinium trifluoromethane-sulfonate (GY-5)*

$^1\text{H}$  NMR (acetone- $\text{d}_6$ )  $\delta$  5.3 (s, 3H), 7.4 (m, 1H), 8.3-9.2 (m, 7H).

*Hexafluoroisopropyl 2-chloro-10-methylacridane-9-carboxylate (GZ-5)*

$^1\text{H}$  NMR (acetone- $\text{d}_6$ )  $\delta$  3.4 (s, 3H), 5.5 (s, 1H), 6.2 (m, 1H), 7.0-7.5 (m, 7H).

**Example 6. Synthesis of GZ-6.**

*2,3,6,7-Tetramethoxyacridine-9-carboxylic acid (GW-6)*

$^1\text{H}$  NMR ( $\text{DMSO-}d_6$ )  $\delta$  3.8 (s, 6H), 3.9 (s, 6H), 7.3 (s, 2H), 7.4 (s, 2H).

*Hexafluoroisopropyl 2,3,6,7-tetramethoxyacridine-9-carboxylate (GX-6)*

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  4.0 (s, 6H), 4.1 (s, 6H), 6.4 (m, 1H), 7.1 (s, 2H), 7.5 (s, 2H).

*9-Hexafluoroisopropoxycarbonyl-2,3,6,7-tetramethoxy-10-methylacridinium trifluoromethanesulfonate (GY-6)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 4.1 (s, 6H), 4.3 (s, 6H), 5.0 (s, 3H), 7.2 (s, 2H), 7.3 (m, 1H), 8.1 (s, 2H).

**Example 7. Synthesis of GZ-8.**

*α,α-Bis(trifluoromethyl)benzyl 2-chloroacridine-9-carboxylate (GX-8)*

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.5-8.3 (m, 12H).

*9-[α,α-Bis(trifluoromethyl)benzyloxycarbonyl]-2-chloro-10-methylacridinium trifluoromethanesulfonate (GY-8)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 5.3 (s, 3H), 7.1-9.3 (m, 12H).

*α,α-Bis(trifluoromethyl)benzyl 2-chloro-10-methylacridane-9-carboxylate (GZ-8)*

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 3.39 (s, 3H), 5.07 (s, 1H), 6.8-7.4 (m, 12H)

<sup>13</sup>C NMR (CDCl<sub>3</sub>) δ 165.6, 141.8, 140.9, 129.9, 129.2, 129.0, 128.7, 128.5, 128.2, 127.8, 126.8, 126.3, 126.0, 125.7, 123.5, 121.1, 120.6, 119.3, 118.5, 115.0, 114.5, 114.0, 112.9, 49.8, 33.2

**Example 8. Synthesis of GZ-9.**

*α,α-Bis(trifluoromethyl)benzyl 2,3-dimethoxyacridine-9-carboxylate (GX-9)*

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 4.0 (s, 3H), 4.1 (s, 3H), 7.5-8.2 (m, 11H).

*9-[α,α-Bis(trifluoromethyl)benzyloxycarbonyl]-2,3-dimethoxy-10-methylacridinium trifluoromethanesulfonate (GY-9)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 4.1 (s, 3H), 4.4 (s, 3H), 5.1 (s, 3H), 7.5-8.9 (m, 11H).

**Example 9. Synthesis of GZ-10.**

*Hexafluoroisopropyl 3-methoxyacridine-9-carboxylate (GX-10)*

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 4.0 (s, 3H), 6.3 (m, 1H), 7.3-8.2 (m, 7H).

*9-Hexafluoroisopropoxycarbonyl-3-methoxy-10-methylacridinium trifluoromethanesulfonate (GY-10)*

<sup>1</sup>H NMR (acetone-d<sub>6</sub>) δ 4.4 (s, 3H), 5.0 (s, 3H), 7.3 (m, 1H), 7.7-9.0 (m, 7H).

