# Characterization of Acridancarboxylic Acid Derivatives as Chemiluminescent Peroxidase Substrates

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A new class of peroxidase substrates has been developed which produces chemiluminescence upon enzymatic oxidation. A wide variety of *N*-alkylacridancarboxylic acid derivatives including esters, thioesters, and sulfonamides are efficiently oxidized by a peroxidase and a peroxide to enzymatically produce the corresponding chemiluminescent acridinium compound. In conjunction with a peroxidase enhancer, continuous light emission with high light intensities and an extended duration are produced. Alternately, an appropriately designed acridan substrate produces a stable acridinium ester intermediate which can be accumulated and the chemiluminescence elicited as a burst of light by raising the pH. The effects of leaving groups and substitution on the acridan ring on the mechanism of light production are discussed. Peroxidase-catalyzed oxidation in the presence of a peroxide permits the detection of enzyme with subattomolar sensitivity and a broad linear dynamic range.

## Introduction

Horseradish peroxidase (HRP) (EC 1.11.1.7) is used widely as a reporter enzyme in various assays. The ready availability, ease of conjugation, and low molecular weight of this enzyme provide many practical advantages. Although many chromogenic and fluorogenic detection reagents, e.g. tetramethylbenzidine, *o*-phenylenediamine, chlorophenol red, and 2,2′-azinobis(3-ethylbenzothiazolone) have been utilized for visualizing peroxidase activity,[1] the only chemiluminescent reagent for detection of HRP which has found significant use is the enhanced luminol detection system.[2] The level of sensitivity with which this enzyme can be detected (ca. 10 amol) is at least 3 orders of magnitude poorer than that of other marker enzymes with chemiluminescent detection and could be improved with new substrates.

We have previously described the development of a new class of peroxidase substrates based on the *N*-alkylacridancarboxylate nucleus.[3] Use of these substrates affords more sensitive chemiluminescent detection of HRP and superior signal duration compared to the luminol system. We report here the spectroscopic characterization of the reaction intermediates and products and discuss the reaction mechanism. A noteworthy feature of this reaction is the ability to enzymatically generate a large number of chemiluminescent acridinium derivatives with each peroxidase molecule. The practical utility of this chemiluminescent reaction is illustrated by the development of highly sensitive enzyme assays using a reagent incorporating the substrate, a peroxide, and a surfactant.

## Results and Discussion

We have discovered that numerous derivatives of *N*-alkylacridancarboxylic acids including esters, thioesters, and sulfonamides are effectively oxidized by peroxidase enzymes in the presence of a peroxide to produce chemiluminescence. The ester compounds reported in the present study were synthesized in good yield using the reaction sequence shown in Scheme 1. The evidence presented below shows that this chemiluminescent reaction, which is catalytic in peroxidase, involves the conversion of the acridan compound to an acridinium ester intermediate. The acridinium ester reacts with peroxide to form a dioxetanone which in turn decomposes to yield the singlet-excited state of the corresponding *N*-methylacridone. Emission from the excited ketone results in intense blue chemiluminescence. Reaction of the continuously generated acridinium species with excess peroxide at moderately alkaline pH produces a continuous light emission which takes advantage of the high efficiency of acridinium ester chemiluminescence.

The well-known chemiluminescent oxidation of acridinium esters with alkaline hydrogen peroxide produces a flash of light which typically decays in 1−2 s.[4−8] While acridinium ester-labeled analytes are employed as chemiluminescent labels in immunoassay and DNA probe tests, they present several practical limitations. Long-term stability is problematic due to hydrolysis of the ester function. Use of esters or other derivatives which are more hydrolytically stable, yet still can be triggered to

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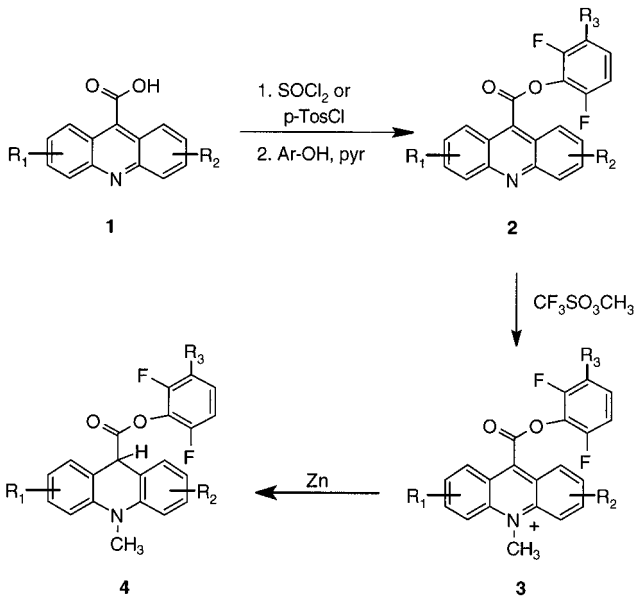
**Scheme 1**



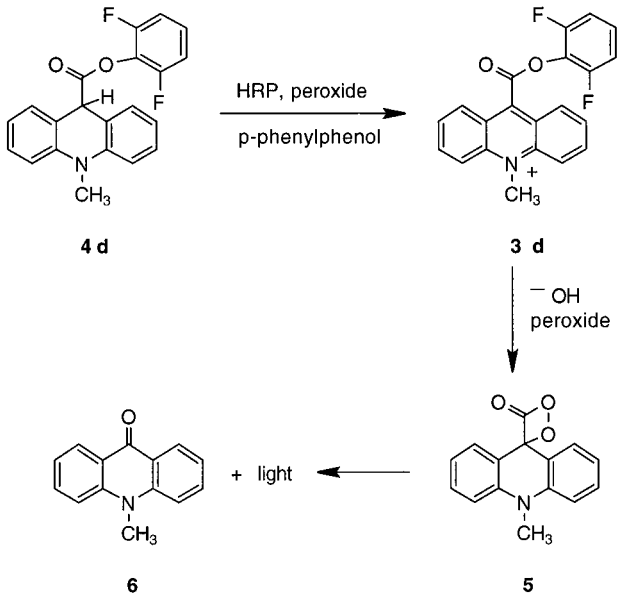

1 2 3 4

**a**, $R_1$ = 3-$OCH_3$ $R_2$ = H $R_3$ = F
**b**, $R_1$ = 1-$OCH_3$ $R_2$ = 6-$OCH_3$ $R_3$ = F
**c**, $R_1$ = H $R_2$ = H $R_3$ = F
**d**, $R_1$ = H $R_2$ = H $R_3$ = H
**e**, $R_1$ = 1-$OCH_3$, 4-$CH_3$ $R_2$ = 6-$OCH_3$ $R_3$ = F

produce useful levels of chemiluminescence, have only partly solved this problem. The electrophilicity of the 9-position leads to pseudobase formation from attack of water or other nucleophiles to form a nonluminescent adduct.[9,10] This can be reversed by a preacidification step prior to reaction with alkaline peroxide; however, this adds additional complexity. Similarly, the approach of deliberately forming a reversible adduct by addition of a nucleophile to the 9-position improves the stability of the acridinium ester label but requires an additional step to remove the addend.[11] A more basic limitation is that at most about 10 acridinium ester labels can usually be incorporated onto a target. Enzymes used as labels are capable of producing much larger amounts of detectable molecules by their ability to catalytically turn over substrate. Even a single enzyme label can produce thousands to millions of detectable molecules. In the present reaction, those detectable molecules are highly chemiluminescent acridinium esters (Schemes 2 and 3). These reactions therefore constitute enzymatically amplified acridinium chemiluminescence. The problems of acridinium ester stability and pseudobase formation are circumvented since, under the proper conditions, the enzymatically produced acridinium derivatives immediately undergo the chemiluminescent reaction.

