

603736

CONVENTION

AUSTRALIA

Patents Act

APPLICATION FOR A STANDARD PATENT

The Board of Governors of Wayne State University
5050 Cass Avenue, Detroit, Michigan, 48202, UNITED STATES OF AMERICA

hereby applies for the grant of a standard patent for an invention entitled:

METHOD AND COMPOSITIONS PROVIDING ENHANCED CHEMILUMINESCENCE FROM
1,2-DIOXETANES

which is described in the accompanying complete specification.

Details of basic application(s):-

224,681 UNITED STATES OF AMERICA 27 July 1988

Address for Service:

PHILLIPS ORMONDE & FITZPATRICK
Patent and Trade Mark Attorneys
367 Collins Street
Melbourne 3000 AUSTRALIA

DATED this TWENTY FIRST day of JUNE 1989

PHILLIPS ORMONDE & FITZPATRICK
Attorneys for:
The Board of Governors of Wayne State University

By:

David B. [Signature]

Our Ref : 136970
POF Code: 507/103078

M 010080 210689

APPLICATION ACCEPTED AND AMENDMENTS

6010

ALLOWED 28 - 8 - 90

AUSTRALIA

Patents Act

DECLARATION FOR A PATENT APPLICATION

▼ INSTRUCTIONS

- (a) Insert "Convention" if applicable
(b) Insert FULL name(s) of applicant(s)

In support of the (a) Convention application made by
(b) The Board of Governors of Wayne State University

- (c) Insert "of addition" if applicable
(d) Insert TITLE of invention

(hereinafter called "applicant(s)" for a patent (c) for an
invention entitled (d) Method and Compositions Providing
Enhanced Chemiluminescence From
1,2-Dioxetanes

- (e) Insert FULL name(s) AND address(es) of declarant(s)
(See headnote*)

I/We (e) Patricia Eames, Vice President of The Board of Governors of Wayne
State University: 5050 Cass Avenue, Detroit, Michigan 48202,
United States of America

do solemnly and sincerely declare as follows:

1. ~~I am/We are the applicant(s).~~
(or, in the case of an application by a body corporate)
1. I am/We are authorized to make this declaration on behalf of the applicant(s).
2. ~~I am/We are the actual inventor(s) of the invention.~~
(or, where the applicant(s) is/are not the actual inventor(s))
2. (f) Arthur Paul Schaap: 1921 Pembridge Place
Detroit, Michigan 48207, USA

- (f) Insert FULL name(s) AND address(es) of actual inventor(s)

- (g) Recite how applicant(s) derive(s) title from actual inventor(s)
(See headnote**)

is/are the actual inventor(s) of the invention and the facts upon which the applicant(s)
is/are entitled to make the application are as follows:

(g)

Applicant is the assignee of the inventor.

(Note: Paragraphs 3 and 4 apply only to Convention applications)

3. The basic application(s) for patent or similar protection on which the application is based
is/are identified by country, filing date, and basic applicant(s) as follows:

(h) UNITED STATES OF AMERICA
27 July, 1988
Arthur Paul Schaap

- (h) Insert country, filing date, and basic applicant(s) for the/or EACH basic application

4. The basic application(s) referred to in paragraph 3 hereof was/were the first application(s)
made in a Convention country in respect of the invention the subject of the application.

- (k) Insert PLACE of signing

Declared at (k) Detroit, Michigan, USA

- (l) Insert DATE of signing

Dated (l) July 10, 1989

- (m) Signature(s) of declarant(s)

(m) Wayne State University

Note: No legalization or other witness required

To: The Commissioner of Patents

By: Patricia Eames
Patricia Eames, Vice President

(12) PATENT ABRIDGMENT (11) Document No. AU-B-36645/89
 (19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 603736

(54) Title
 METHOD AND COMPOSITIONS PROVIDING ENHANCED CHEMILUMINESCENCE FROM
 1,2-DIOXETANES

International Patent Classification(s)
 (51)⁴ C09K 011/07

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(43) Publication Date : 01.02.90

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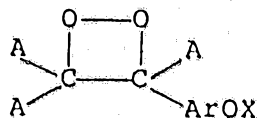
(71) Applicant(s)
 THE BOARD OF GOVERNORS OF WAYNE STATE UNIVERSITY

(72) Inventor(s)
 ARTHUR PAUL SCHAAP

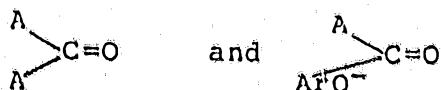
(74) Attorney or Agent
 PHILLIPS ORMONDE & FITZPATRICK, 367 Collins Street, MELBOURNE VIC 3000

(57) Claim

1. A method for generating light which comprises:
 - (a) providing in a setting where the light is to be produced a fluorescent compound dispersed in or on one of a surfactant, a micelle, liposome, reversed micelle, microemulsion, film, including a monolayer or a polymer which brings the fluorescent compound into a closely spaced relationship with and in admixture with a 1,2-dioxetane which is stable in the setting where light is to be produced of the formula



wherein ArOX represents an aryl group substituted with an X-oxy group which forms an oxide intermediate 1,2-dioxetane compound when triggered by removing with an activating agent so that the intermediate 1,2-dioxetane compound decomposes and releases electronic energy to form light and two carbonyl containing compounds of the formula



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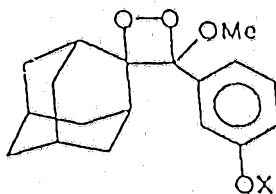
-2-

wherein X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate 1,2-dioxetane and wherein A are passive organic groups which allow the light to be produced by the 1,2-dioxetane when X is removed; and

(b) triggering the stable 1,2-dioxetane with the activating agent wherein the fluorescent compound accepts the electronic energy generated upon decomposition of the oxide intermediate 1,2-dioxetane compound and produces a more intense light than is produced by the triggering of the dioxetane alone.

28. In a method for detecting DNA by hybridization of the DNA with a DNA probe having an enzyme label and then generating a detectable signal by means of the enzyme label, the improvement which comprises:

(a) providing a 1,2-dioxetane of the formula:



wherein X is a group reactive with the enzyme label to produce light;

(b) providing a fluorescent compound which enhances the light from the 1,2-dioxetane in admixture with the hybrid of the probe and the DNA; and

(c) detecting the light from the hybrid produced by the reaction of the enzyme label with the dioxetane.

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COMPLETE SPECIFICATION
(ORIGINAL)

Application Number: Class Int. Class
Lodged:

Complete Specification Lodged:
Accepted:
Published:

Priority

Related Art:

This document contains the
amendments made under
Section 49 and is correct for
printing.

Applicant(s):

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Complete Specification for the invention entitled:

METHOD AND COMPOSITIONS PROVIDING ENHANCED CHEMILUMINESCENCE FROM
1,2-DIOXETANES

Our Ref : 136970
POF Code: 507/103078

The following statement is a full description of this invention, including
the best method of performing it known to applicant(s):

WSU 4...-28
7/21/88

~~METHOD AND COMPOSITIONS PROVIDING ENHANCED
CHEMILUMINESCENCE FROM 1,2-DIOXETANES~~

~~Cross-Reference to Related Application~~

This application is a continuation-in-part of
U.S. application Serial No. 887,139, filed July 17, 1986.

BACKGROUND OF THE INVENTION

5 (1) STATE OF THE INVENTION

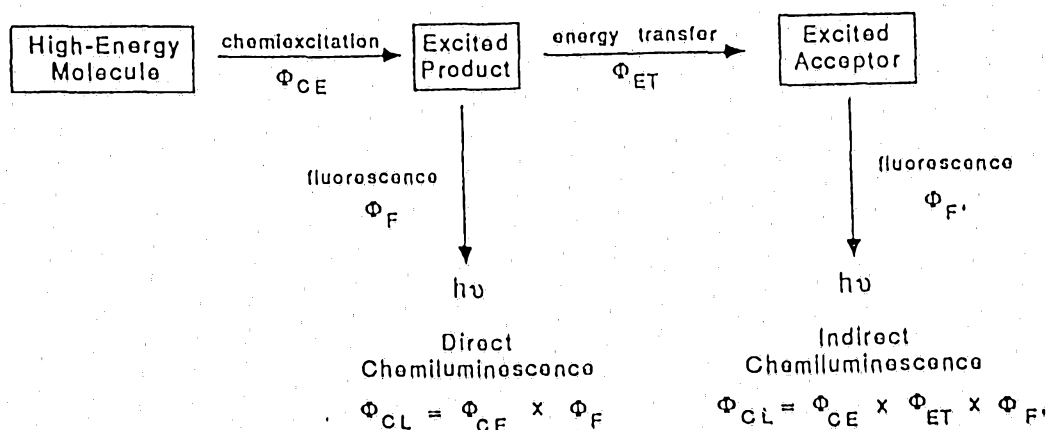
The present invention relates to compositions
containing a fluorescent compound and a stable
1,2-dioxetane which can be triggered by chemical reagents
including enzymes and generate enhanced chemiluminescence.
10 In particular the present invention relates to a method for
significantly enhancing the chemiluminescence which
involves intermolecular energy transfer to a fluorescent
compound in an organized assembly, such as a micelle, which
maintains a close spacing between the dioxetane and the
15 fluorescent compound.

(2) PRIOR ART

1. Mechanisms of Luminescence. Exothermic
chemical reactions release energy during the course of the
reaction. In virtually all cases, this energy is in the
20 form of vibrational excitation or heat. However, a few
chemical processes generate light or chemiluminescence
instead of heat. The mechanism for light production
involves thermal or catalyzed decomposition of a high
energy material (frequently an organic peroxide such as a
25 1,2-dioxetane) to produce the reaction product in a triplet
or singlet electronic excited states. Fluorescence of the
singlet species results in what has been termed direct
chemiluminescence. The chemiluminescence quantum yield is
the product of the quantum yields for singlet
30 chemiexcitation and fluorescence. These quantities are
often expressed as efficiencies where efficient (%) =
 $\phi \times 100$. Energy transfer from the triplet or singlet
product to a fluorescent acceptor can be utilized to give

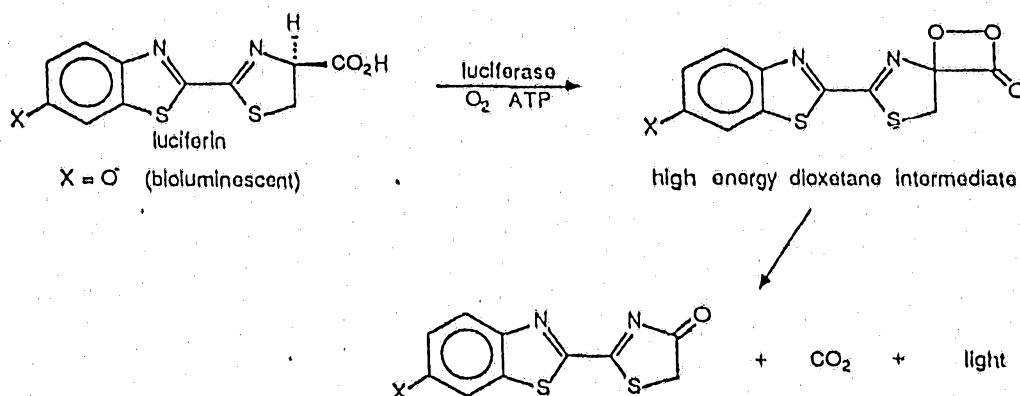


indirect chemiluminescence. The quantum yield for indirect chemiluminescence is the product of the quantum yields for singlet or triplet chemiexcitation, energy transfer, and fluorescence of the energy acceptor.