**Example 10. Synthesis of GZ-11.**

*Perfluoro-*t*-butyl 2-chloroacridine-9-carboxylate (GX-11)*

<sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.6-7.9 (m, 5H), 8.2-8.3 (m, 2H).

*9-Perfluoro-*t*-butoxycarbonyl-2-chloro-10-methylacridinium trifluoromethanesulfonate (GY-11)*

$^1\text{H}$  NMR (acetone- $d_6$ )  $\delta$  5.3 (s, 3H), 8.2-9.3 (m, 7H).

*Perfluoro-t-butyl 2-chloro-10-methylacridane-9-carboxylate (GZ-11)*

$^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.4 (s, 3H), 5.1 (s, 1H), 6.9-7.4 (m, 7H).

$^{13}\text{C}$  NMR ( $\text{CDCl}_3$ )  $\delta$  163.9, 141.7, 140.8, 129.3, 128.9, 128.5, 125.7, 121.4, 121.2, 119.7, 117.8, 117.1, 114.0, 113.0, 49.5, 33.1

#### **Example 11. Chemiluminescence measurements using GZ-8.**

Chemiluminescence (CL) measurements were performed in lumacuvettes using a Lumac Biocounter M2010. A chemiluminogenic signal reagent consisted of a solution of phenylphenol ( $9 \times 10^{-5}$  M), GZ-8 ( $4 \times 10^{-6}$  M) in PBS buffer (pH 7.2, 0.01 M). To this solution was added 250 attomoles of HRP. The lumacuvette was placed in the luminometer and the CL reaction was started by the addition of hydrogen peroxide ( $4 \times 10^{-4}$  M). The CL reaction was followed over a period of 22 minutes. The resulting CL kinetic profile is shown in figure 1.

#### **Example 12. HRP calibration curve using GZ-8.**

In a similar experiment as described in example 12, different amounts of HRP (0-250 amol) were incubated with phenylphenol ( $9 \times 10^{-5}$  M), GZ-8 ( $4 \times 10^{-6}$  M) in PBS buffer (pH 7.2, 0.01 M). To all the tubes was added hydrogen peroxide ( $4 \times 10^{-4}$  M). After 16 minutes the CL was measured. The CL-counts, corrected for background, were plotted against the amounts of HRP to obtain a HRP calibration curve. An example of such a calibration curve is shown in figure 2.

#### **Example 13. Chemiluminescent measurements using GZ-11.**

In a similar experiment as described in example 12, a kinetic curve for the CL-reaction of HRP (25 amol) with GZ-11 was constructed. The results are shown in figure 3. When comparing figures 1 and 3, it can be seen that the use of GZ-11 as part of the CL-signal reagent causes a much more rapid rise of the CL with time. Within 2 minutes the curve reaches half of the maximal value. The results are shown in figure 3.

#### **Example 14. HRP calibration curve using GZ-11.**

A HRP calibration curve was constructed using the same procedure as described in example 13. This gave the calibration curve as shown in figure 4.

#### **Example 15. Comparison of chemiluminescence of GZ-8 and GZ-11 with prior art compound**

Compound 5e of WO 95/23971 (2,3,6-trifluorophenyl 10-methylacridan-9-carboxylate, also denoted as Lumigen PS-3) was synthesized according to the general synthesis procedure starting from 9-acridinecarboxylic acid and 2,3,6-trifluorophenol.