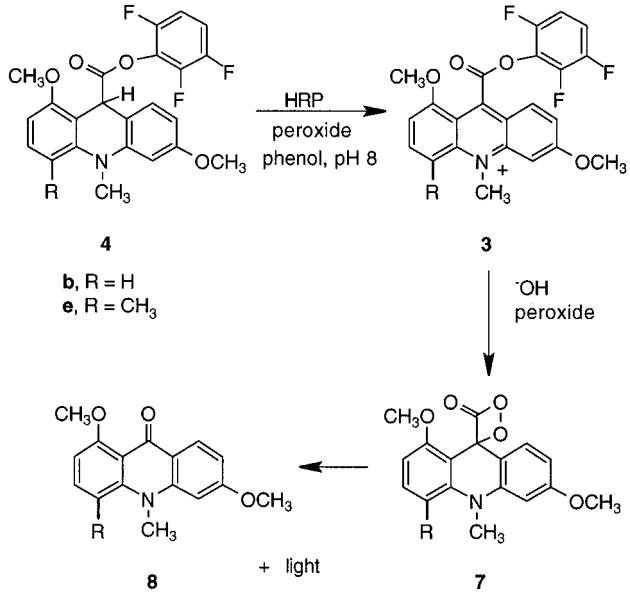
A large number of acridans bearing a wide variety of aromatic leaving groups have been prepared. Substituted phenyl and naphthyl ester groups, aryl and alkyl thioester groups with substituents spanning the range of electron-donating to electron-withdrawing, and sulfonylamino groups are all functional peroxidase substrates and provide chemiluminescence when reacted with a peroxidase and peroxide. Two structural features have been found to be important in determining the kinetics of intermediate formation and light production. Good leaving groups such as a difluorophenoxy or trifluorophenoxy group promote faster reaction. Light emission reaches maximum intensity more quickly and provides higher peak levels. Substitution of one or more ring hydrogens on the acridan nucleus by an alkyl or alkoxy group can reduce peak light intensity and prolong the rise to maximum light intensity. However, the use of ring-substituted acridans provides nearly identical analytical sensitivity for quantitating peroxidase activity since the reduction in peak light intensity is offset by a concomitant decrease in spontaneous, nonenzymatic (background) luminescence from the acridan. The effects of leaving group ability and ring substitution on the kinetic properties of light emission are explainable in terms of the rate of formation and reaction of the acridinium ester intermediate as discussed below.

**Scheme 2**




4 d 3 d 5 6

**Scheme 3**




4 3 7 8

**b**, R = H
**e**, R = $CH_3$

**Continuous Emission of Chemiluminescence from Reaction of HRP with Acridans.** Optimized reagents

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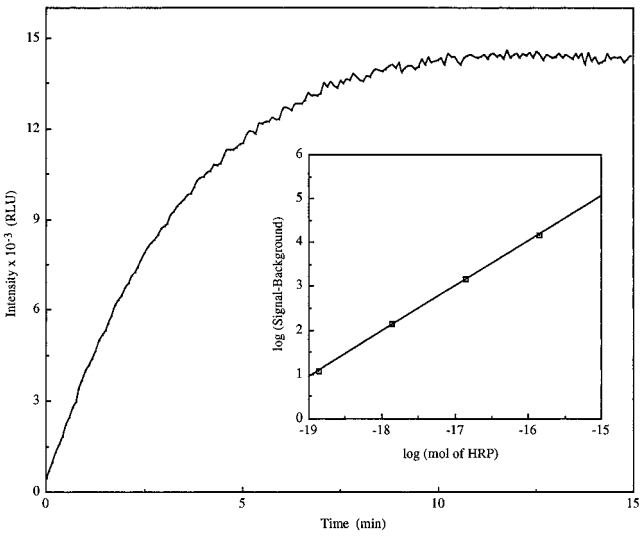


**Figure 1.** Chemiluminescence intensity−time profile from 100 $\mu$L of an enhanced reagent containing acridan **4a** reacted with 140 amol of HRP at room temperature. Inset: detection limit of HRP using this reagent derived from the intensities of enzyme-generated chemiluminescence at 10 min (signal) corrected for reagent background in relation to the amount of HRP.

have been developed for chemiluminescent detection on membranes in blotting applications and for detection of peroxidase conjugates in solution assays and immunoassays. Reaction of HRP in solution with a peroxide, enhancer and acridan **4a**, **4c**, or **4d** produces an intense blue chemiluminescence, reaching a maximum in about 10 min at 25 °C, with a slow decay which can extend over several hours (Figure 1). Light intensity in relative light units (RLU) can be directly correlated to the amount of enzyme. Quantitation can therefore be performed in as little as 2−5 min or at the plateau, in which case precise timing is unnecessary.

We found several variables to be critical to providing extended emission of luminescence at high intensities. Using a 0.01 M Tris-HCl buffer, the optimum reaction pH was between 7 and 9. The choice and concentration of an enhancer of peroxidase activity was investigated and is described in detail below. The concentration of HRP substrate was evaluated with reagents containing various amounts of the acridan compound between 10 and 0.005 mM. In general, a concentration of 0.025−0.1 mM acridan provided the best performance. The use of nonionic surfactants, surprisingly, significantly increased signal/background ratios of the light emission.

Several peroxides including $H_2O_2$, $NaBO_3$, and urea peroxide provided high levels of chemiluminescence. The concentration of peroxide was found empirically to provide optimum signal/background ratios in solution assays at a concentration in the range 10−0.1 mM. Somewhat higher amounts were beneficial in detecting HRP on membranes. Many compounds enhance or promote the activity of peroxidase enzymes in other chromogenic and luminescent reactions.[2,12−15] While we are studying other enhancers, we have limited the present study to the evaluation of a group of phenols including *p*-iodophenol, *p*-bromophenol, 2-naphthol, 6-bromo-2-naphthol, 4-phenylphenol, and *p*-hydroxycinnamic acid, as well as 4-bromophenylboronic acid and 4-aminophenylboronic acid. Each of these compounds increased light output substantially over the amount produced in their absence. In light of these results, we expect that other known HRP enhancers would be effective in promoting the chemiluminescent reaction of acridans by HRP.