2. Dioxetane Intermediates in Bioluminescence.

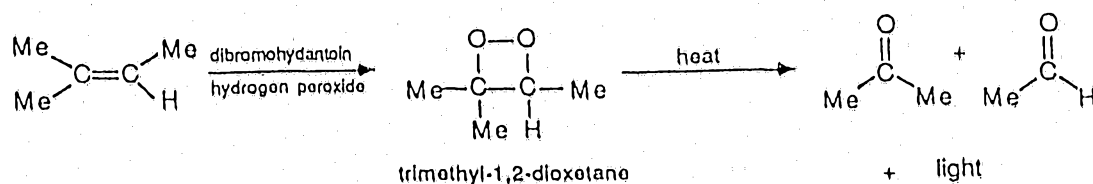
In 1968 McCapra proposed that 1,2-dioxetanes might be the key high-energy intermediates in various bioluminescent reactions including the firefly system. (F. McCapra, Chem. Commun., 155 (1968)). Although this species is apparently quite unstable and has not been isolated or observed spectroscopically, unambiguous evidence for its intermediacy in the reaction has been provided by oxygen-18 labeling experiments. (O. Shimomura and F. H. Johnson, Photochem. Photobiol., 30, 89 (1979)).



3. First Synthesis of Authentic 1,2-Dioxetanes.

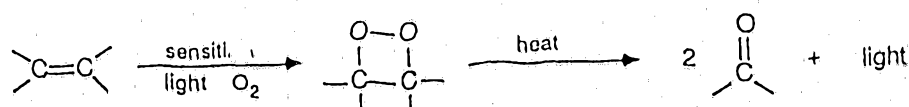
In 1969 Kopecky and Mumford reported the first synthesis of

a dioxetane (3,3,4-trimethyl-1,2-dioxetane) by the base-catalyzed cyclization of a beta-bromohydroperoxide. (K. R. Kopecky and C. Mumford, Can. J. Chem., 47, 709 (1969)). As predicted by McCapra, this dioxetane did, in fact, produce chemiluminescence upon heating to 50°C with decomposition to acetone and acetaldehyde. However, this peroxide is relatively unstable and cannot be stored at room temperature (25°C) without rapid decomposition. In addition, the chemiluminescence efficiency is very low (less than 0.1%).



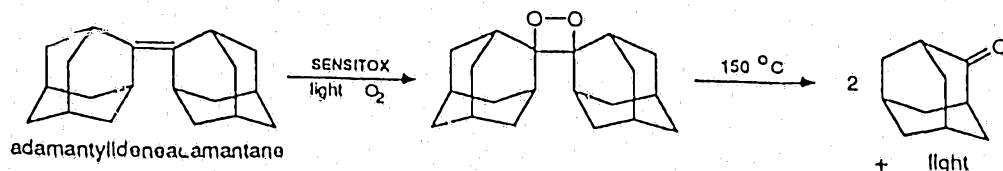
Bartlett and Schaap and Mazur and Foote independently developed an alternative and more convenient synthetic route to 1,2-dioxetanes. Photooxygenation of properly-substituted alkenes in the presence of molecular oxygen and a photosensitizing dye produces dioxetanes in high yields. (P. D. Bartlett and A. P. Schaap, J. Amer. Chem. Soc., 92, 3223 (1970) and S. Mazur and C. S. Foote, J. Amer. Chem. Soc., 92, 3225 (1970)). The mechanism of this reaction involves the photochemical generation of a metastable species known as singlet oxygen which undergoes 2 + 2 cycloaddition with the alkene to yield the dioxetane. Research has shown that a variety of dioxetanes can be prepared using this reaction (A. P. Schaap, P. A. Burns, and K. A. Zaklika, J. Amer. Chem. Soc., 99, 1270 (1977); K. A. Zaklika, P. A. Burns, and A. P. Schaap, J. Amer. Chem. Soc., 100, 318 (1978); K. A. Zaklika, A. L. Thayer, and A. P. Schaap, J. Amer. Chem. Soc., 100, 4916 (1978); K. A. Zaklika, T. Kissel, A. L. Thayer, P. A. Burns, and A. P. Schaap, Photochem. Photobiol., 30, 35 (1979); and A. P. Schaap, A. L. Thayer, and K. Kees, Organic Photochemical Synthesis, II, 49 (1976)). During the course of this research, a polymer-bound sensitizer for photooxygenations

was developed (A. P. Schaap, A. L. Thayer, E. C. Blossey, and D. C. Neckers, J. Amer. Chem. Soc., 97, 3741 (1975); and A. P. Schaap, A. L. Thayer, K. A. Zaklika, and P. C. Valenti, J. Amer. Chem. Soc., 101, 4016 (1979)). This new type of sensitizer has been patented and sold under the tradename SENSITOX™ (U.S. Patent No. 4,315,998 (2/16/82); Canadian Patent No. 1,044,639 (12/19/79)). Over fifty references have appeared in the literature reporting the use of this product.



4. Preparation of Stable Dioxetanes Derived from Sterically Hindered Alkenes. Wynberg discovered that photooxygenation of sterically hindered alkenes such as adamantylideneadamantane affords a very stable dioxetane (J. H. Wieringa, J. Strating, H. Wynberg, and W. Adam, Tetrahedron Lett., 169 (1972)). A collaborative study by Turro and Schaap showed that this dioxetane exhibits an activation energy for decomposition of 37 kcal/mol and a half-life at room temperature (25°C) of over 20 years (N. J. Turro, G. Schuster, H. C. Steinmetzer, G. R. Faler, and A. P. Schaap, J. Amer. Chem. Soc., 97, 7110 (1975)). In fact, this is the most stable dioxetane yet reported in the literature. Adam and Wynberg have recently suggested that functionalized adamantylideneadamantane 1,2-dioxetanes may be useful for biomedical applications (W. Adam, C. Babatsikos, and G. Cilento, Z. Naturforsch., 39b, 679 (1984); H. Wynberg, E. W. Meijer, and J. C. Hummelen, In Bioluminescence and Chemiluminescence, M. A. DeLuca and W. D. McElroy (Eds.) Academic Press, New York, p. 687, 1981; and J. C. Hummelen, T. M. Luider, and H. Wynberg, Methods in Enzymology, 133B, 531 (1986)). However, use of this extraordinarily stable peroxide for chemiluminescent labels requires detection temperatures of 150 to 250°C. Clearly, these conditions are unsuitable for the evaluation of

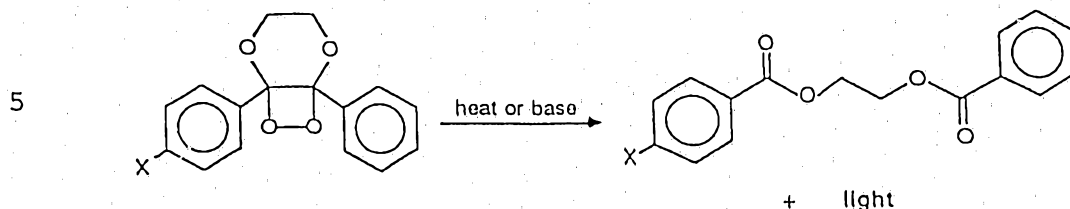
biological analytes in aqueous media. McCapra, Adam, and Foote have shown that incorporation of a spirofused cyclic or polycyclic alkyl group with a dioxetane can help to stabilize dioxetanes that are relatively unstable in the absence of this sterically bulky group (F. McCapra, I. Beheshti, A. Burford, R. A. Hann, and K. A. Zaklika, J. Chem. Soc., Chem. Commun., 944 (1977); W. Adam, L. A. A. Encarnacion, and K. Zinner, Chem. Ber., 116, 839 (1983); G. G. Geller, C. S. Foote, and D. B. Pechman, Tetrahedron Lett., 673 (1983); P. Lechtken, Chem. Ber., 109, 2862 (1976); and P. D. Bartett and M. S. Ho, J. Amer. Chem. Soc., 96, 627 (1974))



5. Effects of Substituents on Dioxetane

Chemiluminescence. The stability and the chemiluminescence efficiency of dioxetanes can be altered by the attachment of specific substituents to the peroxide ring (K. A. Zaklika, T. Kissel, A. L. Thayer, P. A. Burns, and A. P. Schaap, Photochem. Photobiol., 30, 35 (1979); A. P. Schaap and S. Gagnon, J. Amer. Chem. Soc., 104, 3504 (1982); A. P. Schaap, S. Gagnon, and K. A. Zaklika, Tetrahedron Lett., 2943 (1982); and R. S. Handley, A. J. Stern, and A. P. Schaap, Tetrahedron Lett., 3183 (1985)). The results with the bicyclic system shown below illustrate the profound effect of various functional groups on the properties of dioxetanes. The hydroxy-substituted dioxetane (X=OH) derived from the 2,3-diaryl-1,4-dioxene exhibits a half-life for decomposition at room temperature (25°C) of 57 hours and produces very low levels of luminescence upon heating at elevated temperatures. In contrast, however, reaction of this dioxetane with a base at -30°C affords a flash of blue light. Kinetic studies have shown that the deprotonated dioxetane (X=O⁻)

decomposes 5.7×10^6 times faster than the protonated form ($X=OH$) at $25^\circ C$.



$X = O^-$ (chemiluminescent)

10 $X = OH$ (non-chemiluminescent)

The differences in the properties of these two dioxetanes arise because of two competing mechanisms for decomposition (K. A. Zaklika, T. Kissel, A. L. Thayer, P. A. Burns, and A. P. Schaap, Photochem. Photobiol., 30, 35 (1979); A. P. Schaap and S. Gagnon, J. Amer. Chem. Soc., 104, 3504 (1982); A. P. Schaap, S. Gagnon, and K. A. Zaklika, Tetrahedron Lett., 2943 (1982); and R. S. Handley, A. J. Stern and A. P. Schaap, Tetrahedron Lett., 3183 (1985)). Most dioxetanes cleave by a process that involves homolysis of the O-O bond and formation of a biradical. An alternative mechanism is available to dioxetanes bearing substituents such as O^- with low oxidation potentials. The cleavage is initiated by intramolecular electron transfer from the substituent to the antibonding orbital of the peroxide bond.

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6. Chemical Triggering of Dioxetanes. The first example in the literature is described above (A. P. Schaap and S. Gagnon, J. Amer. Chem. Soc., 104, 3504 (1982)). However, the hydroxy-substituted dioxetane and any other examples of the dioxetanes derived from the diaryl-1,4-dioxenes are far too unstable to be of use in any application. They have half-lives at $25^\circ C$ of only a few hours. Neither the dioxetane nor the precursor alkene would survive the conditions necessary to prepare derivatives. Further, these non-stabilized dioxetanes are destroyed by small quantities of amines (T. Wilson, Int. Rev. Sci.: Chem., Ser. Two, 9, 265 (1976)) and metal ions

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(T. Wilson, M. E. Landis, A. L. Baumstark, and P. D. Bartlett, J. Amer. Chem. Soc., 95, 4765 (1973); P. D. Bartlett, A. L. Baumstark, and M.E. Landis, J. Amer. Chem. Soc., 96, 5557 (1974) and could not be used in the aqueous buffers required for enzymatic triggering.