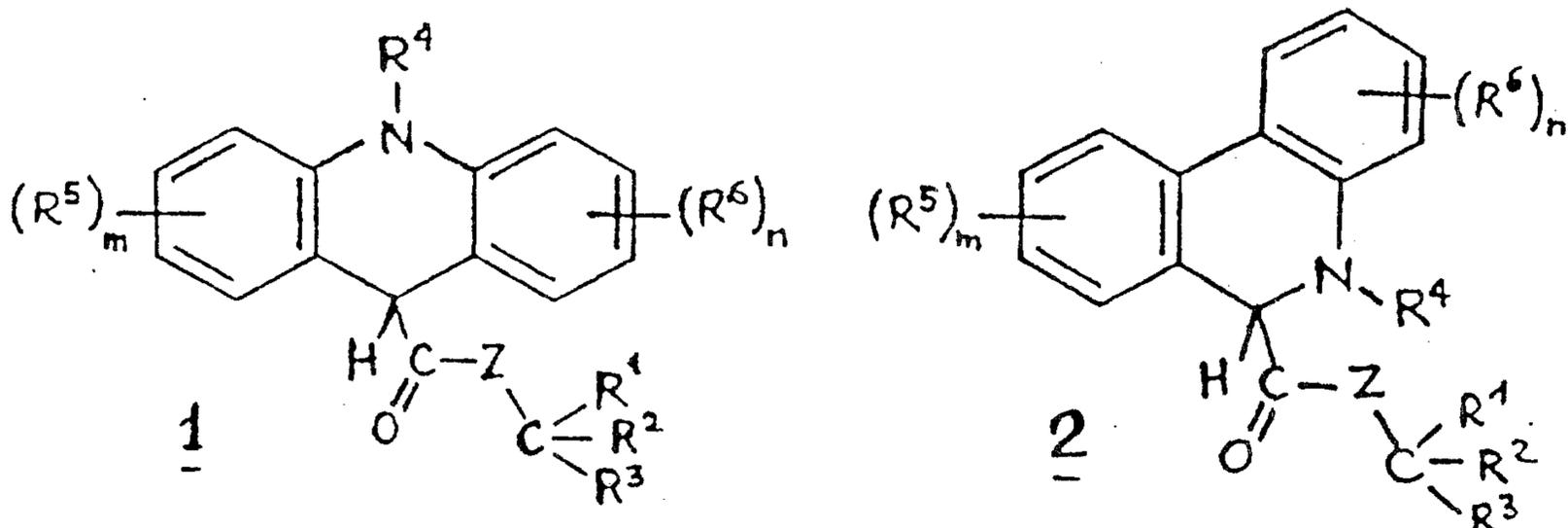
$^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 3.44 (s, 3H), 5.29 (s, 1H), 6.76-7.4 (m, 10H).

The CL measurements of GZ-8 and GZ-11 were performed as described in examples 12 and 14, respectively, using 25 amol of HRP for GZ-8 as well.

The CL measurements of PS-3 were performed using a signal reagent containing PS-3 (0.05 mM), para-iodophenol (1.1 mM), Tween 20 (0.5%), EDTA (1 mM) and  $\text{H}_2\text{O}_2$  (0.4 mM) in Tris buffer (0.1 M, pH 8). Upon addition of 25 amol of HRP, the CL was recorded over a period of 17 minutes. The results are shown in figure 5, wherein S/B denotes signal to background ratio.

## CLAIMS:

1. Dibenzohilhydropyridinecarboxylic ester having formula 1 or 2, wherein:



- $R^1$  is alkyl substituted by halogen, nitro, sulpho, cyano, acyl, sulphonyl or carboxyl, or  $R^1$  is cyano;
- $R^2$ ,  $R^3$  and  $R^4$  are independently hydrogen, alkyl, aryl, heteroaryl, or alkyl or aryl or heteroaryl substituted by halogen, hydroxy, alkoxy, alkanoyl, carboxyl, alkoxycarbonyl, alkanoyloxy, aryl, aryloxy, aroyl, aroyloxy, cyano or nitro;
- $R^5$  and  $R^6$  are independently halogen, alkyl, haloalkyl, hydroxyalkyl, alkoxyalkyl, alkoxy, carboxyl, alkoxycarbonyl, alkanoyl, alkanoyloxy, cyano, nitro, carbamoyl, acylamino, sulpho, phospho, phenyl, pyridyl or other aryl;
- $m$  and  $n$  are integers from 0 to 4; wherein, if  $m$  or  $n$  is at least 2, two groups  $R^5$  or  $R^6$  may be linked together; and
- $Z$  is oxygen or sulphur.

