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Characterization of new fluorescent peroxidase substrates

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Abstract

The reagents Lumigen PS-1 and Lumigen PS-3 were originally developed as chemiluminescent substrates for ultrasensitive detection of horseradish peroxidase (HRP) in homogeneous solution and membrane blotting assays. However, an additional unique feature of these acridan-based reagents is the generation of a fluorescent species on reaction with peroxidase, a property which has been termed as chemifluorescence. These reagents, therefore, represent the first dual-use substrates enabling both chemiluminescent and fluorescent detection. We have developed several additional acridan-based substrates for fluorescent detection of HRP which are capable of subattomole detection sensitivity. By varying several structural parameters within the class of compounds we have produced substrates which either produce fluorescence alone or both chemiluminescence and fluorescence.

Keywords: Fluorescence; Peroxidase; Substrate; Acridan

1. Introduction

Peroxidase enzymes such as horseradish peroxidase (HRP) are frequently used as markers or labels in enzyme-linked assays for biological molecules and other analytes of interest such as drugs, hormones, steroids and cancer markers. Detection of these enzymes can be achieved by the use of substrates which produce a detectable product. Chromogenic substrates such as *o*-phenylenediamine, ABTS or tetramethylbenzidine produce a colored reaction product, fluorogenic substrates produce a fluorescent product, while chemiluminescent substrates produce light as the detectable product. Each of these methods can offer a safe, convenient and sensitive means to provide a quantitative measure of the amount of enzyme in a sample or of the amount of an enzyme-labeled analyte or labeled specific binding partner for an analyte.

Several fluorescent peroxidase substrates have been reported. Among the most widely reported are phenolic compounds [1–4] such as 3-(4-hydroxyphenyl)propionic acid, 2-(4-hydroxyphenyl)-acetic acid, homovanillic acid and tyramine. Various aromatic amines also function as fluorogenic peroxidase substrates [3,5,6] including *o*-phenylenediamine and *N*,*N*′-dicyanomethyl-*o*-

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phenylenediamine, amide and carbamate derivatives of *p*-aminophenol, 3,4-dihydro-2(1H) quinoxalone and related derivatives. Reduced forms of fluorescein, rhodamine and other xanthine dyes and fluorinated derivatives of these dyes have also been reported as fluorogenic substrates of peroxidase [7,8].

Acridinium esters and amides are known in the art of chemiluminescence. These compounds are generally used to label a substance to be detected in an assay. Detection by chemiluminescence comprises reaction of the label with a strongly alkaline hydrogen peroxide solution. A fluorometric assay of acridinium labeled compounds has also been described in which these same chemiluminescent compounds are measured by their inherent fluorescence [9].

The chemiluminescent reaction of peroxidase conjugates with acridan-9-carboxylic acid derivatives is in widespread analytical use in immunoassays and western blot analyses of proteins [10]. We previously reported the results of studies aimed at characterizing the mechanism of the chemiluminescent oxidation of a class of acridancarboxylic esters and presented evidence of the intermediate formation of the corresponding acridinium ester [11]. We have made use of this finding in developing a class of reagents which produce fluorescence during their reaction with peroxide and a peroxidase. Careful consideration of structural parameters has allowed the design of two types of substrates, those which produce both chemiluminescence and fluorescence or substrates which yield a fluorescent product but do not undergo a chemiluminescent reaction.

2. Experimental

2.1. Reagents

Peroxidase (HRP IV, EC 1.11.1.7, 250 U mg^{-1}) was supplied by Biozyme. All buffers and aqueous solutions were prepared with Type 1 water. The synthesis of fluorogenic acridan substrates is fully described in a US patent application (pending). The chemiluminescent reagents Lumigen PS-1 and Lumigen PS-3 are commercial products of Lumigen, Inc. Other reagents were obtained from commercial sources and are of reagent grade.