**Table 1. Maximum Light Intensity from Acridan Compounds in a Standard Solution**

| compd | maximum light intensity (RLU) |
|---|---|
| **4a** | 6500 |
| **4b** | undetectable |
| **4c** | 55400 |
| **4d** | 56880 |
| **4e** | undetectable |

At the present time, the most widely used reagent for chemiluminescent detection of peroxidase activity is luminol. Good sensitivity is achieved when an enhancer substance is used in conjunction with luminol.[15] We have compared peroxidase-catalyzed acridan chemiluminescence with this well-characterized system. A standard assay formulation consisting of 10 mM Tris buffer, pH 8.0 containing 0.1 mM *p*-phenylphenol, 0.05 mM acridan compound, 0.5 mM urea peroxide, 1 mM EDTA, and 0.025% Tween 20 was used to evaluate the chemiluminescence characteristics of each acridan compound. Reagents containing each of the acridan compounds **4a**, **4c**, and **4d** were reacted with varying amounts of HRP. Table 1 summarizes the maximum light intensity from the reaction with 140 amol of HRP. Despite the considerable differences in absolute light intensities, each reagent was capable of detecting 0.1 amol of HRP in a 15 min assay since reagent backgrounds varied proportionately. For example, with **4a**, enzyme detection was linear over more than 4 orders of magnitude (Figure 1 inset).

In a direct comparison with an enhanced luminol chemiluminescent reagent (Amersham ECL), light intensity from the reagent containing **4a** on reaction with 140 amol of HRP at 25 °C was much higher and of significantly longer duration and exhibited lower reagent background. The linearity of detection of HRP using the acridan reagent was investigated by reacting 40 $\mu$L aliquots of the reagent at 25 °C with varying amounts of HRP. Figure 1 (inset) shows the corrected signal-background at 15 min. Light intensity was linearly related to the amount of HRP over a range of 4.5 orders of magnitude with a slope of 1.0. The high peak light intensities, longer signal duration, and extended linearity of the acridan reagent compared to the enhanced luminol detection reagent resulted in an HRP detection sensitivity of 0.1 amol. This represented a 100-fold improvement compared to the detection limit of luminol determined in a similar manner with the ECL luminol reagent (data not shown) and as previously reported.[10] There appeared to be only a very slight dependence of light intensity on reaction temperature so that precise temperature control was not critical. In our experience, there was no improvement in analytical performance to be realized by conducting reactions at 37 °C.

We found the lowest amount of HRP that could be measured with enhanced luminol chemiluminescence to be 10 amol. This level can represent a limitation in some

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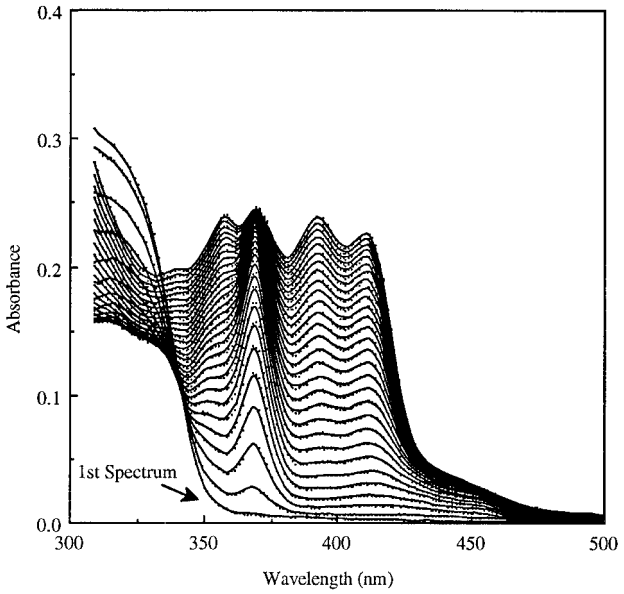


**Figure 2.** UV−visible absorption spectra of the conversion of acridan **4d** by reaction with HRP and $H_2O_2$ to the corresponding acridinium ester **3d** and *N*-methylacridone **6**. Scans were repeated at 30 s intervals.

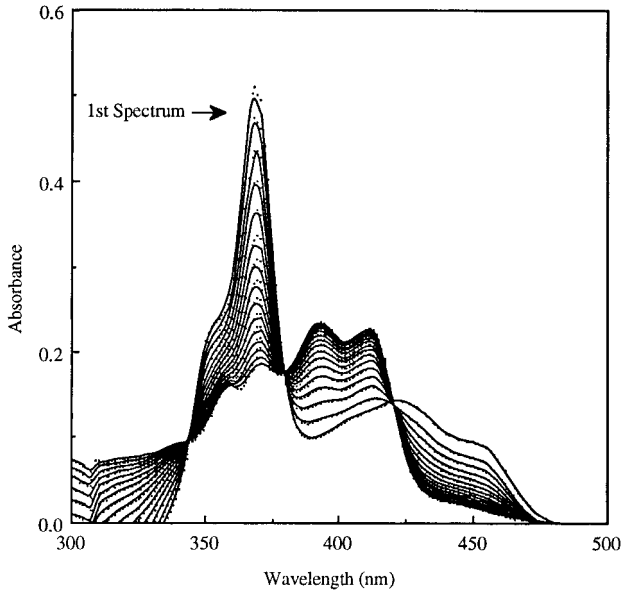


**Figure 3.** UV−visible absorption spectra of the conversion of acridinium ester **3d** by reaction with $H_2O_2$ to *N*-methylacridone **6**. Scans were repeated at 30 s intervals.

enzyme-linked measurements. In addition, the luminol reagent showed lower absolute light intensities than the acridan reagent for a given level of peroxidase. An additional drawback of the luminol system is that the time to reach maximum intensity varies with the amount of HRP and must be determined empirically for each level of enzyme. When low amounts of HRP were used, light intensity dropped rapidly a few seconds after addition of enzyme, probably due to enzyme inactivation. Measurement of peak light intensity was difficult under these conditions.

Reagent stability was also assessed by repeating the HRP assay at various times after preparing the reagent. We found that the reagent containing acridan **4a** and peroxide could be used over a day in an assay for HRP without deterioration of analytical signal or generation of chemiluminescence background.

We have studied the mechanism of the enzyme-catalyzed transformation of acridan derivatives to the acridone by spectroscopic characterization of chemical intermediates. A solution of acridan **4d** in 3 mL of 10 mM Tris buffer, pH 8.0, containing urea peroxide and *p*-phenylphenol was reacted at ambient temperature with 105 fmol of HRP. The spectrum was scanned at 30 s intervals. The spectra show that the absorption of an intermediate with a maximum at 368 nm grows in over the first 10 min (Figure 2). More slowly, additional maxima at 392 and 411 nm appear as the rise of the 368 nm absorptions slows. We believe that these changes are explained by the reaction sequence depicted in Scheme 2: (a) enzymatic oxidation of the acridan **4d** produces acridinium ester **3d**, (b) reaction of **3d** with peroxide generates an unstable dioxetanone **5**, and (c) spontaneous cleavage of **5** yields singlet-excited *N*-methylacridone **6** which decays to produce the observed chemiluminescence. This mechanistic hypothesis is supported by an experiment in which an authentic sample of acridinium ester **3d** was treated with alkaline $H_2O_2$. Reaction of **3d** with $H_2O_2$ in pH 8.0, 10 mM Tris buffer was followed spectroscopically (Figure 3). Acridinium ester **3d** exhibits