2. Dibenzodihydropyridinecarboxylic ester according to claim 1, wherein  $R^1$  is a haloalkyl group.

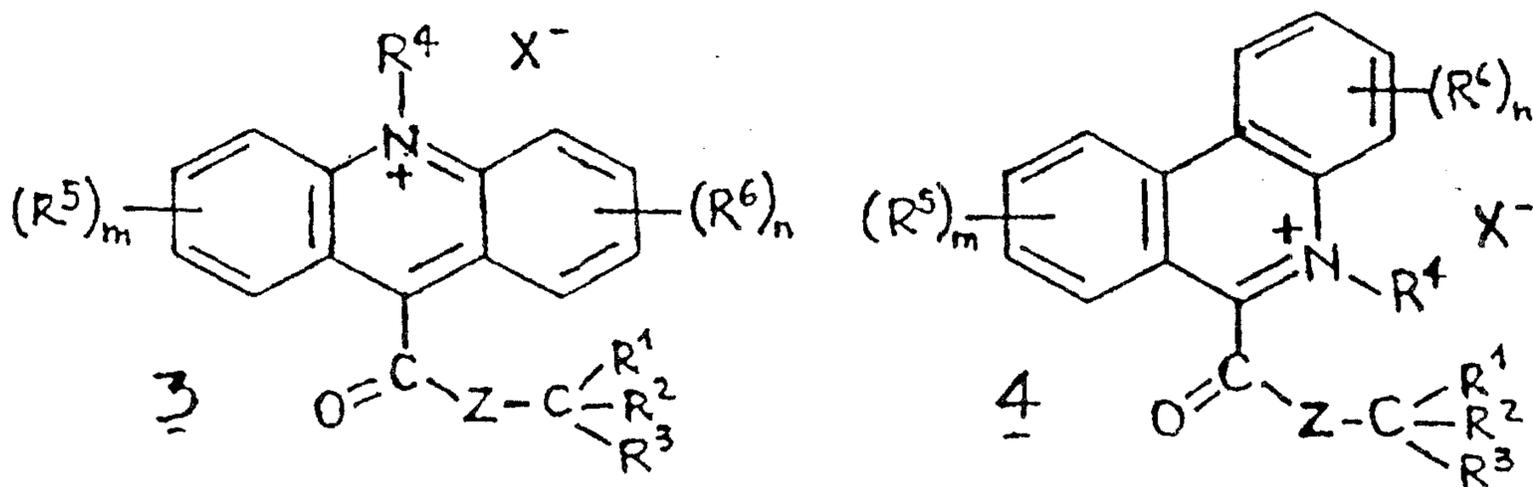
3. Dibenzodihydropyridinecarboxylic ester according to claim 2, wherein  $R^1$  is a trifluoromethyl group.

4. Dibenzodihydropyridinecarboxylic ester according to claim 2 or 3, wherein  $R^2$  is a haloalkyl group.
5. Dibenzodihydropyridinecarboxylic ester according to claim 4 wherein  $R^2$  is a trifluoromethyl group.
- 5 6. Dibenzodihydropyridinecarboxylic ester according to any one of claims 1-5, wherein  $R^3$  is hydrogen, alkyl, haloalkyl, phenyl or halophenyl.
7. Dibenzodihydropyridinecarboxylic ester according to any one of claims 1-6, wherein  $R^5$  and  $R^6$  are independently  
10 methoxy, chloro and/or fluoro, and m and n are independently 0 or 1.
8. Dibenzodihydropyridinecarboxylic ester according to any one of claims 1-7, wherein Z is oxygen.
9. Method for assaying a peroxidase, wherein a  
15 dibenzodihydropyridinecarboxylic ester according to any one of claims 1-8 is used as a chemiluminescent substrate.
10. Method according to claim 9, wherein a pH of 6 - 7.5 is used.
11. Method according to claim 9 or 10, wherein a phenol is  
20 used as an enhancer.
12. Method according to claim 11 wherein said phenol is p-phenyl-phenol.
13. Kit for assaying peroxidase activity, comprising a  
25 dibenzodihydropyridinecarboxylic ester according to any one of claims 1-8.
14. Kit according to claim 13 including a peroxide.
15. Kit according to claim 13 or 14 including a phenolic

enhancer.