2.2. Instrumentation

Fluorescence spectra were measured with a Jobin Yvon/SPEX Fluoroskan spectrofluorometer with the excitation and emission slits set at 1.0 nm bandpass. UV Fluorimeter cuvettes (3.5 ml capacity, Perfector Scientific) were used. Quantitative fluorescence measurements were made with a Labsystems Fluoroskan Ascent microplate spectrofluorometer. Chemiluminescence measurements were performed on a Labsystems Luminoskan microplate luminometer. Data points represent 1 s integrated light intensity values.

2.3. Timed fluorescence spectral acquisition

Three milliliters of the fluorogenic substrate solution containing 0.3 mM 2′,3′,6′-trifluorophenyl 10-methylacridan-9-carboxylate (compound 1), 0.1 mM 4-phenylphenol, 0.5 mM urea peroxide, 1 mM EDTA, and 0.025% Tween 20 in 0.01 M tris buffer, pH 8.0 were pipetted into a cuvette. HRP (13.8 fmol, 4.6×10^{-12} M) was added and mixed thoroughly. Fluorescence emission spectra (excitation at 357 nm) were obtained at 5-min intervals over 1.8 h.

Three milliliters of a fluorogenic substrate solution containing 0.05 mM *N*-methylacridan (compound 6), 0.1 mM 4-phenylphenol, 0.5 mM urea peroxide, 1 mM EDTA, and 0.025% Tween 20 in 0.01 M tris buffer, pH 8.0 were pipetted into a cuvette. HRP (13.8 fmol, 4.6×10^{-12} M) was added and mixed thoroughly. Fluorescence emission spectra (excitation at 357 nm) were obtained at 5-min intervals over 1.8 h.

2.4. Chemiluminescent assay of HRP with a reagent containing compound 2

A stock solution of HRP was serially diluted by tenfold dilution covering the range 1.4×10^{-10} to 1.4×10^{-14} M. Lumigen PS-1 a reagent composition containing 0.05 mM 2′,3′,6′-trifluorophenyl 3-methoxy-10-methylacridan-9-carboxylate (compound 2), 0.1 mM 4-phenylphenol, 0.5 mM urea

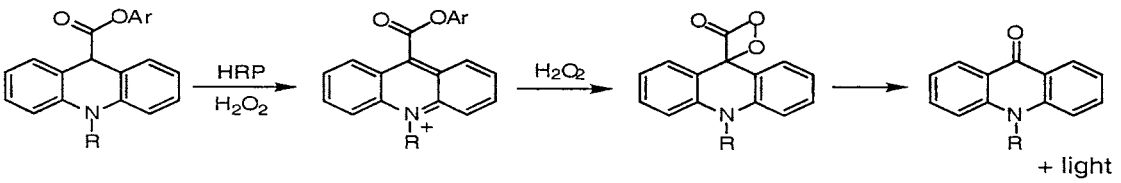
peroxide, 1 mM EDTA, and 0.025% Tween 20 in 0.01 M tris buffer, pH 8.0 was added as 100 µl aliquots to each of three white Microlite 1 FB 12-well strips (Dynatech Laboratories). HRP dilutions (10 µl) were added manually by pipet to the wells. Light intensity was measured on a Labsystems Luminoskan microplate reader after 10 min. Results are the average of triplicate tests.

2.5. Fluorescent assay of HRP with acridan reagent containing substrates 3, 4 or 5

Fluorescence detection limits were obtained on a Labsystems Fluoroskan Ascent microplate fluorimeter using an excitation bandpass filter (380 nm) and an emission bandpass filter (465 or 530 nm). Black MicroFLUOR B 12-well strips (Dynatech Laboratories) were used. HRP was serially diluted in type 1 water and 10 µl of diluted HRP was pipetted into each well. Three detection reagents were prepared. Solutions of compound 3 and 5 contained 0.05 mM substrate in 10 mM acetate buffer (pH 5.0); a solution of compound 4 was 0.05 mM in 10 mM tris buffer (pH 8.0). Each reagent also contained 0.5 mM urea peroxide, 1 mM EDTA, 0.1 mM *p*-phenylphenol and 0.025% (v v^{-1}) Tween-20. Portions of each reagent (100 µl) were pipetted into triplicate wells. The strips were read on the Fluoroskan Ascent with the 380–530 nm filter set after 1 h for compounds 3 and 5 and after 18 min for compound 4.