7. Energy-Transfer Chemiluminescence Involving Dioxetanes in Homogeneous Solution. The first example of energy-transfer chemiluminescence involving dioxetanes was described by Wilson and Schaap (T. Wilson and A. P. Schaap, J. Amer. Chem. Soc., 93, 4126 (1971)). Thermal decomposition of a very unstable dioxetane (cis-diethoxydioxetane) gave both singlet and triplet excited ethyl formate. Addition of 9,10-diphenylanthracene and 9,10-dibromoanthracene resulted in enhanced chemiluminescence through singlet-singlet and triplet-singlet energy-transfer processes, respectively. These techniques have subsequently been used by many other investigators to determine yields of chemiexcited products generated by the thermolysis of various dioxetanes (For a review, see W. Adam, In Chemical and Biological Generation of Excited States, W. Adam and G. Cilento, Eds. Ch. 4, Academic Press, New York, 1982). Energy transfer in homogeneous solution, however, requires high concentrations of the energy acceptor because of the short lifetimes of the electronically excited species. These high concentrations lead to problems of self-quenching and reabsorption. The present invention solves the problem by using the 1,2-dioxetane and a fluorescent energy acceptor which are preferably both incorporated in a micelle affording efficient energy transfer without the need for high concentrations of a fluorescer in bulk solution.

8. Enhanced Chemiluminescence from a Dioxetane Using Intermolecular Energy in Micelles. Rates of various chemical reactions can be accelerated by micelles in aqueous solution (See, for example: E. H. Cordes and R. B. Dunlap, Acc. Chem. Res., 2, 329 (1969)). Catalysis results from solubilization of the substrate in the micellar

pseudophase and from electrostatic, hydrophobic, or polarity factors. Aqueous micelles have been used to increase the rate of chemically triggered dioxetanes (A. P. Schaap, Final Technical Report to the Office of Naval Research, 1987, page 16). No experiments using fluorescent compounds such as co-surfactants to enhance chemiluminescence efficiency are reported.

Several reports describe enhanced chemiluminescence from chemical reactions in micellar environments. However, none of these make use of energy transfer to a fluorescent co-surfactant. No stabilized dioxetanes have been studied in micelles. Goto has investigated the chemical oxidation of a luciferin in the presence of neutral, anionic, and cationic surfactants (T. Goto and H. Fukatsu, Tetrahedron Lett., 4299 (1969)). The enhanced chemiluminescence was attributed to an increase in the fluorescence efficiency of the reaction product in the micelle compared to aqueous solution. The effect of cetyltrimethylammonium bromide micelles on the chemiluminescent reaction of acridan esters in aqueous alkaline solution has been reported (F. McCapra, Acc. Chem. Res., 9, 201 (1976). McCapra indicates, however, that micellar environment does not "assist the excitation reaction". Rather, the micelles are thought to enhance the luminescent yield by decreasing the rate of a competing, non-luminescent hydrolytic reaction. Similarly, Nikokavouras and Gundermann have studied the effect of micelles on chemiluminescent reactions of lucigenin and luminol derivatives, respectively (C. M. Paleos, G. Vassilopoulos, and J. Nikokavouras, Bioluminescence and Chemiluminescence, Academic Press, New York, 1981, p. 729; K. D. Gundermann, Ibid., p. 17). Shinkai observed that chemiluminescence yields from unstable, non-isolable dioxetanes could be enhanced in micelles relative to water (S. Shinkai, Y. Ishikawa, O. Manabe, and T. Kunitake, Chem. Lett., 1523 (1981). These authors suggested that the yield of excited

states may be higher in the hydrophobic core of micelles than in water.

The only reference to enhancement of enzymatically generated chemiluminescence with surfactants involves the work of Kricka and DeLuca on the firefly luciferase system (L. J. Kricka and M. DeLuca, Arch. Biochem. Biophys., 217, 674 (1983)). Nonionic detergents and polymers enhanced the total light yield by increasing the turnover of the enzyme. Cationic surfactants such as (cetyltrimethylammonium bromide, CTAB) actually resulted in complete inhibition of the catalytic activity of the luciferase.

A method for enhancing the chemiluminescent yield of the luminol/peroxidase reaction by addition of 6-hydroxybenzothiazole derivatives or para-substituted phenols (G. H. G. Thorpe, L. J. Kricka, S. B. Moseley, T. P. Whitehead, Clin. Chem., 31, 1335 (1985); G. H. G. Thorpe and L. J. Kricka, Methods in Enzymology, 133, 331 (1986); and L. J. Kricka, G. H. G. Thorpe, and R. A. W. Stott, Pure & Appl. Chem., 59, 651 (1987)). The mechanism for the enhancement is not known but it does not involve intramolecular energy transfer or intermolecular transfer to a co-micellar fluorescent surfactant.

Co-micellar fluorescent probes have been used to study the dynamic properties of micelles (Y. Kubota, M. Kodama, and M. Miura, Bull. Chem. Soc.. Jpn., 46, 100 (1973); N. E. Schore and N. J. Turro, J. Amer. Chem. Soc., 96, 306 (1974); and G. W. Pohl, Z. Naturforsch., 31c, 575 (1976)). However, no examples appear in the literature of using these fluorescent materials to enhance chemiluminescent reactions in micelles through energy-transfer processes.

9. Chemiluminescent Immunoassays. There are no reports of dioxetanes as enzyme substrates or their use in enzyme-linked assays prior to the filing date of Serial No. 887,139. Wynberg has used stable dioxetanes as "thermochemiluminescent" labels for immunoassays (J. C.

Hummelen, T. M. Luider, and H. Wynberg, Methods in Enzymology, 133B, 531 (1986)). These dioxetanes are used to label biological materials such as proteins. Assays are subsequently conducted by heating the sample at 100 to 250°C and detecting the thermally generated chemiluminescence. This technique is distinctly different from the use of triggerable dioxetanes as enzyme substrates.

Luminol derivatives, acridinium esters and lucigenin have been employed as chemiluminescent labels for antigens, antibodies, and haptens (H. R. Schroeder and F. M. Yeager, Anal. Chem., 50, 1114 (1978); H. Arakawa, M. Maeda, and A. Tsuji, Anal. Biochem., 79, 248 (1979); and H. Arakawa, M. Maeda, and A. Tsuji, Clin. Chem., 31, 430 (1985). For reviews, see: L. J. Kricka and T. J. N. Carter, In Clinical and Biochemical Luminescence, L. J. Kricka and T. J. N. Carter (Eds.), Marcel Dekker, Inc., New York, 1982, Ch. 8; L. J. Kricka, Ligand-Binder Assays, Marcel Dekker, Inc., New York, 1985, Ch. 7; F. McCapra and I. Beheshti, In Bioluminescence and Chemiluminescence: Instruments and Applications, Vol. I, K. Van Dyke (Ed.), CRC Press, Inc., Boca Raton, FL, 1985, Ch. 2, Note, in particular, the section on dioxetanes, p. 13; and G. J. R. Barnard, J. B. Kim, J. L. Williams, and W. P. Collins, Ibid, Ch. 7). Assay systems involving the use of enzyme-labeled antigens, antibodies, and haptens have been termed enzyme immunoassays. The enzyme labels have been detected by color or fluorescence development techniques. More recently, luminescent enzyme immunoassays have been based on peroxidase conjugates assayed with luminol/hydrogen peroxide, pyrogallol/hydrogen peroxide, Pholas dactylus luciferin, or luminol under alkaline conditions (L. J. Kricka and T. J. N. Carter, In Clinical and Biochemical Luminescence, L. J. Kricka and T. J. N. Carter (Eds.), Marcel Dekker, Inc., New York, 1982, Ch. 8). No enzyme-linked assays have described dioxetanes as

enzymatic substrates to generate light for detection prior to my application Serial No. 887,139.

10. Photographic Detection of Luminescent Reactions. Instant photographic film and x-ray film have
5 been used to record light emission from several chemiluminescent and bioluminescent reactions (L. J. Kricka and G. H. G. Thorpe, Methods in Enzymology, 133, 404 (1986) and references therein. See also: M. M. L. Leong, C. Milstein, and R. Pannel, J. Histochem. Cytochem., 34, 1645
10 (1986); R. A. Bruce, G. H. G. Thorpe, J. E. C. Gibbons, P. R. Killeen, G. Ogden, L. J. Kricka, and T. P. Whitehead, Analyst, 110, 657 (1985); J. A. Matthews, A. Batki, C. Hynds, and L. J. Kricka, Anal. Biochem., 151, 205 (1985);
15 and G. H. G. Thorpe, T. P. Whitehead, R. Penn, and L. J. Kricka, Clin. Chem., 30, 806 (1984). No examples appear in the literature on the photographic detection of chemiluminescence derived from chemical or enzymatic triggering of stabilized dioxetanes prior to my application
Serial No. 887,139.

20 OBJECTS

It is therefore an object of the present invention to provide a method and compositions for enhancing the chemiluminescence of triggerable
1,2-dioxetanes. Further, it is an object of the present
25 invention to provide a method and compositions which can be used in immunoassays and with enzyme-linked DNA probes. These and other objects will become increasingly apparent by reference to the following description and the drawings.

IN THE DRAWINGS

30 Figure 1 shows Arrhenius plot for the thermal decomposition of phosphate-substituted dioxetane 2c in xylene.

Figure 2 shows a plot of chemiluminescence quantum yield for base-triggered reaction of dioxetane 2b
35 as a function of the concentration of cetyltrimethylammonium bromide (CTAB). Figure 2a shows an

idealized structure of a dioxetane with a fluorescent co-surfactant and CTAB surfactant.

Figure 3 shows a plot of chemiluminescence intensity vs. time for 10^{-5} M dioxetane 2c with 100
5 microliters of human blood serum containing alkaline phosphatase in 2-amino-2-methyl-1-propanol (221) buffer (pH 10.3) at 37°C.

Figure 4 shows a plot of log (light intensity at plateau) vs. log (microliters of serum, 1 - 100) for
10 dioxetane 2c.

Figure 5 shows a plot of log (light intensity at 50 sec) vs. log (microliters of serum, 1 - 100) for dioxetane 2c.

Figure 6 shows chemiluminescence spectra: (Curve
15 A) chemiluminescence from enzymatic triggering of dioxetane 2c in 221 buffer in the absence of CTAB and fluorescent co-surfactant 3; (Curve B) energy-transfer chemiluminescence from enzymatic triggering of dioxetane 2c in the presence of CTAB and 3.

Figure 7 shows a plot of light intensity vs. time
20 for dioxetane 2c in 3 mL of 221 buffer with CTAB/fluorescer and 2.7×10^{-15} moles of alkaline phosphatase (Experiment D). Reagent background in the absence of enzyme is equal to intensity at time zero.

Figure 8 shows plot of log (integrated light
25 intensity) for time period of zero to 3 minutes vs. log (moles of alkaline phosphatase).

Figure 9 shows a plot of light intensity vs. time
30 for dioxetane 2c in 200 microliters of 221 buffer with CTAB/fluorescer and 2.3×10^{-17} moles of alkaline phosphatase. Reagent background in the absence of enzyme is equal to the intensity at time zero.

Figure 10 shows a plot of log (integrated light
35 intensity) for time period of zero to 30 minutes vs. log (moles of alkaline phosphatase).

Figure 11 is a photographic detection of chemiluminescence from dioxetane 2c using ASA 3000 Polaroid

Type 57 film. Solutions of 221 buffer (100 microliters) containing alkaline phosphatase, dioxetane, $\text{Mg}(\text{OAc})_2$, CTAB, and fluorescein surfactant 3 were incubated in Dynatech Immulon™ wells for 1 hour at 37°C and then photographed at that temperature for 15 minutes. Quantities of alkaline phosphatase: A, 2700 attomol; B, 250 attomol; C, 23 attomol; and D, reagent control with no enzyme (not visible).

Figure 12 is a photographic detection of chemiluminescence from dioxetane 2c using ASA 3000 Polaroid Type 57 film. Solutions of 221 buffer (100 microliters) containing alkaline phosphatase, dioxetane, $\text{Mg}(\text{OAc})_2$, CTAB, and fluorescein surfactant 3 were incubated in Dynatech Immulon™ wells for 1 hour at 37°C and then photographed at that temperature for 30 minutes. Quantities of alkaline phosphatase: A, 250 attomol; B, 23 attomol; C, 2 attomol; and D, reagent control with no enzyme (not visible).