16. Kit according to any one of claims 13 to 15 including a peroxidase.

17. Dibenzopyridinium compound having formula 3 or 4,



5 wherein

$R^1$  is haloalkyl or cyano;

$R^2$ ,  $R^3$  and  $R^4$  are independently hydrogen, alkyl, aryl,  
heteroaryl, or alkyl or aryl or heteroaryl substituted  
by halogen, hydroxy, alkoxy, alkanoyl, carboxyl,  
10 alkoxy carbonyl, alkanoyloxy, aryl, aryloxy, aroyl,  
aroyloxy, cyano or nitro;

$R^5$  and  $R^6$  are independently halogen, alkyl, haloalkyl,  
hydroxyalkyl, alkoxyalkyl, alkoxy, carboxyl,  
alkoxy carbonyl, alkanoyl, alkanoyloxy, cyano, nitro,  
15 carbamoyl, acylamino, sulpho, phospho, phenyl, pyridyl  
and other aryl;

$m$  and  $n$  are integers from 0 to 4; wherein, if  $m$  or  $n$  is at  
least 2, two groups  $R^5$  or  $R^6$  may be linked together;

and

20  $X$  is an inert counterion;

$Z$  is oxygen or sulphur.

18. Dibenzopyridinium compound according to claim 17  
wherein at least one of  $m$  and  $n$  is 1 or 2.

1/3

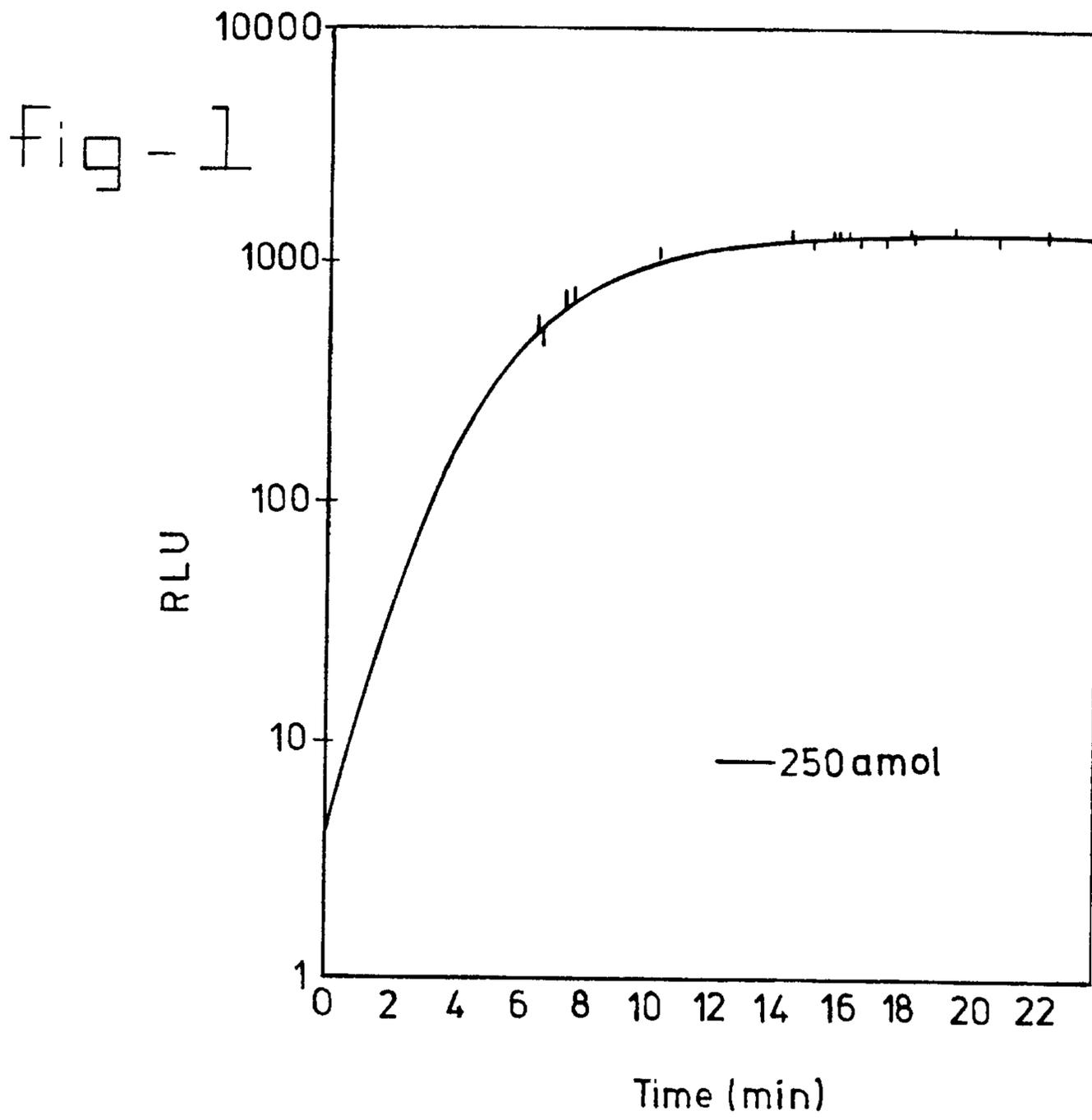
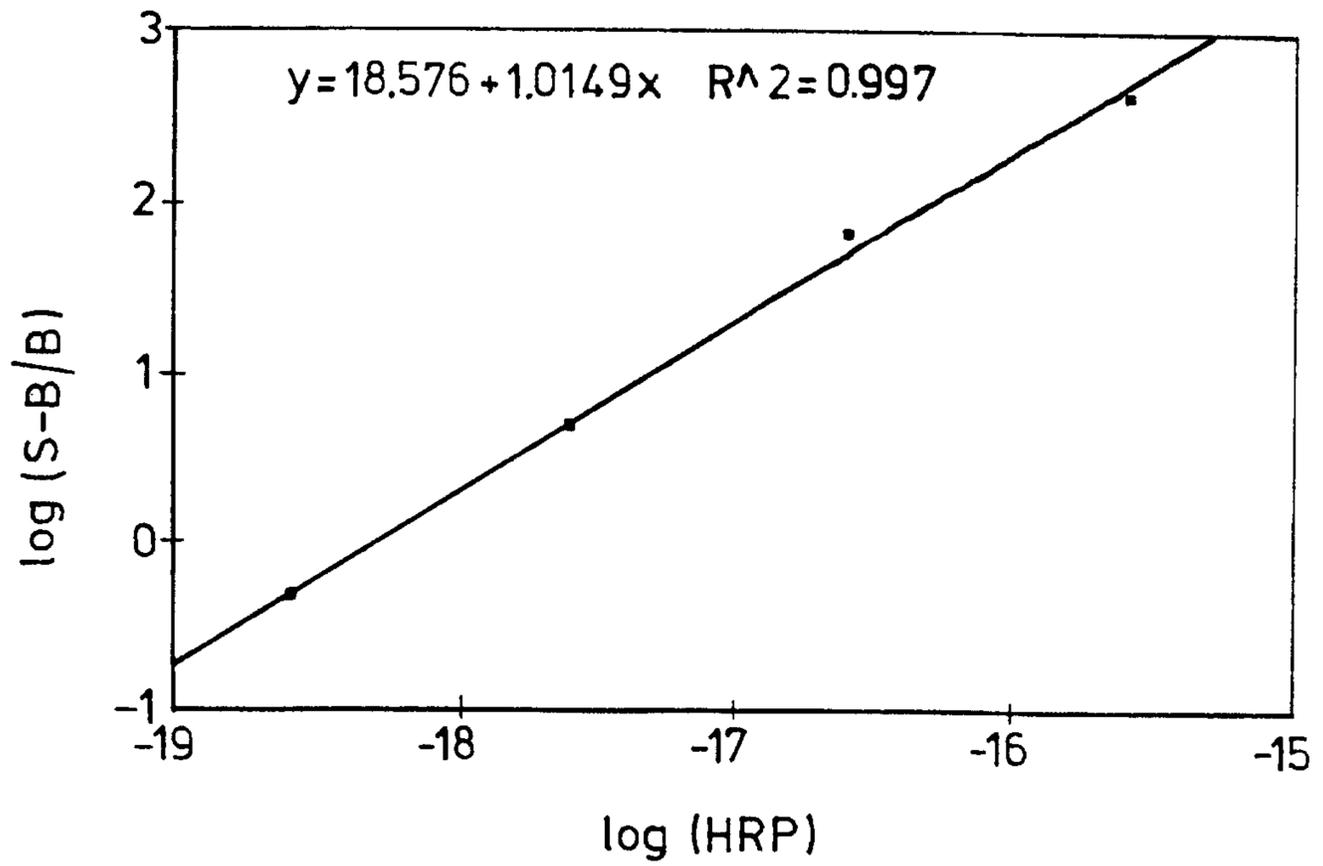