3. Results and discussion

The chemiluminescent reaction of peroxidase enzymes with acridan-9-carboxylic acid derivatives has been applied to many kinds of analysis because of the excellent analytical features of this reaction in terms of sensitivity and linear dynamic range [10]. We have previously postulated a mechanistic picture of the transformation of the acridan substrate involving formation of a ring oxidized acridinium intermediate by two electron oxidation followed by reaction with peroxide and cyclization to an unstable dioxetanone intermediate [11]. The latter species immediately cleaves off CO_2 and produces electronically excited acridone. This mechanistic scheme, coupled with the knowledge that acridinium salts often exhibit efficient fluorescence, suggested the ability to design fluorogenic peroxidase substrates built on the acridan ring system.



Lumigen PS-3 is a chemiluminescent reagent designed for ultrasensitive detection of peroxidase conjugates in blotting applications such as western blot analysis of proteins. The use of this reagent for fluorogenic detection of peroxidase-labeled antibodies has also been reported [12]. The identity of the fluorescer(s) in this reaction has not been established. Uncertainty arises because there are at least two fluorescent species formed during the reaction, an acridinium ester intermediate and *N*-methylacridone, the end product responsible for

the chemiluminescence. We sought to characterize the nature of the fluorescent emitters in the reaction of acridan-based peroxidase substrates and compare the analytical performance of the two means of detection with the same substrate.

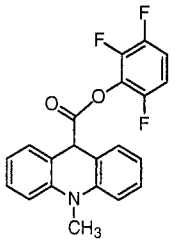
3.1. Time dependence of fluorescence from a chemiluminescent acridan

The time course of the formation of fluorescent species was investigated using the commercial reagent Lumigen PS-3. This reagent which contains the acridan compound 2′,3′,6′-trifluorophenyl 10-methylacridan-9-carboxylate (compound 1) in 0.01 M tris buffer, pH 8.0 was reacted with 13.8 fmol of HRP (4.6×10^{-12} M) at room temperature. Emission spectra (excitation at 357 nm) were obtained at 5-min intervals over 1.8 h. The set of emission spectra obtained are shown in Fig. 1. The spectra over the course of the reaction depict two overlapping spectra which indicate the growth of acridinium fluorescence with a maximum at approximately 490 nm and gradual predominance of *N*-methylacridone fluorescence at 430–460 nm. The spectra of the individual components were not resolvable at any time point or by changing excitation wavelength.

reaction scheme above in which two consecutive reactions take place. The steps not only occur at different rates, but only the first is directly a function of peroxidase concentration. It was, therefore, of interest to determine whether a linear correlation could be established between peroxidase concentration and fluorescence at a given wavelength at which two fluorescent species both contribute. We have compared the sensitivity of detection of peroxidase and the linear range of response with an acridan compound as substrate by both chemiluminescent and fluorescent assays under identical reaction conditions.

Chemiluminescent HRP detection was performed using Lumigen PS-1, a commercially available reagent containing an acridan ester compound and peroxide. Light intensity was measured at 10 min a point at which light intensity is near the maximum value. The assay produced a linear response over a four log span. Higher enzyme concentrations could not be measured due to photomultiplier saturation.