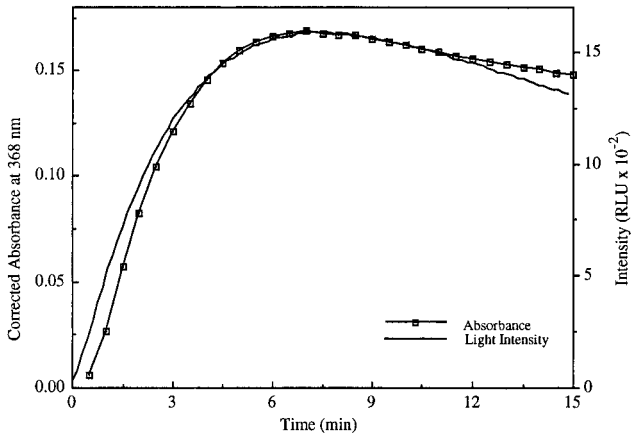


**Figure 4.** Formation of acridinium ester **3d** from reaction of acridan **4d** with HRP and peroxide compared to the chemiluminescence profile. The amount of the acridinium ester was determined by measuring the absorbance at 368 nm and correcting for the absorbance of the acridone product.

a maximum at 368 nm which was replaced by maxima at 392 and 411 nm as **3d** was converted to *N*-methylacridone. An isosbestic point at 380 nm demonstrates that this reaction produces the acridone as the sole product. An authentic sample of *N*-methylacridone exhibited an absorption spectrum which was identical with that obtained from the reaction of **3d** with $H_2O_2$.

Further support for the mechanism shown in Scheme 2 comes from a comparison of the time course of the formation of acridinium ester **3d** in the reaction of acridan **4d** with HRP and peroxide with the chemiluminescence time profile (Figure 4). Acridinium ester **3d** was quantified by measuring the change in absorbance at 368 nm with time and correcting for absorbance of the acridone product. The acridan starting material **4d** had negligible absorbance at this wavelength. The similarity of the two curves in Figure 4 strongly supports a mechanism in which the acridinium ester is formed as an intermediate in the chemiluminescent reaction. The concentration of the acridinium ester increases with time

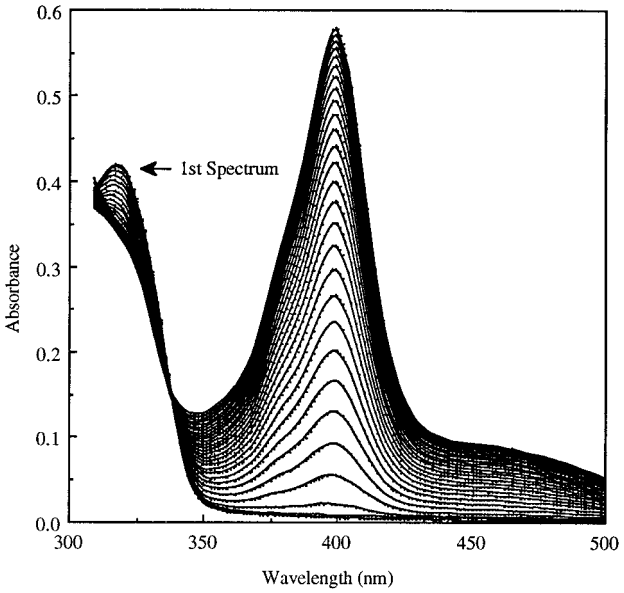


**Figure 5.** UV−visible absorption spectra of the conversion of acridan **4b** by reaction with HRP and $H_2O_2$ to the corresponding acridinium ester **3b**. Scans were repeated at 30 s intervals.

until a pseudoplateau is reached, at which point the rate of formation of the acridinium ester is equal to the rate of its subsequent chemiluminescent reaction with peroxide.

**Two-Step Chemiluminescent Enzyme Assays.** We have discovered that acridan peroxidase substrates such as **4b** and **4e** which have a substituent in a position peri to the carboxylic ester produce a *stable* acridinium ester intermediate. This property has been utilized to develop a chemiluminescent system which separates the enzymatic reaction of the acridan derivative from the light-producing step. We observed in evaluating the reaction of acridans **4b** and **4e** with peroxide and HRP that the kinetics of light emission were significantly slowed compared to that of compounds **4a** or **4c**. This finding suggested that the acridinium ester intermediate might be more stable and could therefore be accumulated.

This idea was confirmed by a spectroscopic study of the peroxidase-catalyzed oxidation of acridan **4b** with excess $H_2O_2$, which showed clean conversion to a single new species with a $\lambda_{max}$ of 399 nm as evidenced by an isosbestic point at 337 nm (Figure 5). Conversion was essentially complete in 15 min and produced a yellow solution. An identical reaction run in parallel and monitored by light production showed negligible chemiluminescence. *These results taken together show that a stable acridinium ester is formed which does not react in the presence of excess hydrogen peroxide at pH 8.* Addition of base produced a flash of light, indicative of the presence of acridinium ester **3b**. An absorption spectrum of the solution after the emission revealed an absorption at 372 nm, which was identified as the corresponding acridone product **8**.

These results lead to an alternate way to detect HRP in which the enzymatic step is conducted at a pH <9. A luminescent flash of 2−3 s is subsequently generated by simply raising the pH above about 12 according to the reaction depicted in Scheme 3. This mode of signal generation could be advantageous since the light emission can be postponed until the desired time. A further advantage is that higher light intensities can be produced from a given quantity of peroxidase due to the time compression of the chemiluminescent signal.

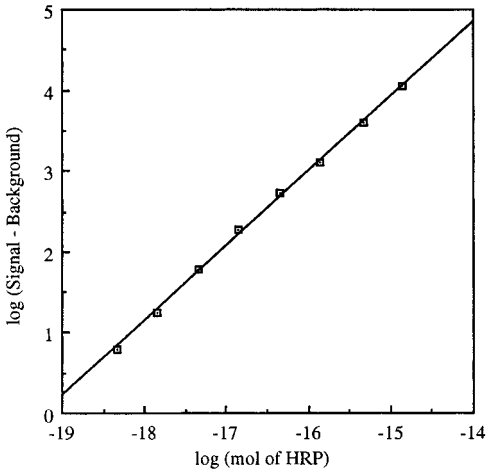


**Figure 6.** Detection limit of HRP using chemiluminescent peroxidase-catalyzed oxidation of acridan **4b** in a two-step assay. Acridan **4b** was incubated with HRP for 5 min and light output integrated for 2 s following addition of 1 M NaOH.

The success of this approach rests on several factors in addition to the stability of the acridinium intermediate toward excess peroxide at neutral pH. Importantly, the acridan itself does not undergo significant chemiluminescent autoxidation at high pH, as is known to occur for other acridan compounds.[4] Had autoxidation of the unreacted acridan occurred, there would be no way to distinguish the nonenzymatic chemiluminescence due to the acridan from the emission of the enzymatically generated acridinium intermediate. The hydrolytic stability of the acridinium intermediate also prevented hydrolysis of the ester to the nonluminescent carboxylate anion[16] and formation of the pseudobase by attack of hydroxide at the 9-position.