Fig - 2



2/3

fig - 3

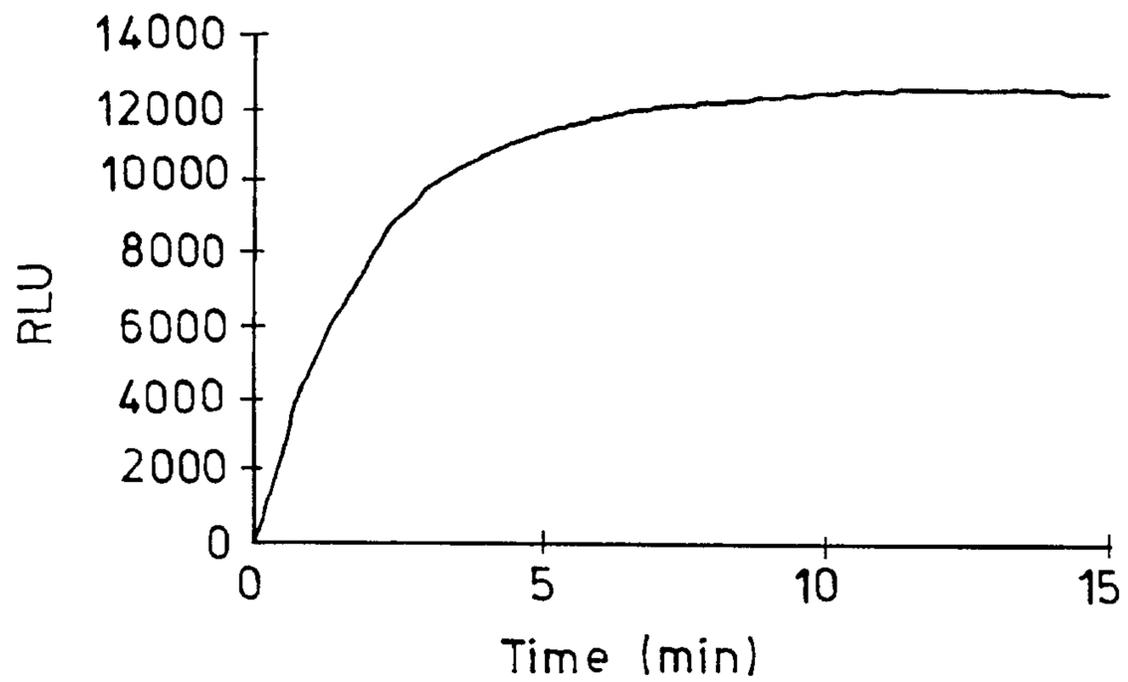


fig - 4