After making the chemiluminescence measurement described above, the strips were transferred to the microplate fluorimeter and fluorescence intensity determined at 530 nm with excitation at 380 nm. The wavelengths used represented the best



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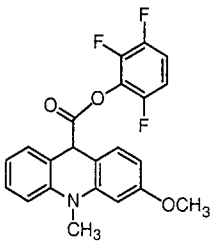

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3.2. Analytical sensitivity of peroxidase detection by chemiluminescence and fluorescence

The ratio of acridinium/acridone fluorescence varies with time and with the extent of reaction with peroxidase. This behavior is explained by the

available filter set and not the spectral maxima. Fig. 2 compares the chemiluminescent and fluorescent assay results and shows that the fluorescent method, like the chemiluminescent method, was able to detect less than 1 amol (1×10^{-14} M) of HRP. The linear least squares best fit of the

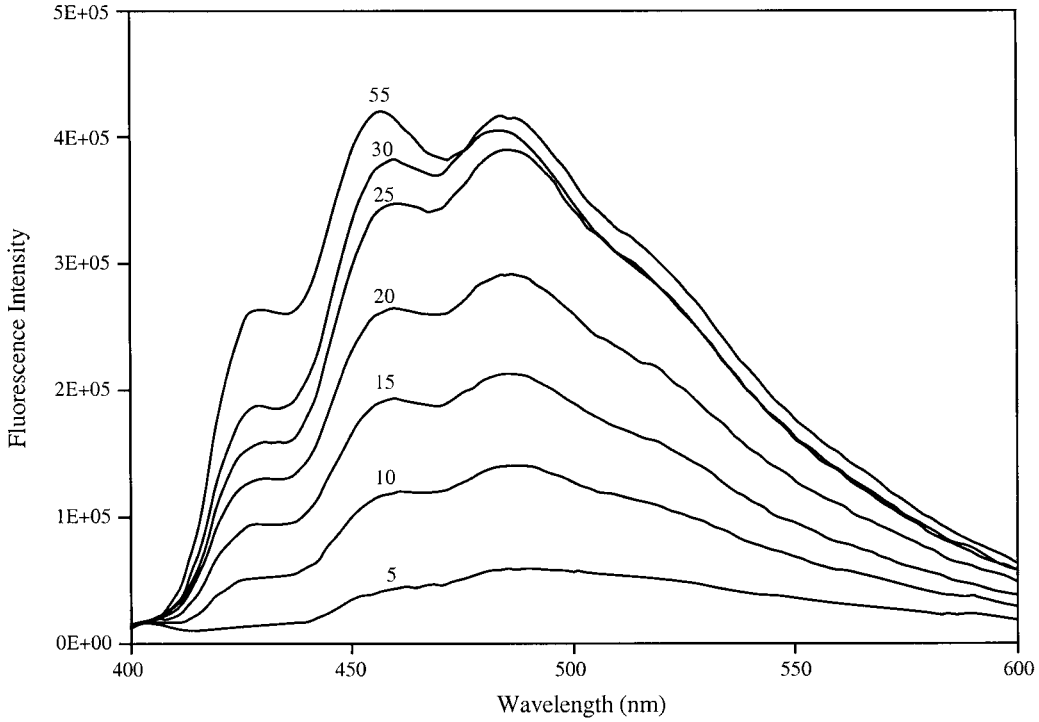


Fig. 1. Fluorescence spectra of the products of the reaction of compound 1 with 4.6×10^{-12} M HRP in 0.01 M tris buffer, pH 8.0, at room temperature. Emission spectra excited at 357 nm were obtained at 5-min intervals over 1.8 h. The spectra depict two overlapping spectra which indicate the growth of acridinium fluorescence and gradual predominance of *N*-methylacridone fluorescence.

chemiluminescence data was described by a line with the equation log(intensity) = 0.941(log(moles of HRP))+17.65, $r^2 = 0.999$. The best fit of the fluorescence data was log(intensity) = 0.870 (log(moles of HRP))+15.94, $r^2 = 1.000$. The sensitivity was equivalent to chemiluminescent detection with the same substrate. A survey of published data (Table 1) concerning the sensitivity of detecting peroxidase enzymes by fluorimetric assay reveals that the sensitivity demonstrated with Lumigen PS-1 and additional acridan compounds discussed below is superior to most other substrates and on a par with the best reported result which required a 2-h incubation [4].