Figure 13 shows a plot of light intensity vs. time for dioxetane 2c in 100 microliters of 221 buffer with CTAB/fluorescer and 1.3 ng S-antigen and antibody-alkaline phosphatase conjugate. Reagent background in the absence of enzyme is equal to the intensity at time zero.

Figure 14 shows a plot of log (integrated light intensity) for time period of zero to 15 minutes vs. log (ng of S-antigen coated on the microwell).

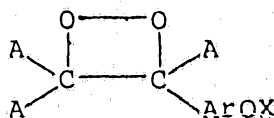
Figure 15 is a chemiluminescent assay for S-antigen using dioxetane 2c, $\text{Mg}(\text{OAc})_2$, CTAB, and fluorescein surfactant 3 in 221 buffer (100 microliters). Following the luminometer experiments the 7 Immulon™ wells were incubated for 30 minutes at 37°C and then photographed at that temperature for 15 minutes with ASA 3000 Polaroid Type 57 film. Quantities of S-antigen from lower left to second from upper right: 112, 56, 28, 14, 7, 3.5, and 1.3 ng with well containing only reagents in upper right (not visible).

Figure 16 is a chemiluminescent assay for S-antigen. Four wells were coated with 50 ng of S-antigen,

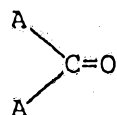
reacted with MAbA9-C6 monoclonal antibody, reacted with antimouse IgG-alkaline phosphatase conjugate, and then assayed with dioxetane 2c, $Mg(OAc)_2$, CTAB, and fluorescein surfactant 3 in 221 buffer (100 microliters). The wells were incubated for 1 hour at 45°C and then photographed at that temperature for 30 seconds with ASA 3000 Polaroid Type 57 film. A control well in the center (not visible) contained only the dioxetane in the CTAB/fluorescein buffer.

GENERAL DESCRIPTION

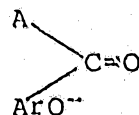
The present invention relates to a method for generating light which comprises providing a fluorescent compound in closely spaced relationship with a stable 1,2-dioxetane compound of the formula



wherein ArOX is an aryl group having an aryl ring substituted with an X-oxy group which forms an unstable oxide intermediate 1,2-dioxetane compound when trigger to remove X by an activating agent so that the unstable 1,2-dioxetane compound decomposes and releases electronic energy to form light and two carbonyl containing compounds of the formula

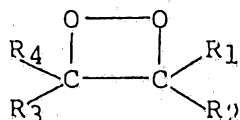


and

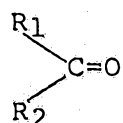


wherein X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate 1,2-dioxetane and wherein A are passive organic groups which allow the light to be produced and decomposing the stable 1,2-dioxetane with the activating agent wherein the fluorescent compound accepts the electronic energy generated upon decomposition of the unstable oxide intermediate and produces a more intense light than is produced by the triggering of the dioxetane alone.

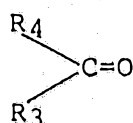
In particular the present invention relates to a method for generating light which comprises providing a fluorescent compound in closely spaced relationship with a stable 1,2-dioxetane compound of the formula



wherein R₁ and R₂ together and R₃ and R₄ together can be joined as spirofused alkylene groups which can contain hetero atoms (N, S, O or P) and aryl rings, wherein at least one of R₁ and R₂ or R₃ and R₄ is an aryl group, having an aryl ring substituted with an X-oxy- group which forms an unstable oxide intermediate 1,2-dioxetane compound when triggered to remove X by an activating agent selected from acids, bases, salts, enzymes, inorganic and organic catalysts and electron donors so that the unstable 1,2-dioxetane compound decomposes and releases electronic energy to form light and two carbonyl containing compounds of the formula:



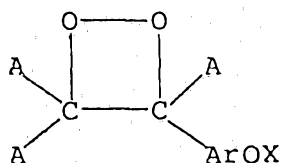
and



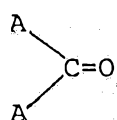
wherein those of R₁, R₂, R₃ or R₄ which are unsubstituted by an X-oxy group are carbon or hetero atom containing organic groups which provide stability for the stable 1,2-dioxetane compound and wherein X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate; and decomposing the stable 1,2-dioxetane with the activating agent wherein the fluorescent compound accepts the electronic energy generated upon decomposition of the unstable oxide intermediate and produces a more intense light than is produced by the triggering of the dioxetane alone.

The present invention also relates to compositions which generate light upon triggering which comprises a fluorescent compound and a stable 1,2-dioxetane of the formula

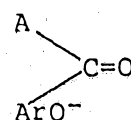




wherein ArOX represents an aryl group substituted with an X-oxy group which forms an unstable oxide intermediate 1,2-dioxetane compound when triggered to remove X by an activating agent so that the unstable 1,2-dioxetane compound decomposes and releases electronic energy to form light and two carbonyl containing compounds of the formula



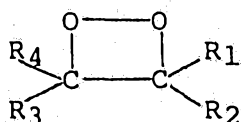
and



wherein X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate 1,2-dioxetane and wherein A are passive organic groups which allow the light to be produced wherein the stable 1,2-dioxetane is decomposed with the activating agent and wherein the fluorescent compound accepts the electronic energy generated upon decomposition of the unstable oxide intermediate and produces a more intense light than is produced by the triggering of the dioxetane alone.

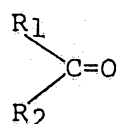
The compositions use the same preferred dioxetanes as the method.

Further the present invention relates to a method for generating light which comprises providing a fluorescent compound in closely spaced relationship with a stable dioxetane compound of the formula:

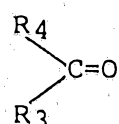


wherein R₁ is selected from alkyl, alkoxy, aryloxy, dialkyl or aryl amino, trialkyl or aryl silyloxy and aryl groups including spirofused aryl groups with R₂, wherein R₂ is an aryl group which can include R₁ and is substituted with an

X-oxy group which forms an unstable oxide intermediate 1,2-dioxetane compound when activated by an activating agent to remove X selected from acids, bases, salts, enzymes, inorganic and organic catalysts and electron donors so that the unstable 1,2-dioxetane compound decomposes and releases electronic energy to form light and two carbonyl containing compounds of the formula:



and

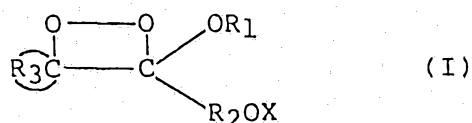


wherein X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate and wherein R₃ and R₄ are selected from aryl, heteroalkyl and alkyl groups which can be joined together as spirofused polycyclic alkyl and polycyclic aryl groups and decomposing the stable 1,2-dioxetane with the activating agent wherein the fluorescent compound accepts the electronic energy generated upon decomposition of the unstable oxide intermediate and produces a more intense light than is produced by the triggering of the dioxetane alone.

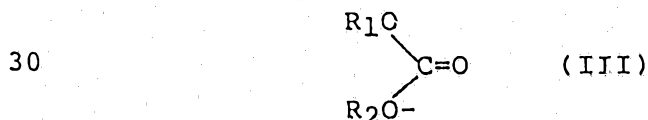
Any fluorescent compound which has a lower energy for its singlet excited states compared to the excited state of the dioxetane product can be used to enhance the chemiluminescence efficiency. A group such as a long hydrocarbon chain (preferably 8 to 20 carbon atoms) is preferably attached to the fluorescer so that it acts as a co-surfactant in order to incorporate the material into the organized assembly. Examples of fluorescers include: any fluorescent dye; aromatic compounds including naphthalenes, anthracenes, pyrenes, biphenyls; acridine; coumarins; xanthenes; phthalocyanines; stilbenes; furans; oxazoles; oxadiazoles; and benzothiazoles. Most preferably a surfactant which forms micelles with the fluorescent compound is used so that the 1,2-dioxetane is adjacent to the fluorescent compound. Possible surfactants are described in Chapter 1, pages 1 to 18 of Catalysis

in Micellar and Macromolecular Systems published by Academic Press, (1975). These include: zwitterion; cationic (ammonium, pyridinium, phosphonium, sulfonium salts); anionic (sulfate, sulfonate, carboxylate salts); neutral (polyoxyethylene derivatives, cyclodextrins, long chain esters, long chain amides); and naturally occurring surfactants (lipids).

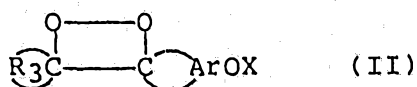
Specifically the present invention relates to a method and compositions which use a stable 1,2-dioxetane compound of the formula:



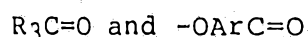
wherein R_1 is selected from lower alkyl containing 1 to 8 carbon atoms, R_2 is selected from aryl, biaryl and fused ring polycyclic aryl groups which can be substituted or unsubstituted, and $\text{R}_3\text{C}-$ is selected from polycyclic alkyl groups containing 6 to 30 carbon atoms, wherein OX is an oxy group substituted on an aryl ring which forms an unstable oxide intermediate 1,2-dioxetane compound when triggered to remove X by an activating agent selected from acid, base, salt, enzyme, inorganic and organic catalysts and electron donor sources and X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate and wherein (I) decomposes in the presence of an activating agent to produce light and carbonyl containing compounds of the formula



Also the present invention relates to a method and compositions which uses a stable, 1,2-dioxetane compound of the formula:

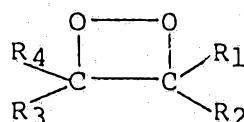


wherein ArOX is a spirofused aryl group containing a ring substituted X-oxy group, wherein ArOX forms an unstable oxide intermediate 1,2-dioxetane compound when triggered by an activating agent to remove X selected from acids, bases, salts, enzymes, inorganic and organic catalysts and electron donors, wherein X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate 1,2-dioxetane so that the unstable 1,2-dioxetane compound decomposes to form light and two carbonyl containing derivatives of the formula



and wherein R_3C- is selected from polycyclic alkyl groups containing 6 to 30 carbon atoms. In this structure R_1 and R_2 are joined together.

In reference to the structure:



(1) When R_1 is not combined with R_2 the group is preferably alkyl, alkoxy, dialkyl or arylamino trialkyl or aryl silyloxy. The alkyl groups preferably contain 1 to 8 carbon atoms. R_1 can also be cyclic aliphatic or aryl groups, including fused ring aryl compounds, containing 6 to 14 carbon atoms. When R_1 is combined with R_2 they provide an aryl group containing 6 to 30 carbon atoms.

(2) R_2 is an aryl group substituted with an X oxy (OX) group. The aryl containing group can be phenyl, biphenyl, fused phenyl and other aryl groups and can contain between 6 and 30 carbon atoms and can include other substituents. X is any labile group which is removed by an activating agent. The OX group can be for instance selected from hydroxyl, alkyl or aryl carboxyl ester, inorganic oxy acid salt, particularly a phosphate or sulfate, alkyl or aryl silyloxy and oxygen pyranoside groups.

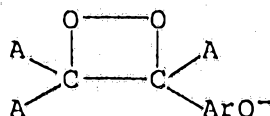
(3) R_3 and R_4 can be the same as R_1 . In the following Examples, R_3 and R_4 are combined together to form

a polycyclic alkylene group, particularly for ease of synthesis and comparison; however any organic group can be used. Preferably the polycyclic alkylene group contains 6 to 30 carbon atoms.