Measuring peak light intensities allowed subattomole quantitation of HRP in an assay in which a 5 $\mu$M solution of **4b** was incubated with HRP for 5 min followed by addition of 100 $\mu$L of 0.1 M NaOH. Light output was integrated for 2 s immediately following addition of NaOH. The assay was linear from 0.5 amol to 2 fmol of HRP (Figure 6). The identical assay using **4e** had a linear range of 0.2 amol to 2 fmol of HRP. Assays using this type of acridan derivative represent the first example, to the best of our knowledge, of a peroxidase assay based on the accumulation of an intermediate, followed by the rapid, controlled release of signal.

**The Reaction Mechanism.** A plausible mechanism for the chemiluminescent reaction of the acridan substrates is shown in Scheme 4. Enzymatic oxidation of, for example, acridan substrate **4d** proceeds by two-electron oxidation of **4d** by HRP compound **I** and/or compound **II**, mediated by the phenol enhancer, with concomitant loss of a proton to produce acridinium ester **3d**. Nucleophilic attack of peroxide at C-9 produces a peroxyacridan which cyclizes to the dioxetanone with expulsion of the aryloxide leaving group. Exothermic fragmentation of the four-membered ring produces $CO_2$ and the singlet-excited *N*-methylacridone **6**.

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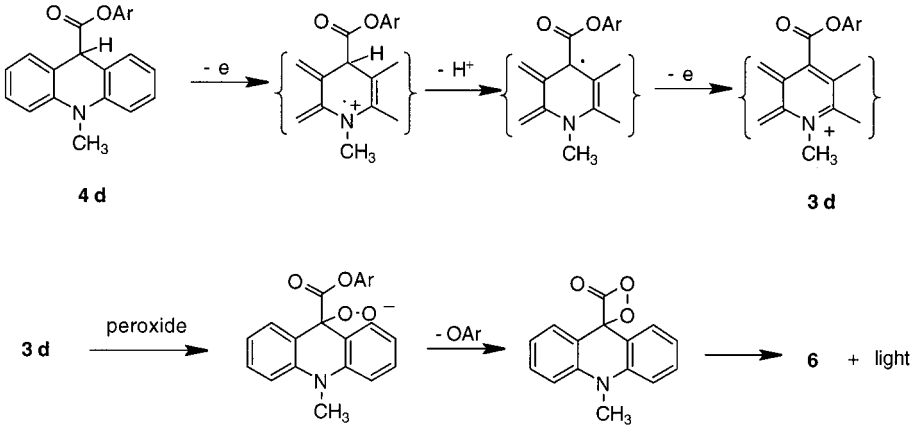
**Scheme 4**




Enzymatically produced peak light intensity correlates with the lability of the leaving group. Thioesters generally produced higher signal and background than esters. In the series of both esters and thioesters, light intensities decreased with substitution on the benzene ring of the leaving group in the order F > Cl > H > $OCH_3$, although steric effects had some influence when the leaving group bore substituents at the two ortho positions. These leaving group effects suggest that the cyclization step also is important in the kinetic rate expression.

## Conclusions

We have found that a peroxidase enzyme catalyzes the efficient chemiluminescent oxidation of a class of *N*-alkylacridancarboxylic acid derivatives. A key feature of this reaction is the enzymatic generation of chemiluminescent acridinium intermediates which can be induced to emit light in a continuous mode or can be accumulated for later detection as a flash of light. Insight into the reaction mechanism was gained by UV−visible spectroscopic studies which identified an acridinium ester as the intermediate responsible for generating chemiluminescence. A mechanistic picture of the reaction has been developed which rationalizes the observed substituent and leaving group effects on enzymatic and nonenzymatic light production. Enhanced reagents incorporating surfactants and enhancers have been developed which permit the detection of HRP with subattomolar sensitivity and provide convenient and sensitive detection in enzyme assays, immunoassays and blotting assays. Nonionic surfactants provided significantly increased signal/background ratios. The use of phenol compounds prolonged the enzymatic reaction similar to other peroxidase-catalyzed reactions. We are currently exploring the properties of chemiluminescent acridans with other aromatic and nonaromatic leaving groups and different ring substitution. The full details of these effects will be reported in future publications.

## Experimental Section

**General Procedures.** Acridan compounds **4a**−**e** were prepared by standard synthetic methods[17,18] and were characterized by high field NMR ($^1$H, $^{13}$C and $^{19}$F) and mass spectrometry. HRP type IV was purchased from Biozyme and used without further purification. All other reagents were obtained from commercial sources and were of the highest purity available. The enhanced luminol reagent was from Amersham (Amersham ECL) and was prepared according to the manufacturer's directions by combining solutions 1 and 2 in a 1:1 ratio.

**Luminescence and Absorbance Measurement.** Light intensity measurements were made using either a Turner Designs TD-20e single tube luminometer or Labsystems Luminoskan microplate luminometer. Data collection, analysis, and display were software-controlled. All analytical results are the average of at least triplicate measurements. Measurements of absorption spectra were performed using a diode array spectrophotometer.

**3-Methoxyacridine-9-carboxylic Acid (1a).** Condensation of 3-methoxydiphenylamine (Aldrich) with oxalyl chloride and $AlCl_3$ followed by base-catalyzed rearrangement of the *N*-arylisatin according to the method of Stollé[18] produced 3-methoxyacridinecarboxylic acid **(1a)**: $^1$H NMR ($D_2O$/NaOD) $\delta$ 3.126 (s, 3H), 5.873 (s, 1H), 6.50−6.58 (d, 1H), 6.85−6.95 (m, 1H), 7.00−7.07 (m, 2H), 7.47 (d, 1H), 7.58−7.63 (m, 1H); $^{13}$C NMR ($D_2O$/NaOD) $\delta$ 54.93, 102.56, 116.46, 119.34, 120.52, 125.17, 125.62, 126.20, 127.08, 130.54, 146.20, 147.08, 148.78, 160.11, 174.74.

**General Procedure for Esterification of Acridinecarboxylic Acids. 2′,3′,6′-Trifluorophenyl 3-Methoxyacridine-9-carboxylate (2a).** 3-Methoxyacridine-9-carboxylic acid (**1a**) (1.5 g) was suspended in excess $SOCl_2$ (10 mL) and the reaction mixture was refluxed for 3 h. The solvent was removed under reduced pressure to obtain a yellow solid which was dissolved in $CH_2Cl_2$ and pyridine (0.7 mL) under argon. A solution of the phenol (0.878 g) in $CH_2Cl_2$ was added dropwise. The solution was stirred overnight at room temperature and then diluted with more $CH_2Cl_2$ (100 mL) and washed with water (3 × 50 mL). The organic layer was dried over $Na_2SO_4$ and concentrated. Compound **2a** was isolated by column chromatography on silica with 20% ethyl acetate/hexane: $^1$H NMR ($CDCl_3$) $\delta$ 4.043 (s, 3H), 7.08−8.25 (m, 9H); $^{19}$F NMR ($CDCl_3$) $\delta$ −146.16 (m), −139.87 (m), −129.85 (m). Anal. Calcd for $C_{21}H_{12}F_3NO_3$: C, 65.80; H, 3.16; N, 3.65. Found: C, 65.61; H, 2.99; N, 3.63.