It was not possible with this substrate to devise a fluorescent assay having kinetic characteristics of a chemiluminescent assay in which a signal builds up during the course of the reaction and eventually decays to zero. One of the chief advantages of chemiluminescent detection in enzyme assays is the low background in the absence of enzyme that results from this property. A fluorescent assay based on measurement of an enzymatically produced intermediate would benefit from the lack of fluorescence background signal in the same manner.

3.3. New fluorogenic substrates

We have also prepared numerous additional acridan derivatives to explore the scope of structural variation which permits fluorescent peroxidase detection. In particular, we sought to develop assays in which the acridan substrate would be converted to a stable acridinium derivative which accumulates. This mode of detection differs from the assay described above by producing only a single, persistent fluorescent product. Compounds containing various substituents on the ring nitrogen atom including and at the 9-position hydrogen, methyl, phenyl, benzyl, carboxylate, ester, hydrazide, sulfonamide and acridan

Table 1
Comparison of reported limits of detection (LOD) of HRP with fluorescent substrates

Reference	Substrate	Reported LOD	LOD (moles of HRP)
[13]	Tyramine	0.1 mU	2.3×10^{-14} mol
[13]	Homovanillic acid	0.5 mU	1.1×10^{-13} mol
[4]	MHPMC	0.5 μmol	5×10^{-19} mol
[2]	*p*-Hydroxyphenylpropionic acid	7.8 μU	6.5×10^{-16} mol
[2]	Tyrosol	15.6 μU	1.3×10^{-15} mol
[2]	Tyramine	0.5 mU	4.2×10^{-14} mol
[2]	Homovanillic acid	1 mU	8.3×10^{-14} mol
[14]	*o*-Phenylenediamine	0.56 μU ml^{-1} (2 ml)	1.1×10^{-16} mol
[15]	Amino aluminum phthalocyanine	0.6 pM (10 ml)	6×10^{-15} mol
[5]	DCM-OPA	~20 pM (5 ml)	1×10^{-13} mol
	Lumigen PS-1	10^{-14} M	$< 10^{-18}$ mol
	Compound 4	4.6×10^{-14} M	4.6×10^{-19} mol

MHPMC, *N*-methyl-*N*-(4-hydroxyphenyl)methyl carbamate; DCM-OPA, *N*,*N*′-dicyanomethyl *o*-phenylenediamine.

groups were prepared. A preliminary evaluation of these compounds was made to screen for enzymatic production of fluorescent species. Fluorescence excitation and emission maxima were determined for the fluorescent reaction product formed as the result of peroxidase-catalyzed oxidation. Most compounds exhibited an emission maximum at around 490–495 nm. Additional