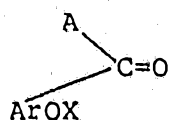
5 The stable 1,2-dioxetane compounds have relatively long $1/2$ lives at room temperatures ($20-35^{\circ}\text{C}$) even though they can be triggered by the activating agent. All of the prior art compounds are either unstable at room temperatures or require temperatures of 50°C or above in
10 order to be thermally decomposed which is impractical for most applications.

 The activating agent may be chemical or enzymatic. In some cases (F^-) 1 equivalent is required and in others (enzymatic) only a very small amount is used. The agents
15 are described in any standard chemical treatise on the subject and include acids, bases, salts, enzymes and other inorganic, organic catalysts. The agent used will depend upon the conditions under which the stable 1,2-dioxetane is to be activated and how labile the X group is on a
20 particular 1,2-dioxetane. Electron donors can be used to remove X which can include reducing agents as well as electrical sources of electrons.

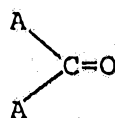
 The 1,2-dioxetane decomposes to form carbonyl containing compounds and light. An unstable 1,2-dioxetane
25 intermediate is formed of the formula:



 In general an $-\text{ArOX}$ substituted 1,2-dioxetanes
30 are formed by addition of oxygen to the appropriate alkene. These alkenes are synthesized through alkyl and/or aryl substituted carbonyl containing compounds of the formula:



and



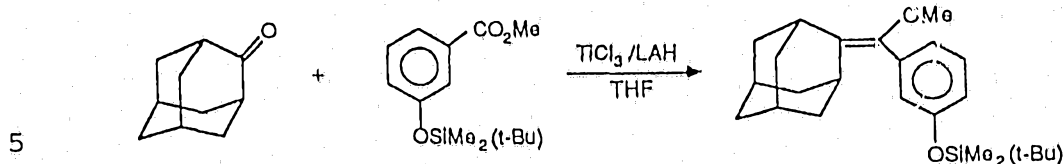
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These materials are reacted in the presence of lithium aluminum hydride or other metal hydride in a polar organic

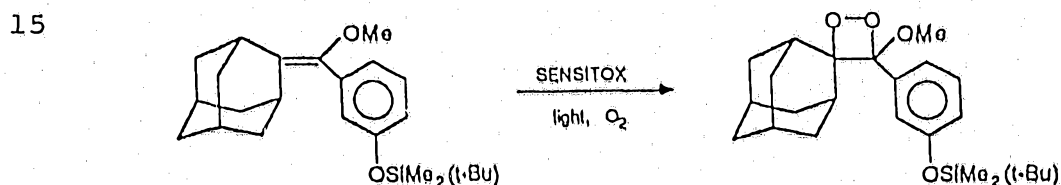
solvent, particularly tetrahydrofuran, with a transition metal halide salt, particularly titanium chloride, and a tertiary amine base. The reaction is generally conducted in refluxing tetrahydrofuran and usually goes to completion in about 4 to 24 hours.

Preparation of and Chemical Triggering of Stabilized 1,2-Dioxetanes. It was discovered that thermally stable dioxetanes can be triggered by chemical and enzymatic processes to generate chemiluminescence on demand (A. P. Schaap, patent application Serial No. 887,139 filed July 17, 1986, A. P. Schaap, R. S. Handley, and B. P. Giri, Tetrahedron Lett., 935 (1987); A. P. Schaap, T.S. Chen, R. S. Handley, R. DeSilva, and B. P. Giri, Tetrahedron Lett., 1155 (1987); and A. P. Schaap, M. D. Sandison, and R. S. Handley, Tetrahedron Lett., 1159 (1987)). To do this, new synthetic procedures were developed to produce dioxetanes with several key features: (1) the stabilizing influence of spirofused adamantyl groups has been utilized to provide dioxetanes that have "shelf lives" of years at ambient temperature; (2) a moiety has been incorporated in the structure so that direct chemiluminescence from the carbonyl cleavage product is obtained; and (3) new methods for triggering the chemiluminescent decomposition of the stabilized dioxetanes were provided.

The required alkenes have been prepared by reaction of 2-adamantanone with aromatic esters or ketones using titanium trichloride/LAH in THF (A. P. Schaap, patent application Serial No. 887,139, filed July 17, 1986). This is the first report of the intermolecular condensation of ketones and esters to form vinyl ethers using the McMurry procedure. Although McMurry had earlier investigated the intramolecular reaction of ketone and ester functional groups, cyclic ketones and not vinyl ethers were prepared by this method (J. E. McMurry and D. D. Miller, J. Amer. Chem. Soc., 105, 1660 (1983)).

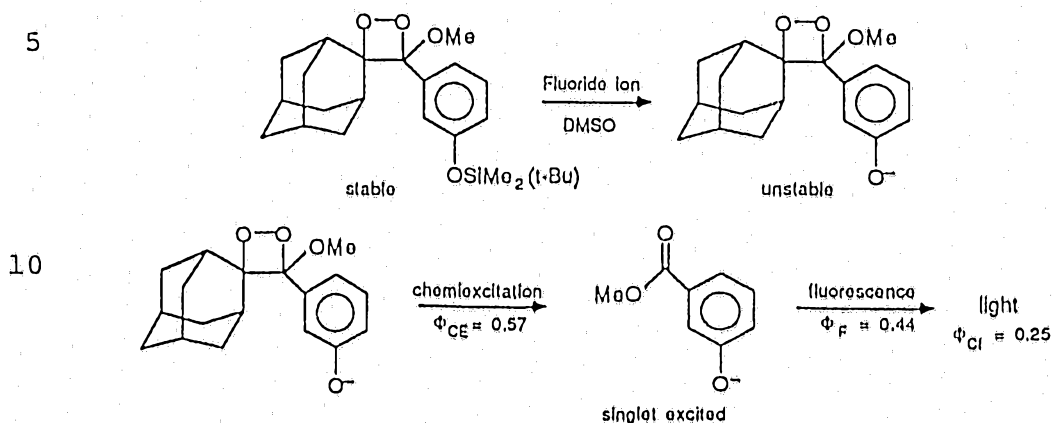


Photooxygenation of these vinyl ethers affords dioxetanes that are easily handled compounds with the desired thermal stability. For example, the dioxetane shown below exhibits an activation energy of 28.4 kcal/mol and a half-life at 25°C of 3.8 years. Samples of this dioxetane in o-xylene have remained on the laboratory bench for several months with no detectable decomposition.



20 However, the chemiluminescent decomposition of this dioxetane can be conveniently triggered at room temperature by removal of the silyl-protecting with fluoride ion to generate the unstable, aryloxy form which cleaves to yield intense blue light. The half-life of the aryloxy-substituted dioxetane is 5 seconds at 25°C. The spectrum of the chemiluminescence in DMSO exhibited a maximum at 470 nm which is identical to the fluorescence of the anion of the ester cleavage product (methyl 3-hydroxybenzoate) and the fluorescence of the spent dioxetane solution under these conditions. No chemiluminescence derived from adamantane fluorescence appears to be produced. Chemiluminescence quantum yields for the fluoride-triggered decomposition measured relative to the luminol standard was determined to be 0.25 (or a chemiluminescence efficiency of 25%). Correction for the fluorescence quantum yield of the ester under these conditions ($\Phi_F=0.44$) gave an efficiency for the formation

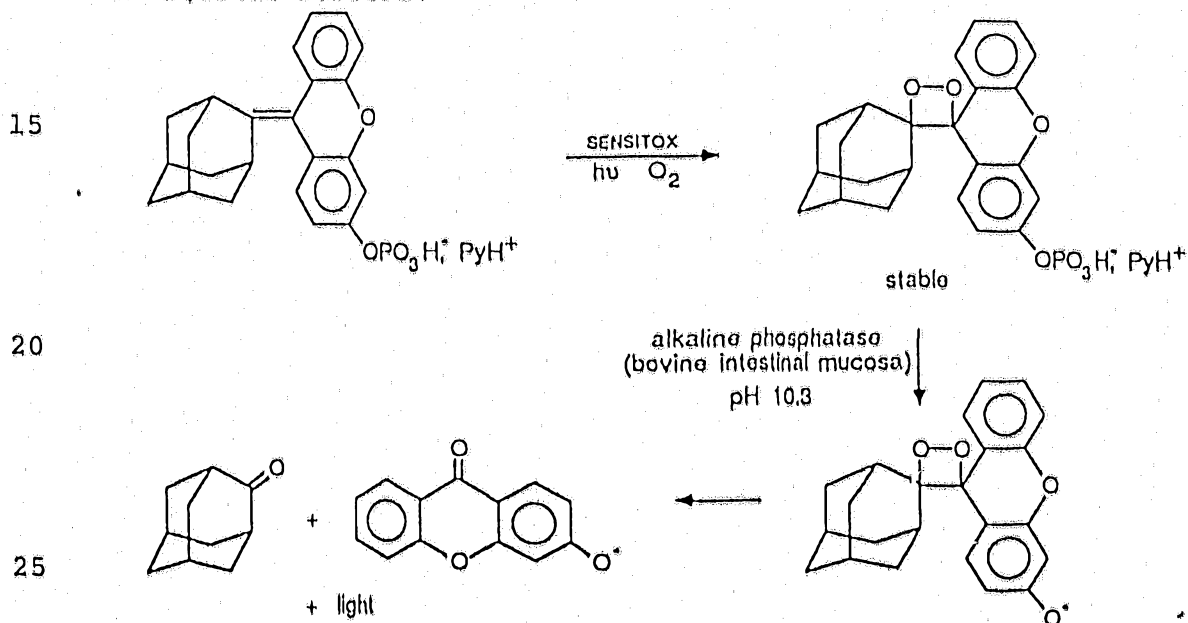
of the singlet excited ester of 57%, the highest singlet chemiexcitation efficiency yet reported for a dioxetane prepared in the laboratory.



15 Enzymatic Triggering of 1,2-Dioxetanes. Biologi-
cal assays such as immunoassays and nucleic acid probes
involving enzymes utilize a wide variety of substrates
which either form a color (chromogenic) or become fluore-
scent (fluorogenic) upon reaction with the enzyme. Appli-
cation Serial No. 887,139 describes the first dioxetanes
20 which can function as chemiluminescent enzyme substrates
(A. P. Schaap, patent application filed July 17, 1986; A.
P. Schaap, R. S. Handley, and B. P. Giri, Tetrahedron Lett.,
935 (1987); A. P. Schaap, T. S. Chen, R. S. Handley, R.
25 DeSilva, and B. P. Giri, Tetrahedron Lett., 1155 (1987);
and A. P. Schaap, M. D. Sandison, and R. S. Handley,
Tetrahedron Lett., 1159 (1987)). Use of these peroxides in
biological systems requires dioxetanes which are thermally
stable at the temperature of the enzymatic reaction and don't
30 undergo rapid spontaneous decomposition in the aqueous
buffers. The spirofused adamantyl dioxetanes described in
the previous paragraph meet these requirements.
1,2-dioxetanes were prepared bearing functional groups
which can be enzymatically modified to generate the
35 aryloxide form. Decomposition of this unstable
intermediate provides the luminescence. 1,2-dioxetanes
were synthesized which can be triggered by various enzymes

including aryl esterase, acetylcholinesterase, and alkaline phosphatase. The phosphatase example is particularly significant because this enzyme is used extensively in enzyme-linked immunoassays and nucleic acid probes.

For example, enzymatic triggering by alkaline phosphatase was observed with the phosphate-substituted 1,2-dioxetane derived from 3-hydroxy-9H-xanthen-9-one and 2-adamantanone. The dioxetane is thermally stable with an activation energy of 30.7 kcal/mol and a half-life at 25°C of 12 years. The dioxetane is not only stable in organic solvents but also shows very slow spontaneous decomposition in aqueous buffers.