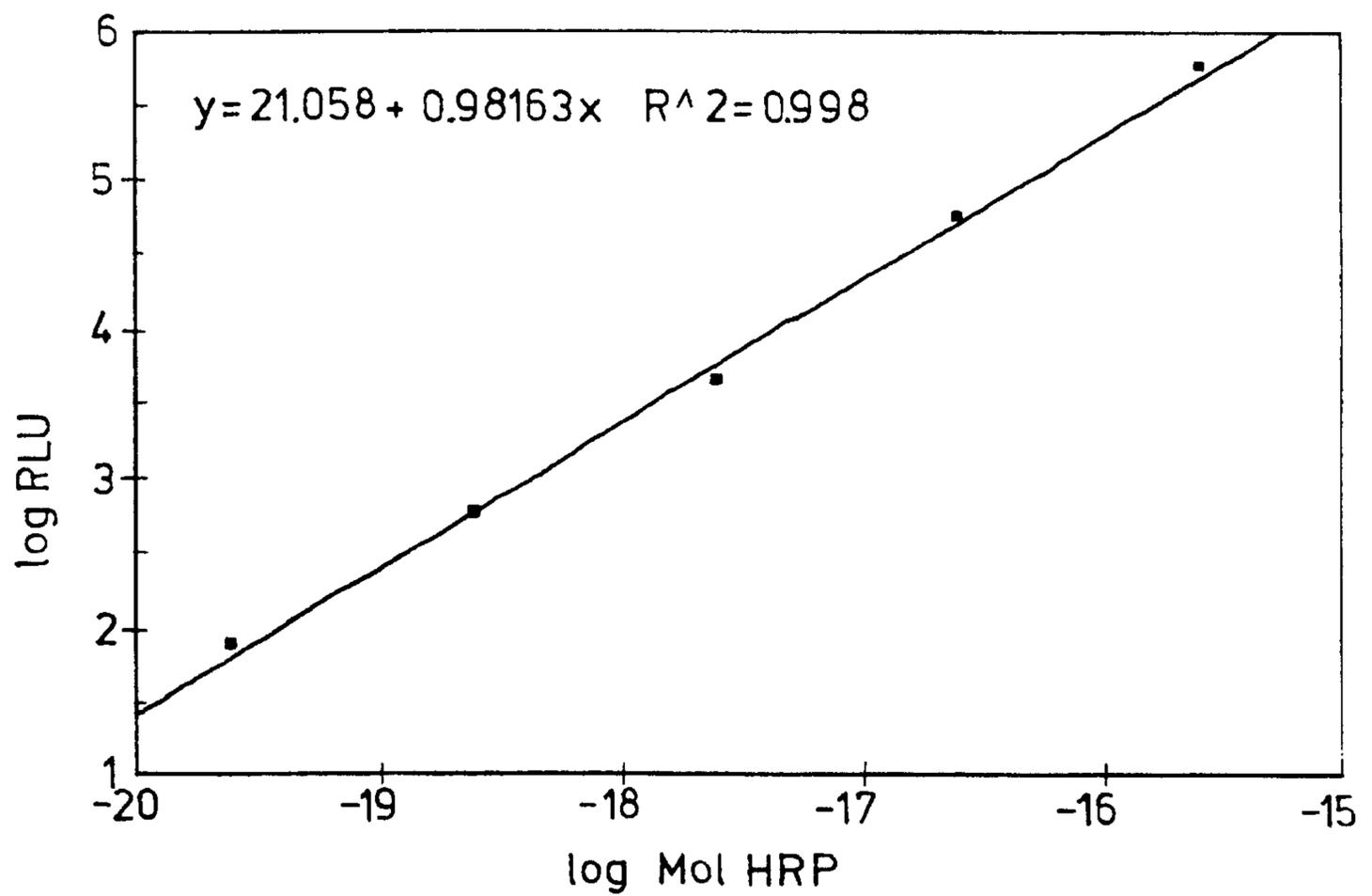


fig-5

DETECTION OF HRP (25 amol)