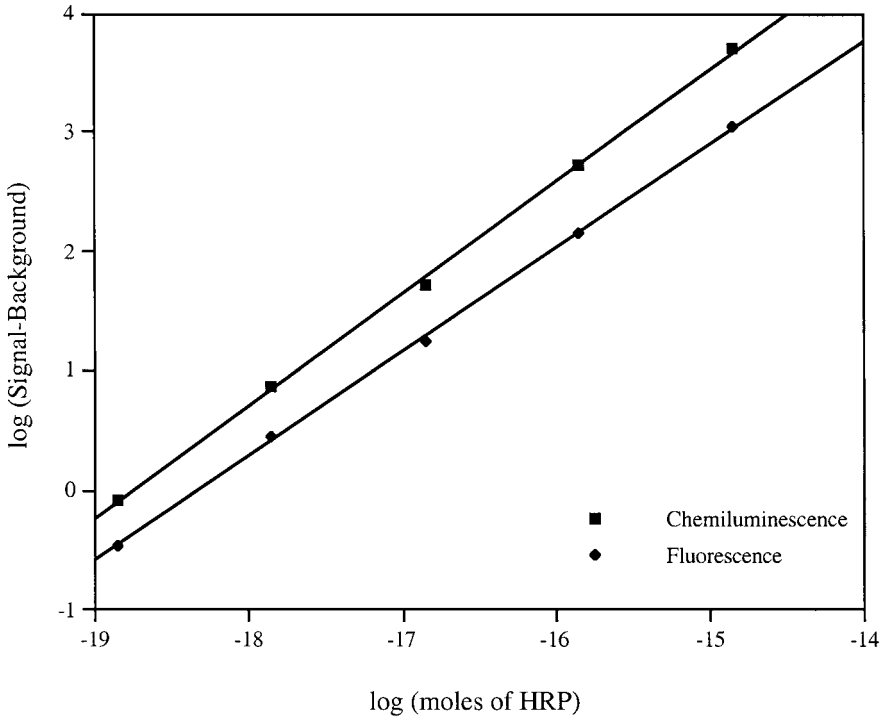


Fig. 2. Comparison of fluorescence and chemiluminescence detection of HRP using Lumigen PS-1. Chemiluminescence intensity was measured 10 min after the start of each reaction. Fluorescence intensity was measured immediately thereafter, exciting at 380 nm and detecting at 530 nm.

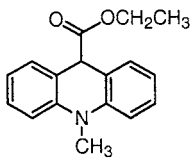
substitution of the acridan ring at any of ring positions 1–8 shifted the emission to around 510 nm. Interestingly, an alkyl ester compound designated compound 3 below had a maximum at 524 nm and showed only a single broad fluorescence maximum. All other compounds tested revealed two shoulders on the main band at longer and shorter wavelengths. Fluorescence emission spectra of reactions conducted at pH 5.0 and 8.0 were identical in every case studied.

3.4. Components of enhanced detection reagent

Reaction of acridan-ring compounds as peroxidase substrates proceeds in the absence of phenolic peroxidase activity enhancers at markedly diminished rates compared to the rates in the presence of an enhancer. All fluorescence spectral and quantitative measurements were made with reagent formulations containing an enhancer. Enhanced acridan peroxidase substrates were prepared in either pH 8.0 tris buffer (Basic Formulation) or pH 5.0 acetate (Acidic Formulation) described below. The basic formulation contained 10 mM tris buffer (pH 8), 0.05 mM substrate, 0.5 mM urea peroxide, 1 mM EDTA, 0.1 mM *p*-phenylphenol and 0.025% (v v^{-1}) Tween-20. The acidic formulation contained 10 mM Na acetate buffer (pH 5.0), 0.05 mM substrate, 0.5 mM urea peroxide, 1 mM EDTA, 0.1 mM *p*-phenylphenol and 0.025% (v v^{-1}) Tween-20. The presence of the surfactant aids in solubilizing the acridan compound in aqueous solution.

3.5. Preliminary evaluation of fluorogenic substrates

Each of the compounds was tested for production of a fluorescent product by reaction of 3 ml of a 0.05 mM solution of the substrate in the acidic formulation with 13.8 fmol of HRP (4.6×10^{-12} M) followed by spectrofluorometric analysis. Excitation and emission spectra were recorded. The increase in fluorescence at the fluorescence maximum wavelength was recorded over 15 min. We selected four compounds with the highest signal/background for further analysis. The compound 9,9′-bis(*N*-phenylacridan) was also effective as a fluorogenic peroxidase substrate producing a highly fluorescent product.



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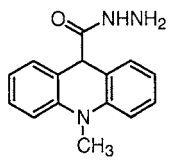

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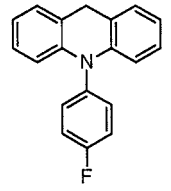

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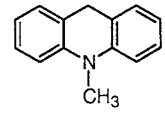

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3.6. Time course of fluorescence from nonchemiluminescent acridan

We examined the evolution of the reaction of compound 6 with HRP by progressive fluorescence scans of the reaction mixture. Three milliliter of a 0.05 mM solution of compound 6 in the acidic formulation were mixed with 13.8 fmol of HRP (4.6×10^{-12} M). Emission spectra (excitation at 357 nm) were obtained at 5-min intervals over 1.8 h. The set of emission spectra obtained (Fig. 3) show the simple oxidation of the acridan ring to the acridinium compound as identified by comparison with an authentic sample of a 0.05 mM solution of *N*-methyl acridinium triflate (shown as dashed lines) in the acidic formulation (excitation at 357 nm). The identity of the reaction spectra