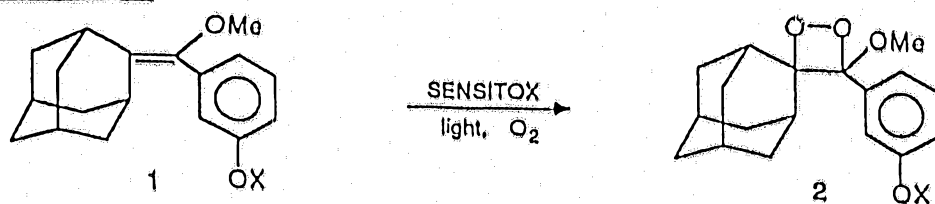


Triggering experiments were conducted using alkaline phosphatase from bovine intestinal mucosa [suspension of 5.3 mg of protein (1100 units/mg protein) per mL in 3.2 M (NH₄)₂SO₄] and the phosphate-protected dioxetane at pH 10.3 in 0.75 M 2-amino-2-methyl-1-propanol buffer. A 50 µL aliquot (0.013 µmol) of a phosphate-dioxetane stock solution was added to 3 mL of the buffer at 37°C to give a final dioxetane concentration of 4.2 x 10⁻⁶ M. Injection of 1 µL (final conc of protein = 1.8 µg/mL) of alkaline phosphatase to the solution resulted in burst of chemiluminescence that decayed over a period of

3 min. Over this period of time, the background luminescence from slow non-enzymatic hydrolysis of the dioxetane in the buffer was only 0.2% of that produced by the enzymatic process. The total light emission was found to be linearly dependent on the dioxetane concentration. The rate of decay of the emission is a function of enzyme concentration while the total light emission is independent of the enzyme concentration because of turnover of the enzyme. The chemiluminescence spectrum for the phosphatase-catalyzed decomposition was obtained at room temperature in the buffer solution. A comparison of this chemiluminescence spectrum with the fluorescence spectrum of the spent reaction mixture and the fluorescence spectrum of the hydroxyxanthanone cleavage product in the buffer indicates that the emission is initiated by the enzymatic cleavage of the phosphate group in dioxetane to yield the unstable aryloxide dioxetane which generates the singlet excited anion of hydroxyxanthanone.

SPECIFIC DESCRIPTION

Synthesis of 1,2-Dioxetane Compounds and Fluorescent Surfactants



(a) X = H

(b) X = Ac

(c) X = PO₃Na₂

Instrumentation

Nuclear magnetic resonance (NMR) spectra were obtained on either a Nicolet NT300™ or a General Electric QE300™ spectrometer as solutions in CDCl₃ with tetramethylsilane as internal standard unless noted otherwise. Infrared (IR) spectra were obtained on either a Nicolet™ or a Beckman Acculab 8™ spectrometer. Mass spectra were obtained on either a Kratos™ or an AEI MS-90™.

spectrometer. Ultraviolet and visible absorption spectra were obtained on a Varian Cary 219[™] spectrophotometer. Fluorescence spectra were recorded on a Spex Fluorolog[™] spectrophotofluorometer. Chemiluminescence spectra were measured using the Spex Fluorometer. Chemiluminescence kinetic and quantum yield measurements were made with luminometers constructed in this laboratory. The instruments which use RCA A-31034A gallium-arsenide photomultiplier tubes cooled to -78°C and Ortec photon-counting electronics are interfaced to Apple IIe[™] and Macintosh[™] computers. Elemental analyses were performed by Midwest Microlabs, Indianapolis. Melting points were measured in a Thomas Hoover[™] capillary melting apparatus and are uncorrected. Precision weights were obtained on a Cahn model 4700/[™] electrobalance.

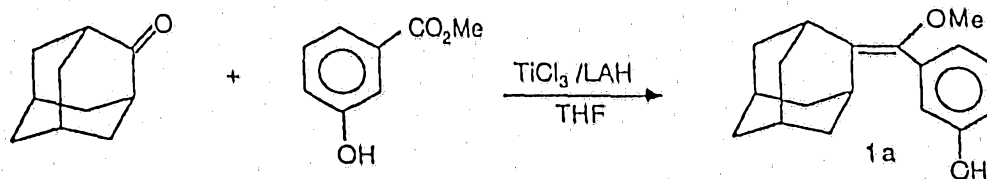
Materials

o-Xylene was obtained from Burdick and Jackson Laboratories and used as received for kinetic and spectroscopic measurements. Dry DMF and DMSO were obtained by vacuum distillation from calcium hydride. Deuterium oxide, 1,4-dioxane-d₈, chloroform-d, fluorescein amine (isomer 1), and other chemical reagents were purchased from Aldrich Chemical Co. Samples of alkaline phosphatase were purchased from Sigma Chemical Co. Silica, alumina and the other solid supports were obtained from various commercial sources and used without further purification.

Syntheses of Alkenes

[(3-Hydroxyphenyl)methoxymethyleneladamantane (1a)]. A 500-mL flask was fitted with a reflux condenser, a 125-mL addition funnel, and nitrogen line. The apparatus was dried by means of a hot air gun and nitrogen purging. Dry THF (40 mL) was added and the flask cooled in an ice bath. TiCl₃ (1.5 g, 10 mmol) was added rapidly followed by LAH (0.19 g, 5 mmol) in portions with stirring. The cooling bath was removed and the black mixture was allowed to warm to room temperature. Triethylamine (0.7 mL, 5 mmol) was added to the stirred suspension and refluxed for

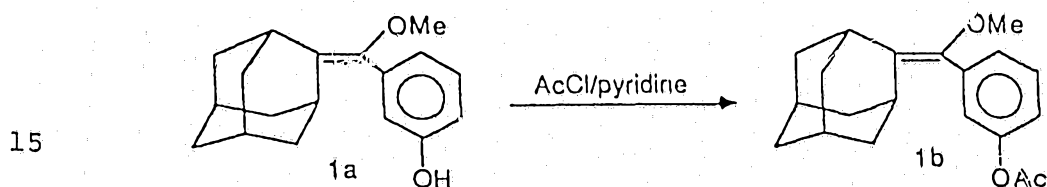
15 minutes. After this period, a solution of methyl 3-hydroxybenzoate (152 mg, 1 mmol) and 2-adamantanone (300 mg, 2 mmol) in 20 mL of dry THF was added dropwise to the refluxing mixture over 15 minutes. Refluxing was continued for an additional 15 minutes after which the reaction was cooled to room temperature and diluted with 100 mL of distilled water. The aqueous solution was extracted with 3 x 50 mL portions of ethyl acetate. The combined organic layer was washed with water, dried over MgSO_4 , and concentrated. Chromatography over silica with 15% ethyl acetate/hexane gave 240 mg (89%) of 1a as a white solid: mp 133 - 4°C; ^1H NMR (CDCl_3) δ 1.64 - 1.96 (m, 12H), 2.65 (s, 1H), 3.24 (s, 1H), 3.32 (s, 3H), 5.25 (s, 1H, OH exchange with D_2O), 6.70 - 7.30 (m, 4H), ^{13}C NMR (CDCl_3) δ 28.45, 30.36, 32.36, 37.30, 39.18, 39.33, 57.82, 114.60, 116.16, 122.19, 129.24, 137.24, 155.62; MS m/e (rel intensity) 271 (20, M + 1), 270 (100, M), 253 (7.3), 213 (35.1), 121 (41.7), 93 (9.4); Exact mass: cal d 270.1619, found 270.1616.



[(3-Acetoxyphenyl)methoxymethylene]adamantane

(1b). Hydroxy alkene 1a (0.75g, 2.9 mmol) was dissolved in 10 mL of CH_2Cl_2 and pyridine (5.2 g, 65.8 mmol) under N_2 . The solution was cooled in an ice bath and a solution of acetyl chloride (2.6 g, 33 mmol) in 1 mL of CH_2Cl_2 was added dropwise via syringe. After 5 minutes at 0°C, TLC on silica with 20% ethyl acetate/hexane showed complete acetylation of 1a. After removal of the solvent, the solid residue was washed with 30 mL of ether. The ether was washed with 3 x 25 mL of water, dried over MgSO_4 , and evaporated to dryness. The product was chromatographed on

silica using 20% ethyl acetate/hexane affording 0.45g of 1b as an oil: ^1H NMR (CDCl_3) δ 1.79 - 1.96 (m, 12H), 2.27 (s, 3H), 2.66 (s, 1H), 3.26 (s, 1H), 3.29 (s, 3H), 6.99 - 7.36 (m, 4H); ^{13}C NMR (CDCl_3) δ 20.90, 28.13, 30.07, 31.99, 36.99, 38.89, 39.01, 57.59, 120.34, 122.14, 126.55, 128.66, 132.19, 136.90, 142.59, 150.42, 169.04; MS m/e (rel intensity) 312 (100, M), 270 (25), 255 (19.3), 213 (20.7), 163 (12.2), 121 (30.7), 43 (30); IR (neat) 3006, 2925, 2856, 1725, 1600, 1438, 1362, 1218, 1100 cm^{-1} ; Anal. Calcd. for $\text{C}_{20}\text{H}_{24}\text{O}_3$: C, 76.92; H, 7.69, Found: C, 76.96; H, 7.85.



[(3-Phosphatephenyl)methoxymethylene]adamantane, disodium salt (1c).

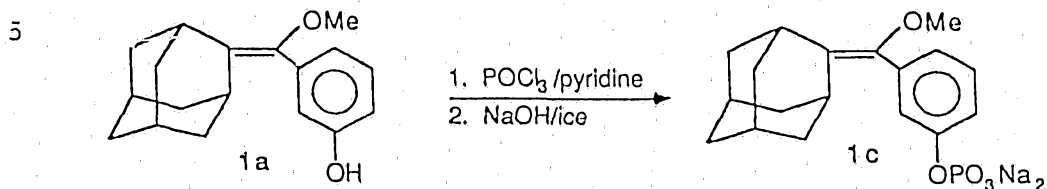
20 Hydroxy alkene 1a (500 mg, 1.58 mmol) was dissolved in 5 mL of dry pyridine (dried over basic alumina). This solution was slowly added to a cold mixture of 1 mL (10.7 mmol) of phosphoryl chloride and 5 mL of dry pyridine at such a rate that the temperature of the

25 reaction remained below 5°C . After 30 minutes the reaction was terminated and the phosphoryl dichloridate product was poured onto a mixture of 20 g of ice and 1mL of 10 N sodium hydroxide. The mixture was transferred to a separatory

30 funnel and washed with 5 x 30 mL portions of CH_2Cl_2 . The product precipitated from the aqueous fraction after overnight refrigeration. The solid material was washed with 3 x 10 mL portions of CH_2Cl_2 followed by 3 x 10 mL portions of cold water. The white solid was then dried under reduced pressure to give 400 mg (1.02 mmol, 64%) of

35 phosphorylated alkene 1c: ^1H NMR ($\text{D}_2\text{O}/p$ -dioxane- d_8) δ 1.67-1.83 (m, 12H), 2.5 (s, 1H), 3.04 (s, 1H), 3.19 (s, 3H), 6.7-7.2 (m, 4H); ^{13}C NMR (D_2O) δ 28.29, 30.44, 32.45,

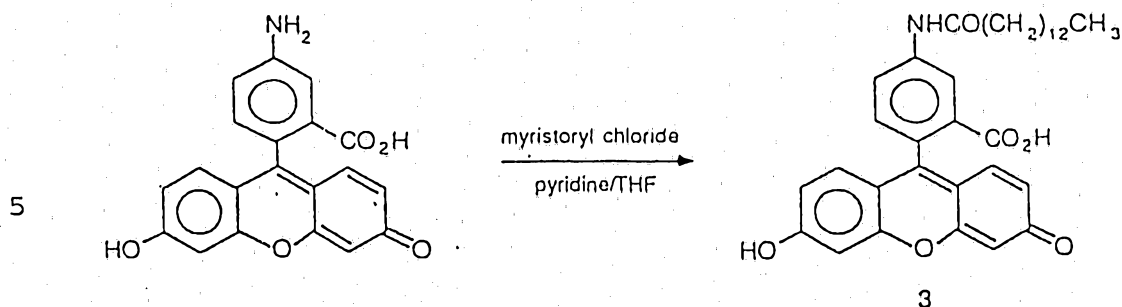
36.99, 38.89, 57.98, 120.16, 120.85, 123.74, 128.39, 133.15, 136.11, 142.68, 154.45; ^{31}P NMR ($\text{D}_2\text{O}/p$ -dioxane- d_8) δ 1.586.