**General Procedure for *N*-Methylation of Acridinecarboxylic Esters. 2′,3′,6′-Trifluorophenyl 3-Methoxy-10-methylacridinium-9-carboxylate Trifluoromethanesulfonate (3a).** Compound **2a** (0.24 g) was dissolved in $CH_2Cl_2$ (3 mL) under argon and methyl trifluoromethanesulfonate (0.10 mL) was added. The solution was stirred overnight at room temperature to yield a thick yellow precipitate. This precipitate was filtered, washed with ether and dried to obtain **3a** as yellow crystals: $^1$H NMR (DMSO-$d_6$) $\delta$ 4.288 (s, 3H), 4.837 (s, 3H), 7.64−8.89 (m, 9H); $^{19}$F NMR (acetone-$d_6$) $\delta$ −146.49 (m), −139.92 (m), −129.35 (m), −77.75 (s). Anal.

(17) Zomer, G.; Stavenuiter, J.; Van Den Berg, R.; Jansen, E. In *Luminescence Techniques in Chemical and Biochemical Analysis*; Baeyens, W., De Keukeleire, D., Korkidis, K., Eds., Dekker: New York, 1991; pp 505−21.

(18) Stollé, R. *J. Prakt. Chem.* **1922**, *105*, 137.

Calcd for $C_{23}H_{15}F_6NO_6S$: C, 50.46; H, 2.76; N, 2.56. Found: C, 50.39; H, 2.91; N, 2.55.

**General Procedure for Reduction of *N*-Methylacridiniumcarboxylic Esters. 2′,3′,6′-Trifluorophenyl 3-Methoxy-10-methylacridan-9-carboxylate (4a).** Compound **3a** (0.035 g) was suspended in absolute ethanol (15 mL) and the solution was refluxed for 10 min to obtain a clear solution. Excess $NH_4Cl$ (4.0 g), was added by portions to the solution followed by zinc (4.0 g) causing immediate decolorization of the solution. The colorless solution was refluxed for 30 min. The cooled solution was filtered and the precipitate washed with ethanol (3 × 20 mL). The solution was concentrated to obtain an off-white solid which was redissolved in $CH_2Cl_2$ and washed with water (3 × 50 mL). The crude material obtained after evaporation of $CH_2Cl_2$ was chromatographed on silica gel (ethyl acetate/hexane) to yield **4a** as a white solid: $^1$H NMR ($CDCl_3$) $\delta$ 3.422 (s, 3H), 3.847 (s, 3H), 5.25 (s, 1H), 6.54−7.39 (m, 9H); $^{19}$F NMR ($CDCl_3$) $\delta$ −147.55 (m), −141.18 (m), −131.15 (m). HRMS (EI) *m*/*z* calcd for $C_{22}H_{16}NO_3F_3$ ($M^+$) 399.1082, found 399.1084; MS (EI) *m*/*z* (rel int) 399.1 (5), 224.1 (100), 209 (7), 181 (17), 166 (8), 148 (4). Anal. Calcd for $C_{22}H_{16}F_3NO_3$: C, 66.16; H, 4.04; N, 3.51. Found: C, 65.92; H, 4.03; N, 3.47.

**3-Methoxyacetanilide.** Acetic anhydride (20 mL) was added to a solution of *m*-anisidine (20 g) in 20 mL of acetic acid at 0 °C. The solution was stirred overnight at room temperature and then poured into 100 g of ice and 100 mL of water. The light pink solid was filtered and air-dried, yielding 21.5 g of the product: $^1$H NMR ($CDCl_3$) $\delta$ 2.18 (s, 3H), 3.81 (s, 3H), 6.65−6.68 (d, 1H), 6.94−6.97 (d, 1H), 7.15 (br s, 1H), 7.18−7.28 (m, 2H); $^{13}$C NMR ($CDCl_3$) $\delta$ 24.43, 55.15, 105.77, 109.84, 112.12, 129.51, 139.19, 159.95, 168.91.

**Bis(3-methoxyphenyl)amine.** 3-Methoxyacetanilide (20 g) was condensed with 3-bromoanisole (38.3 mL) in the presence of 18.4 g of $K_2CO_3$ and 2.3 g of CuI at 220 °C for 17 days. After 8 days, 2.76 g of $K_2CO_3$, 3.45 g of CuI, and 10 mL of bromoanisole were added. After 12 days, an additional 1.9 g of $K_2CO_3$ and 2.3 g of CuI were added. TLC (30% ethyl acetate/hexane) indicated complete conversion of the acetanilide to the diphenylamine compound. The excess bromoanisole was distilled away under vacuum and the product used without further purification. The acetanilide compound was dissolved in 100 mL of absolute ethanol containing 13.46 g of KOH. The mixture was refluxed for 1.5 h. The cooled mixture was concentrated, dissolved in ether, and washed with 3 × 100 mL of water. The organic layer was dried and concentrated to a brown oil. The diphenylamine product (33 g) was isolated by column chromatography using 40% ethyl acetate/hexane: $^1$H NMR ($CDCl_3$) $\delta$ 3.77 (s, 6H), 6.48−6.51 (dd, 2H), 6.65−6.68 (m, 4H), 7.16 (t, 2H); $^{13}$C NMR ($CDCl_3$) $\delta$ 55.18, 103.71, 106.41, 110.57, 130.06, 144.20, 160.62.

**1,6-Dimethoxyacridine-9-carboxylic Acid (1b).** A solution of 10 g of bis(3-methoxyphenyl)amine in 50 mL of $CH_2Cl_2$ was added dropwise over 35 min to a warm solution of 4.95 mL of oxalyl chloride in 50 mL of $CH_2Cl_2$. The solution was refluxed for 1 h, after which time the volatiles were evaporated, and the residue was redissolved in 200 mL of $CH_2Cl_2$. The ice-cooled solution was treated with 20.36 g of $AlCl_3$ added in portions. Reflux was started after 25 min. The volatiles were removed after 3 h and the black residue quenched with a mixture of 250 g of ice and 250 mL of 0.5 N HCl. The orange isatin compound was filtered and air-dried: $^1$H NMR ($CDCl_3$) $\delta$ 3.85, 3.87 (2 s, 6H), 6.38 (d, 1H), 6.60−6.64 (dd, 1H), 6.93−7.01 (m, 3H), 7.46 (t, 1H), 7.68 (d, 1H). Without further purification, the isatin was refluxed with 200 mL of 10% KOH for 22 h, cooled, evaporated, and neutralized with 200 mL of 5 N HCl and 300 g of ice. The product, isolated by filtration as a yellow solid, contained a mixture of 1,6-dimethoxyacridine-9-carboxylic acid and 3,6-dimethoxyacridine-9-carboxylic acid, which were separated after conversion to the 2,3,6-trifluorophenyl esters.