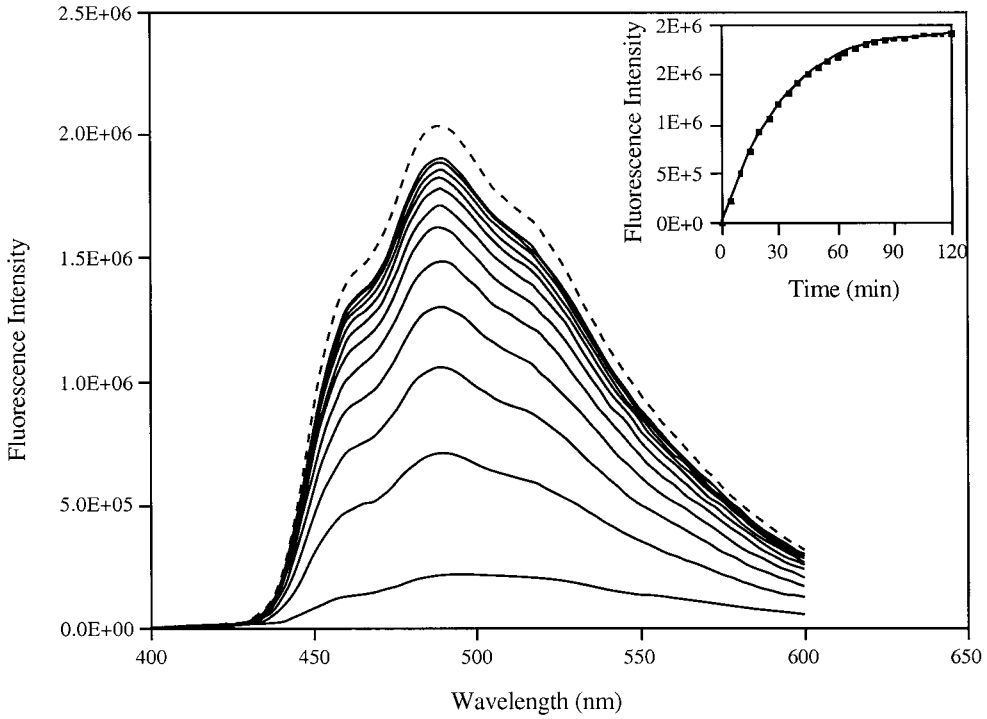

Fig. 3. Fluorescence spectra of the products of the reaction of compound 6 with 4.6×10^{-12} M HRP in 0.01 M acetate buffer, pH 5.0, at room temperature. Emission spectra excited at 357 nm were obtained at 5-min intervals over 1.8 h. The spectra depict the growth of acridinium fluorescence. The dashed line is an emission spectrum of authentic *N*-methylacridinium triflate. The inset is a plot of fluorescence intensity at 490 nm vs. time.

with the acridinium compound demonstrates the clean enzymatic conversion of compound 6 and substantiates our earlier proposed mechanism. The inset depicts the time course of the reaction as indicated by fluorescence intensity at 490 nm.