10 Synthesis of fluorescein Surfactant:

5-(N-tetradecanoylamino)fluorescein (3)

A solution of myristoyl chloride (2.18 g, 8.83 mmol) in THF was added to a solution of fluoresceinamine-isomer 1 from Aldrich Chemical Co. (3.07 g, 8.85 mmol) in dry pyridine (dried over basic alumina) dropwise with stirring at room temperature over a 12 hour period. TLC on silica with 20% MeOH/benzene showed conversion to a less polar product. The reaction was poured into ice water and the solid precipitate isolated by filtration to give 4 g of solid orange material. Column chromatography with 10% MeOH/benzene afforded 500 mg of the pure product as an orange solid: mp 185-190°C; ^1H NMR (CDCl_3) δ 0.88 (t, 3H), 1.27 (m, 20H), 1.74 (m, 2H), 2.42 (t, 2H), 6.54-8.32 (m, 9H); ^{13}C NMR (CDCl_3) δ 13.05, 22.27, 25.34, 28.90, 29.01, 29.17, 29.31, 31.61, 36.65, 101.00, 110.25, 112.43, 114.93, 124.41, 126.58, 127.86, 128.77, 140.41, 147.00, 152.89, 160.28, 169.96, 173.65; MS (FAB) m/e (rel intensity) 558 (10, M+1), 402 (14.7), 388 (14.2), 374 (12.5), 348 (28.1), 302 (35.2), 213 (36.4), 165 (36.4), 133 (50). The properties of 3 are in agreement with those described for the commercially available material from Molecular Probes, Inc.



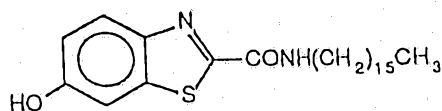
Synthesis of 1-Hexadecyl-6-hydroxy-2-benzothiazamide (4).

10 Methyl 6-hydroxy-2-benzothiazate (60 mg, 0.30 mmol) [see F. McCapra and Z. Razani, Chem. Commun., 153 (197,)] and 1-hexadecylamine (430 mg, 1.8 mmol) were dissolved in methanol. After refluxing the solution for two days, thin layer chromatography with 40% EtOAc/hexane showed conversion of the benzothiazate to a less polar material.

15 The methanol was evaporated and the residue was then chromatographed with 2% EtOAc/hexane to remove the excess 1-hexadecylamine. The chromatography was then continued with 50% EtOAc/hexane and afforded 4 as a white solid: 33 mg (27%); mp 82-4°C; ¹H NMR (acetone-d₆) δ 0.83 (t, 3H), 1.32 (br. 26H), 1.66 (m, 2H), 3.43 (t, 2H), 7.10-7.88 (m, 3H), 8.15 (br. 1H, OH exchanges with D₂O); ¹³C NMR (benzene-d₆) δ 14.29, 23.05, 27.08, 29.58, 29.66, 29.75, 29.86, 30.13, 32.28, 39.98, (acetone-d₆) δ 107.51, 117.69, 125.61, 139.33, 147.80, 157.63, 160.48, 162.12; MS (m/e (rel. intensity) 418 (M⁺, 33.7), 360 (14.1), 240 (61.1), 178 (77.3), 151 (39.1), 97 (28.6), 83 (34.7); Exact mass: calcd. 418.2653, found 418.2659 for C₂₄H₃₈N₂O₂S.

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Preparation of 1,2-Dioxetanes

Photooxygenation procedure. Typically a 5-10 mg sample of the alkene was dissolved in 5 mL of CH₂Cl₂ in the photooxygenation tube. Approximately 40 mg of polystyrene-bound Rose Bengal (Sensitox I) [reference to this type of sensitizer: A. P. Schaap, A. L. Thayer, E. C. Blossey, and D. C. Neckers, J. Amer. Chem. Soc., 97, 3741 (1975)] was added and an oxygen bubbler connected. Oxygen was passed slowly through the solution for 5 minutes and the apparatus immersed in a half-silvered Dewar flask containing dry ice/2-propanol. The sample was irradiated with either a 250 W or 1000 W sodium lamp (General Electric Lucalox) and a UV cutoff filter while oxygen was bubbled continuously. Progress of the reaction was monitored by TLC. A spot for the highly stable dioxetanes could usually be detected and had a R_f slightly less than that of the alkene. The adamantyl-substituted dioxetanes were filtered at room temperature, evaporated on a rotary evaporator, and recrystallized from a suitable solvent.

4-(3-Hydroxyphenyl)-4-methoxyspiro[1,2]-dioxetane-3,2'-adamantane](2a).

Hydroxy alkene 1a (100 mg) was irradiated with the 1000W Na lamp in 8 mL of CH₂Cl₂ at -78°C in the presence of Sensitox I. The alkene and dioxetane on TLC using 20% ethyl acetate/hexane exhibit the same R_f value. Therefore, the reaction was stopped when a trace of the

cleavage product began to appear. The sensitizer was removed by filtration and the solvent evaporated. ^1H NMR was used to check that all of the starting material had been oxidized. Dioxetane 2a was recrystallized from pentane/benzene to give a white solid: mp 135°C: ^1H NMR (CDCl₃) δ 1.04 - 2.10 (m, 12H), 2.21 (s, 1H), 3.04 (s, 1H), 3.24 (s, 3H), 6.48 (s, 1H, OH exchange with D₂O), 6.93 - 7.30 (m, 4H). ^{13}C NMR (CDCl₃) δ 25.81, 25.95, 31.47, 31.57, 32.27, 32.86, 33.07, 34.58, 36.30, 49.83, 95.88, 112.08, 116.46, 129.34, 136.1, 156.21.

4-(3-Acetoxyphenyl)-4-methoxyspiro[1,2-dioxetane-3,2'-adamantane](2b). Alkene 1b (140 mg, 0.45 mmol) was photooxygenated in 30 mL of CH₂Cl₂ at -78°C with the 1000 W high pressure sodium lamp using 400 mg of Sensitox I. TLC analysis on silica gel with 20% ethyl acetate/hexane showed clean conversion to a more polar material in 2.5 h. Filtration and removal of solvent produced 2b as an oil: ^1H NMR (CDCl₃) δ 0.90 - 1.90 (m, 12H), 2.15 (s, 1H), 2.31 (s, 3H), 3.03 (s, 1H), 3.23 (s, 3H), 6.61 - 7.45 (m, 4H), ^{13}C NMR (CDCl₃) δ 21.00, 25.82, 25.97, 31.50, 31.65, 32.21, 32.80, 33.09, 34.71, 36.32, 49.92, 95.34, 111.50, 122.58, 129.16, 136.42, 150.72, 169.11.

4-Methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2'-adamantane], disodium salt (2c). Alkene 1c (50mg) was photooxygenated in 2 mL of D₂O/p-dioxane-d₈ (1:1 v/v) at 10°C with the 1000 W high pressure sodium lamp using Sensitox I. ^1H NMR analysis showed clean conversion to dioxetane 2c in 45 minutes. The sensitizer was removed by filtration and the filtrate used as a stock solution for chemiluminescence experiments. ^1H NMR (D₂O/p-dioxane-d₈) δ 0.91-1.70 (m, 12H), 2.08 (s, 1H), 2.80 (s, 1H), 3.07 (s, 3H), 7.00-7.26 (m, 4H); ^{13}C NMR (D₂O/p-dioxane-d₈) δ 28.95, 30.95, 32.98, 37.65, 39.53, 58.31, 120.62, 121.64, 123.55, 129.31, 132.45, 136.57, 143.98, 155.30.

Chemiluminescence Kinetics Procedures

Rates for thermal decomposition of the stable dioxetanes were monitored by the decay of chemiluminescence of aerated solutions. A cylindrical Pyrex vial equipped with magnetic stir bar was filled with 3-4 mL of the reaction solvent, sealed with a Teflon-lined screw cap and placed in the thermostatted sample block of the chemiluminescence-measuring luminometer. Temperature control was provided by an external circulating water bath. Appropriate values for the instrument gain and optical slit size were selected. When thermal equilibrium was reached (ca. 3 minutes), an aliquot of the dioxetane stock solution sufficient to achieve a final concentration not greater than 10^{-4} M was added via pipette by opening the top of the luminometer or via syringe through a light-tight rubber septum located in the cover directly above the vial. The vial was sealed with a Teflon-lined screw cap to prevent evaporation when high temperatures were used. Measurement of the signal was begun by opening the shutter. The chemiluminescent decay was generally recorded for at least three half-lives. Calculation of the first-order rate constant (k) from the $\ln$ (Intensity) vs. time data was performed by a computer program utilizing a standard least-squares treatment. The correlation coefficient (r) was typically at least 0.999 and k varied less than 5% between replicate samples.

Activation parameters for decomposition of the dioxetanes were calculated from plots of $\ln k$ vs. $1/T$ (Arrhenius eq.) or $\ln k/T$ vs. $1/T$ (Eyring eq.) by a standard least-squares linear regression analysis. The results of replicate runs at 5 to 10 temperatures encompassing a temperature range of 80 to 120°C were found to yield a straight line with a correlation coefficient of 0.99 or better. For example, Figure 1 shows an Arrhenius plot for the thermal decomposition of phosphate-substituted dioxetane 2c in o-xylene with $E_a = 32.5$ kcal/mol and $r =$

0.999. The half-life for 2c at 25°C is calculated from the Arrhenius equation to be 19 years.

Activation Energies for Thermal Decomposition of Dioxetanes in Xylene.

5	Dioxetane (X)	E_a	Log A	$k(\text{sec}^{-1})$ at 25°C	$t_{1/2}$ at 25°C
	2a (OH)	28.3	12.5	5.38×10^{-9}	4.1 yrs
	2b (OAc)	30.4	13.6	1.73×10^{-9}	13 yrs
	2c (PO ₃ Na ₂)	32.5	14.9	1.19×10^{-9}	19 yrs

10 The above results demonstrate the extremely high stability (long half-life) that these types of dioxetanes exhibit before triggering with the appropriate chemical agent or enzyme.

Determination of Chemiluminescence Quantum Yields

15 The chemiluminescence quantum yield (Φ_{CL}) for the decomposition of dioxetanes is defined as the ratio of einsteins of chemiluminescence emitted to moles of dioxetane decomposed. Sufficient energy is released during the reaction from the reaction enthalpy (ΔH_R) plus the Arrhenius activation energy (E_a) to populate the singlet
20 excited state of one of the carbonyl cleavage products. Therefore, the maximum quantum yield is 1.0. Another parameter of interest is the chemiexcitation quantum yield (Φ_{CE}) which is defined as the ratio of excited states formed to dioxetane decomposed. The chemiexcitation
25 quantum yield is related to the chemiluminescence quantum yield via the fluorescence quantum yield of the dioxetane cleavage (Φ_F) through the equation: $\Phi_{CL} = \Phi_{CE} \times \Phi_F$.