**2′,3′,6′-Trifluorophenyl 1,6-Dimethoxyacridine-9-carboxylate (2b).** A mixture of the 3,6- and 1,6-dimethoxyacridinecarboxylic acids (100 mg) in pyridine was reacted wtih *p*-toluenesulfonyl chloride (280 mg) for 1 h. 2,3,6-Trifluorophenol (260 mg) was added to the solution, which was stirred at room temperature until the reaction was complete. The pyridine was removed under reduced pressure and the products separated by preparative TLC. Compound **2b** was isolated as the minor isomer. Compound **2b**: $^1$H NMR ($CDCl_3$) $\delta$ 4.03 (s, 3H), 4.04 (s, 3H), 6.86−6.88 (d, 1H), 7.04−7.24 (m, 2H), 7.33−7.37 (dd, 1H), 7.49−7.50 (d, 1H), 7.71−7.77 (m, 1H), 7.81−7.83 (m, 1H), 8.08−8.11 (d, 1H).

**2′,3′,6′-Trifluorophenyl 1,6-Dimethoxy-10-methylacridinium-9-carboxylate Trifluoromethanesulfonate (3b).** *N*-Methylacridinium ester **3b** (45 mg) obtained by the standard procedure was used without purification in the next step.

**2′,3′,6′-Trifluorophenyl 1,6-Dimethoxy-10-methylacridan-9-carboxylate (4b).** Following the standard protocol, 12 mg of the acridan ester was isolated: $^1$H NMR ($CDCl_3$) $\delta$ 3.39 (s, 3H), 3.84 (s, 3H), 3.90 (s, 3H), 5.90 (s, 1H), 6.50−7.43 (m, 8H).

**2′,3′,6′-Trifluorophenyl Acridine-9-carboxylate (2c).** The commercially available acridine-9-carboxylic acid (0.5 g) was refluxed with 5 mL of $SOCl_2$ for 3 h, the solution evaporated, and the acid chloride reacted with 0.365 g of 2,3,6-trifluorophenol and 0.53 g of pyridine using the general procedure described above to produce ester **2c**: $^1$H NMR ($CDCl_3$) $\delta$ 7.08−7.28 (m, 2H) 7.71−8.42 (m, 8H); $^{19}$F NMR ($CDCl_3$) $\delta$ −146.13 (m), −139.85 (m), −129.82 (m). Anal. Calcd for $C_{20}H_{10}F_3NO_2$: C, 67.99; H, 2.85; N, 3.96. Found: C, 67.91; H, 2.92; N, 3.97.

**2′,3′,6′-Trifluorophenyl 10-Methylacridan-9-carboxylate (4c).** Because of the low reactivity of **2c** with methyl triflate, an alternate procedure was used to prepare **4c**. Ester **2c** was reduced with zinc and $NH_4Cl$ in 2-propanol. The crude product was then methylated in $CH_2Cl_2$ with methyl triflate as described above. Ester **4c** was purified by chromatography on silica gel with 25% $CH_2Cl_2$/hexane: $^1$H NMR ($CDCl_3$) $\delta$ 3.44 (s, 3H), 5.29 (s, 1H), 6.76−6.84 (m, 2 H) 6.99−7.39 (m, 8H); $^{19}$F NMR ($CDCl_3$) $\delta$ −147.57, −141.15, −131.19. HRMS (EI) *m*/*z* calcd for $C_{21}H_{14}NO_2F_3$ ($M^+$) 369.0976, found 369.0966; MS (EI) *m*/*z* (rel int): 369 (5), 194 (100), 179 (17). Anal. Calcd for $C_{21}H_{14}F_3NO_2$: C, 68.29; H, 3.82; N, 3.79. Found: C, 68.18; H, 3.76; N, 3.80.

**2′,6′-Difluorophenyl Acridine-9-carboxylate (2d).** Acridine-9-carboxylic acid (0.5 g) was refluxed with 10 mL of $SOCl_2$ for 3 h, the solution evaporated and the acid chloride reacted with 0.32 g of 2,6-difluorophenol and 0.44 g of pyridine using the general procedure described above to produce ester **2d**: $^1$H NMR ($CDCl_3$) $\delta$ 7.13−7.39 (m, 3H), 7.68−8.35 (m, 8H); $^{19}$F NMR ($CDCl_3$) $\delta$ −125.33 (s). Anal. Calcd for $C_{20}H_{11}F_2NO_2$: C, 71.64; H, 3.31; N, 4.18. Found: C, 71.42; H, 3.30; N, 4.14.

**2′,6′-Difluorophenyl 10-Methylacridinium-9-carboxylate (3d).** Compound **2d** (0.40 g) was dissolved in $CH_2Cl_2$ (5 mL) under argon and methyl trifluoromethanesulfonate (0.945 mL) was added. The solution was stirred overnight at room temperature to yield a thick yellow precipitate. This precipitate was filtered, washed with ether, and dried to obtain **3d** as yellow crystals: $^1$H NMR (acetone-$d_6$) $\delta$ 5.28 (s, 3H), 7.44−7.68 (m, 3H), 8.32−9.13 (m, 8H); $^{19}$F NMR (acetone-$d_6$) $\delta$ −125.57 (m), −77.73 (s). Anal. Calcd for $C_{22}H_{14}F_5NO_5S$: C, 52.91; H, 2.83; N, 2.80. Found: C, 53.23; H, 2.94; N, 2.82.

**2′,6′-Difluorophenyl 10-Methylacridan-9-carboxylate (4d).** Compound **3d** (0.10 g) was reduced with 0.65 g of Zn and 0.535 g of $NH_4Cl$ in 15 mL of ethanol according to the general procedure described above to produce acridan **4d** as a white solid: $^1$H NMR ($CDCl_3$) $\delta$ 3.49 (s, 3H), 5.29 (s, 1H), 6.82−7.10 (m, 7 H) 7.29−7.41(m, 4H); $^{19}$F NMR ($CDCl_3$) $\delta$ −126.65 (m). HRMS (EI) *m*/*z* calcd for $C_{21}H_{15}NO_2F_2$ ($M^+$) 351.1071, found 351.1074; MS (EI) *m*/*z* (rel int): 351 (5), 194 (100), 179 (16). Anal. Calcd for $C_{21}H_{15}F_2NO_2$: C, 71.79; H, 4.30; N, 3.99. Found: C, 71.68; H, 4.24; N, 3.96.

**5-Methoxy-2-methylacetanilide.** 5-Methoxy-2-methylaniline (9.77 g, Aldrich) was converted to the acetamide derivative by reaction with 8.7 mL of acetic anhydride, 8.2 mL of acetic acid, and 43 mg of zinc at reflux for 7 h. The reaction mixture was poured into 250 mL of ice water and stirred. The light brown solid was filtered and air-dried yielding 6.37 g of the product: mp 95−95.7 °C; $^1$H NMR ($CDCl_3$) $\delta$ 2.19 (s, 3H),

2.21 (s, 3H), 3.79 (s, 3H), 6.62−6.66 (m, 1H), 6.93 (br s, 1H), 7.05−7.08 (d, 1H), 7.55 (s, 1H); $^{13}$C NMR ($CDCl_3$) $\delta$ 16.81, 24.25, 52.30, 108.54, 111.03, 120.68, 130.79, 136.40, 158.23, 168.42. Anal. Calcd for $C_{10}H_{13}NO_2$: C, 67.02; H, 7.31; N, 7.82. Found: C, 66.66; H, 7.11; N, 7.75.