3.7. Analytical sensitivity of fluorescent peroxidase detection with nonchemiluminescent acridans

Fluorescent assays of HRP using reagents containing each of the nonchemiluminescent acridans 3–5 as peroxidase substrate were conducted over a range of peroxidase concentration spanning five orders of magnitude. Fluorescence intensities were recorded using a microplate fluorimeter with an excitation bandpass filter (380 nm) and an emission bandpass filter (465 or 530 nm). Fluorescence was measured after 1-h incubation for compounds 3 and 5 and after 18 min for compound 4. Fig. 4 demonstrates the linear response to peroxidase obtained with each of the three substrates. The analytical parameters for each substrate were compound 3: $\log(\text{intensity}) = 0.877(\log(\text{moles of HRP})) + 15.709$, $r^2 = 0.997$; compound 4: $\log(\text{intensity}) = 0.989(\log(\text{moles of HRP})) + 16.90$, $r^2 = 0.999$; and compound 5: $\log(\text{intensity}) = 0.739(\log(\text{moles of HRP})) + 13.42$, $r^2 = 0.998$. Nonenzymatic background fluorescence varied among the substrates in the order: compound $1 > 5 > 3 > 4$. The hydrazide compound 4 showed a signal/background of 2 at [HRP] = 0.5 amol making it along with Lumigen PS-1 among the most sensitive substrates for fluorescent peroxidase detection.

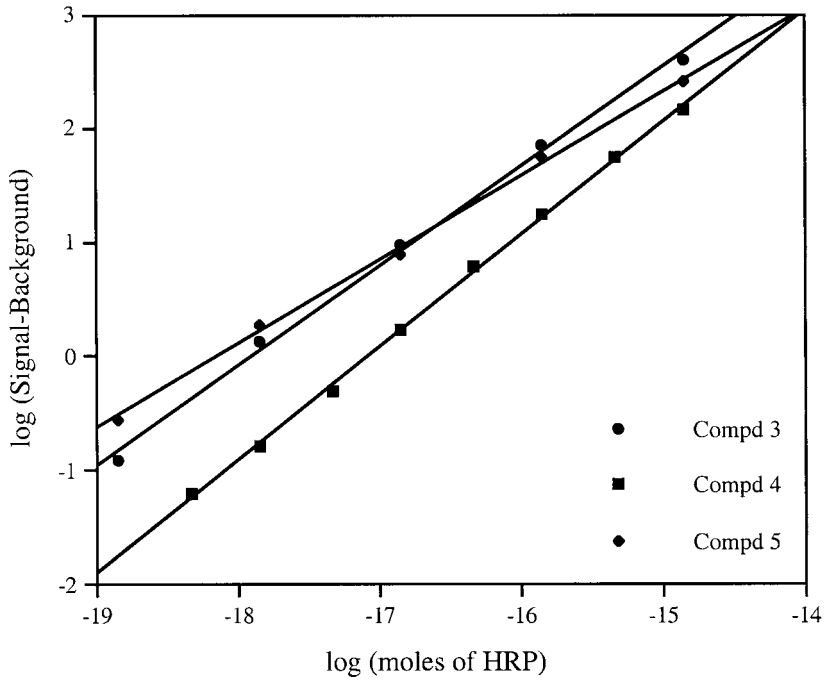

Fig. 4. Comparison of fluorescence detection of HRP using nonchemiluminescent compounds 3,4, and 5 as peroxidase substrates. Fluorescence intensities were recorded with an excitation bandpass filter (380 nm) and an emission bandpass filter (465 or 530 nm). Fluorescence was measured after 1-h incubation for compounds 3 and 5 and after 18 min for compound 4.

4. Conclusions

Careful consideration of structural parameters has allowed the design of two types of substrates, those which produce both chemiluminescence and fluorescence and substrates which yield a fluorescent acridinium product that do not undergo a chemiluminescent reaction. Both types of substrates allowed highly sensitive, rapid assays of peroxidase enzyme to be performed. Analytical sensitivity was at or below 1 amol of enzyme, equivalent to the best sensitivity reported in the literature. The principal advantage of detecting a reaction intermediate product is the minimal reagent background buildup over time due to the lack of accumulation of the signal producing species. This characteristic could not be fully exploited in the present generation of substrates because of significant spectral overlap between the acridinium and acridone product fluorescence spectra. Work is underway to develop compounds in which there is greater spectral separation between the two fluorescence spectra. The final goal is to develop substrates which would permit background-free detection by producing a fluorescent intermediate that is converted to a non-fluorescent product.

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