**5-Methoxy-2-methylphenyl-3′-methoxyphenylamine.** 5-Methoxy-2 methylacetanilide (6.37 g) was condensed with 3-bromoanisole (11.1 mL) in the presence of 5.06 g of $K_2CO_3$ and 0.74 g of CuI at reflux for 8 h. After standing overnight, the mixture was heated and then extracted with toluene (3 × 100 mL) and evaporated to a brown oil. The oil was dissolved in 150 mL of ethanol, 4 g of KOH was added, and the mixture was refluxed for 9 h. The ethanol was evaporated and the red-brown solid taken up in 300 mL of water and extracted with ethyl acetate. The ethyl acetate was evaporated and the crude solid partially purified by passing a $CH_2Cl_2$ solution through a plug of silica. Final purification was effected by column chromatography on silica (5% ethyl acetate/hexane) to produce 7.63 g of the diphenylamine compound: $^1$H NMR ($CDCl_3$) $\delta$ 2.194 (s, 3H), 3.752 (s, 3H), 3.783 (s, 3H), 5.404 (s, 1H), 6.47−6.52 (m, 2H), 6.55−6.62 (m, 2H), 6.85−6.86 (d, 1H), 7.08−7.10 (d, 1H), 7.14−7.20 (t, 1H).

**1,6-Dimethoxy-4-methylacridine-9-carboxylic Acid (1e).** Reaction of 5-methoxy-2-methylphenyl-3′-methoxyphenylamine (7.63 g) with 3.12 mL of oxalyl chloride in 110 mL of $CH_2Cl_2$ followed by reaction with 8.36 g of $AlCl_3$ produced the isatin which was converted by base-catalyzed rearrangement with 100 mL of 10% KOH and neutralization to 1,6-dimethoxy-4-methylacridine-9-carboxylic acid (**1e**) (8.75 g): mp 172.8−173.5 °C (dec); $^1$H NMR ($D_2O$/NaOD) $\delta$ 2.091 (s, 3H), 3.580 (s, 3H), 3.824 (s, 3H), 6.49 (d, 2H), 6.72 (s, 1H), 6.93 (d, 1H), 7.11 (d, 1H), 7.76 (d, 1H); $^{13}$C NMR ($D_2O$/NaOD) $\delta$ 18.14, 55.30, 55.72, 102.55, 103.50, 112.69, 116.94, 120.19, 126.41, 127.54, 130.88, 144.47, 148.21, 149.30, 153.82, 160.65, 176.71. Anal. Calcd for $C_{17}H_{17}NO_5$ (monohydrate): C, 64.76; H, 5.43; N, 4.44. Found: C, 64.51; H, 5.25; N, 4.44.

**2′,3′,6′-Trifluorophenyl 1,6-Dimethoxy-4-methylacridine-9-carboxylate (2e).** Acid **1e** (1.0 g) was dissolved in pyridine (20 mL) and reacted with *p*-toluenesulfonyl chloride (1.28 g) for 1 h. 2,3,6-Trifluorophenol (1.0 g) in 5 mL of pyridine was added and the solution was stirred overnight at room temperature. The ester product was isolated by chromatography on silica gel (20−40% ethyl acetate/hexane). A second chromatographic purification using 50% $CH_2Cl_2$/hexane yielded 110 mg of ester **2e**: mp 187 °C; $^1$H NMR ($CDCl_3$) $\delta$ 2.841 (s, 3H), 4.003 (s, 3H), 4.051 (s, 3H), 6.75−6.78 (d, 1H), 7.04−7.23 (m, 2H), 7.31−7.36 (dd, 1H), 7.53−7.57 (m, 2H), 8.08−8.11 (d, 1H); $^{19}$F NMR ($CDCl_3$) (rel. to $CFCl_3$) $\delta$ −145.27 to −145.17 (m), −140.44 to −140.27 (m), −128.50 to −128.37 (m). Anal. Calcd for $C_{23}H_{16}F_3NO_4$: C, 64.64; H, 3.77; N, 3.28. Found: C, 64.61; H, 3.85; N, 3.32.

**2′,3′,6′-Trifluorophenyl 1,6-Dimethoxy-4-methylacridan-9-carboxylate.** To a slurry of ester **2e** (110 mg) and $NH_4$-Cl (138 mg) in Ar-purged 2-propanol (25 mL) was added zinc (168 mg), causing immediate decolorization of the solution. The colorless solution was stirred at room temperature for 2.5 h shielded from light. $CH_2Cl_2$ was added to the solution which was then filtered. The solvents were removed from the filtrate under reduced pressure. The crude material was purified by preparative TLC (20% ethyl acetate/hexane) to yield 29.5 mg of acridan: $^1$H NMR ($CDCl_3$) $\delta$ 2.204 (s, 3H), 3.799 (s, 3H), 3.850 (s, 3H), 5.534 (s, 1H), 6.025 (s, 1H), 6.31−6.32 (d, 1H), 6.35−6.39 (d, 1H), 6.50−6.54 (dd, 1H), 6.80−7.03 (m, 3H), 7.42−7.45 (d, 1H).

**2′,3′,6′-Trifluorophenyl 1,6-Dimethoxy-4,10-dimethylacridan-9-carboxylate (4e).** The acridan from the previous step (29.5 mg) was reacted with methyl triflate (0.5 mL) in 6 mL of $CH_2Cl_2$ under Ar with protection from light. After 48 h, another 1 mL of methyl triflate was added and stirring continued for another day. The volatiles were evaporated and the product purified by preparative TLC using 20% ethyl acetate/hexane, yielding **4e** as a white solid: mp 127.2−127.6 °C; $^1$H NMR ($CDCl_3$) $\delta$ 2.373 (s, 3H), 3.478 (s, 3H), 3.838 (s, 3H), 3.876 (s, 3H), 5.447 (s, 1H), 6.53−6.64 (m, 3H), 6.77−7.01 (m, 2H), 7.07−7.10 (d, 1H), 7.30−7.34 (d, 1H); $^{19}$F NMR ($CDCl_3$) (relative to $CFCl_3$) $\delta$ −147.67 to −147.58 (m), −141.46 to −141.29 (m), −131.26 to −131.17 (m), HRMS (EI) *m*/*z* calcd for $C_{24}H_{20}F_3NO_4$ ($M^+$) 443.1344, found 443.1351; MS (EI) *m*/*z* (rel int) 443.1 (6), 268.1 (100), 253 (13), 238 (11). Anal. Calcd for $C_{24}H_{20}F_3NO_4$: C, 65.01; H, 4.55; N, 3.16. Found: C, 64.98; H, 4.53; N, 3.16.

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**Supporting Information Available:** $^1$H and $^{13}$C NMR spectra of selected compounds (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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