30 The same procedure as those employed in the measurement of the decay kinetics was used for the determination of chemiluminescence quantum yields with the following modifications. An accurately measured aliquot of a dioxetane stock solution of known concentration was added to 3 mL of the pre-thermostatted organic solvent or aqueous
35 buffer. The reaction was then triggered by adding the appropriate chemical reagent or enzyme. The total light intensity was integrated by a photon-counting luminometer using an RCA A-31034A gallium-arsenide PMT cooled to -78°C.

Light intensity was converted to photons by reference to a calibration factor based on the accurately known quantum yield of the chemiluminescent reaction of luminol with base in aerated DMSO. The luminol reaction has been determined to have a chemiluminescence quantum yield of 0.011 (1.1%) (J. Lee and H. H. Seliger, Photochem. Photobiol., 15, 227 (1972); P. R. Michael and L. R. Faulkner, Anal. Chem., 48, 1188 (1976)).

Acquisition of Chemiluminescence Spectra

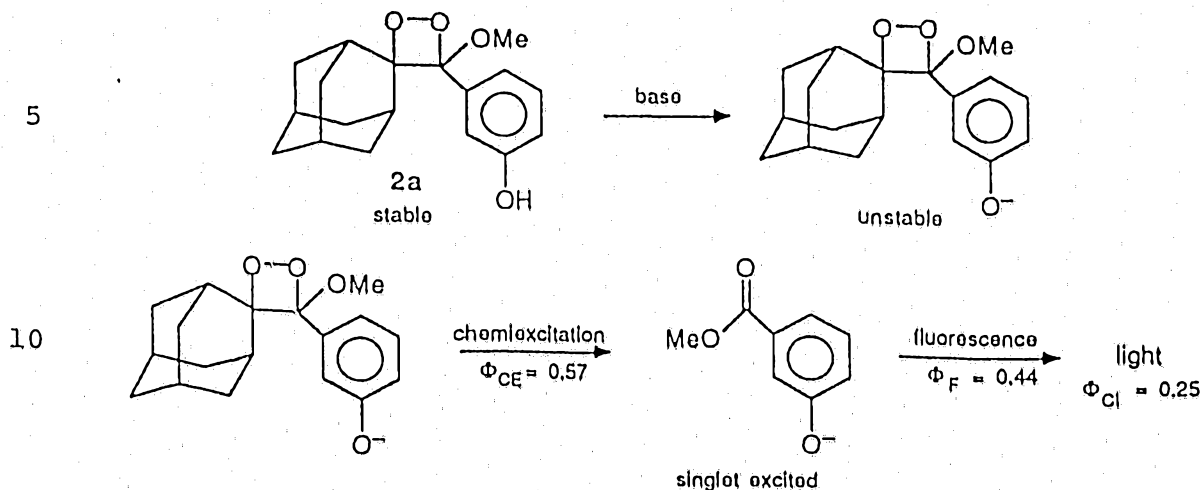
Spectra of the chemiluminescence from chemically or enzymatically triggered dioxetanes were obtained by conducting the reaction in a 1-cm square quartz cuvette in the sample compartment of a Spex Fluorolog spectrofluorometer at ambient temperature. Correction for the decay of the chemiluminescence intensity during the wavelength scan was made by accumulating the spectrum in a ratio mode so that the observed spectrum was divided by the signal from an auxiliary detector (EMI 9781B) which measures the total signal as a function of time. The monochromator bandpass was typically 18nm. For weakly emitting samples, several identical scans were performed and added together to improve the signal-to-noise ratio.

Chemical Triggering of Dioxetanes

1. Triggering the Chemiluminescence of

Hydroxy-Substituted Dioxetane 2a with Base. Treatment of a 10^{-4} M solution of dioxetane 2a in DMSO at room temperature with an excess of tetra-n-butylammonium hydroxide resulted in intense blue chemiluminescence which decayed over a period of several minutes. The emission maximum for the chemiluminescence is 470 nm. The fluorescence of the anion of the cleavage product (methyl 3-hydroxybenzoate, MHB) is identical to the chemiluminescence spectrum. These results demonstrate that the chemiluminescence process involves: (a) base triggering to yield the unstable aryloxide form of the dioxetane, (b) subsequent cleavage of this species to generate MHB in the singlet excited state, and (c)

fluorescence of MHB to yield the luminescence with an overall quantum yield (ϕ_{Cl}) of 0.25.



2. Catalysis of the Base-Triggered

15 Chemiluminescence of Acetoxy-Substituted Dioxetane 2b in Aqueous Micelles: Enhanced Chemiluminescence Efficiency via Intermolecular Energy Transfer. Cationic surfactants such as cetyltrimethylammonium bromide (CTAB) can be used to increase rates for chemical triggering of

20 chemiluminescence from appropriately substituted dioxetanes in aqueous solution. For example, CTAB catalyzes the base-induced luminescent cleavage of the

25 acetoxy-substituted dioxetane 2b. The dioxetane is solubilized in the micelles formed by the surfactant and NaOH is added to initiate the chemiluminescence. The electrostatic attraction of the cationic head group and the hydroxide anion provides the observed micellar catalysis. Typically, the experiments were carried out with $[2b] = 9.1 \times 10^{-5} \text{ M}$, $[CTAB] = 2 \times 10^{-3} \text{ M}$, and $[OH^-] = 9.1 \times 10^{-5} \text{ M}$ at

30 37°C .

The micellar environment can lead to higher chemiluminescence efficiencies. Although the light yields of 2a and 2b with base in DMSO are extremely high at 0.25, the yield for these dioxetanes in water is only 8.9×10^{-6} .

35 The principal reason for this large decrease results from the fact that the cleavage product (MHB) is only weakly fluorescent in water. However, as demonstrated by the

following experiment, the luminescence can be enhanced by triggering the dioxetane in a micelle. The conditions for base-triggering of 2b were the same as described above except that the concentration of CTAB was varied from 0 to 5×10^{-3} M. Figure 2 shows a 19-fold increase in the chemiluminescence quantum yield ($\phi_{CL}=1.7 \times 10^{-4}$) above the critical micelle concentration for CTAB ($cmc \approx 1 \times 10^{-3}$ M). Enhanced chemiluminescence efficiency in the micellar environment is the result of increases in ϕ_F and/or ϕ_{CE} .

Incorporation in the micelle of a co-surfactant with a fluorescent head group provides dramatically higher chemiluminescence yields for both chemically and enzymatically triggered dioxetanes. Energy transfer from the excited cleavage product to the fluorescent surfactant can be very efficient because both the triggerable dioxetane and the energy acceptor (fluorescer) are held in close proximity in the micelle as illustrated in Figure 2a.

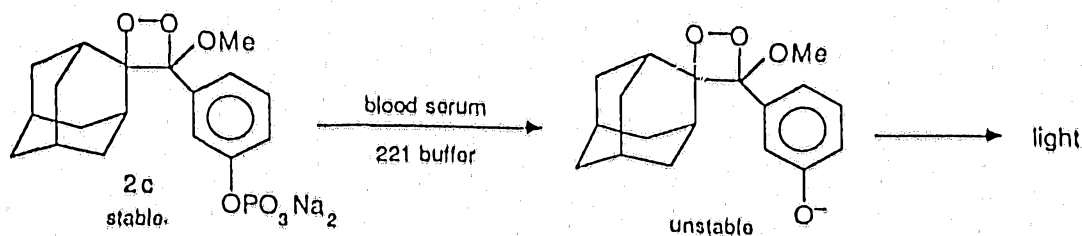
For example, CTAB micelles containing fluorescein surfactant 3 and dioxetane 2b were prepared in aqueous solution with final concentrations of: CTAB (1.5×10^{-3} M), 3 (9×10^{-5} M), and 2b (9×10^{-5} M). Addition of base at 37°C resulted in intense yellow chemiluminescence rather than the normal blue emission with a chemiluminescence efficiency of 1.4% ($\phi_{CL} = 0.014$), an increase of 500-fold over the luminescence of 2b in the absence of CTAB and 3.

Similar experiments were conducted with the benzothiazamide surfactant 4. The chemiluminescence efficiency was 0.3% with λ_{max} at 506 nm.

Enzymatic Triggering of Phosphate-Substituted Dioxetane 2c.

1. Triggering the Chemiluminescence of 2c with Alkaline Phosphatase from Human Blood Serum. A stock solution of dioxetane 2c was prepared in dioxane/water (1:1 v/v) by photooxygenation of 1c. A sample of fresh blood was drawn from a healthy donor and the red blood cells removed by centrifugation to provide serum for the experiments. Treatment of 3 mL of a 10^{-5} M solution of 2c

in 0.75 M 2-amino-2-methyl-1-propanol (221) buffer (pH 10.3) at 37°C with 100 µL of serum led to the typical intensity vs. time profile shown in Figure 3 where the serum is injected at time zero. Under these conditions the light intensity reaches a constant level of approximately 2.3×10^4 counts/sec. The background luminescence signal from non-enzymatic hydrolysis of 2c is less than 0.05% of the enzyme-generated value. The light intensity at the plateau is directly proportional to the enzyme concentration as shown by a series of experiments using 100 to 1 µL of serum (Figure 4). The light intensity at any other time point can also be conveniently used to provide a direct measure of the enzyme concentration (Figure 5).



2. Triggering the Chemiluminescence of 2c with Alkaline Phosphatase from Bovine Intestinal Mucosa.

Alkaline phosphatase from bovine intestinal mucosa was obtained from Sigma Chemical Co. as a suspension of 5.1 mg of protein per mL of 3.2 M (NH₄)₂ SO₄ solution. In a typical experiment, 50 µL of a 2.56×10^{-3} M stock solution of dioxetane 2c in 221 buffer was added to 3 mL of 221 buffer (0.75 M, pH 9.1) containing 8.0×10^{-4} M Mg(OAc)₂ giving a final dioxetane concentration of 4.3×10^{-5} M. Injection of a 10 µL aliquot of diluted enzyme into the solution at 37°C resulted in chemiluminescence, the quantum yield of which was 3.1×10^{-5} . As with chemical triggering, the addition of CTAB (1.13×10^{-3} M) results in a modest increase in ϕ_{Cl} to 2.1×10^{-4} . The kinetics of the enzymatic triggering were not significantly altered by the presence of the surfactant.

3. Triggering the Chemiluminescence of 2c with Alkaline Phosphatase: Enhanced Chemiluminescence Efficiency via Intermolecular Energy Transfer in Aqueous Micelles.

The efficiency of the enzyme triggered chemiluminescence of 2c can be dramatically enhanced by incorporation of the fluorescein co-surfactant 3 in the micelles. Alkaline phosphatase experiments with dioxetane 2c were conducted at 37°C with 3 mL of a solution containing: 2c (4.3×10^{-5} M), 221 buffer (0.75 M, pH 9.1), $\text{Mg}(\text{OAc})_2$ (8.0×10^{-4} M), CTAB (1.13×10^{-3} M), and fluorescein surfactant 3 (5.6×10^{-5} M). Addition of alkaline phosphatase (Sigma, bovine intestinal mucosa) to give a final concentration of 12 pg/mL of protein resulted in chemiluminescence over a 45 minute period. Integration of the light intensity over the entire course of light emission gave $\Phi_{\text{CL}} = 0.015$ (or 1.5% chemiluminescence efficiency, a 500-fold increase compared to the enzymatic reaction in the absence of CTAB and 3). As in the case of chemical triggering of 2b, the chemiluminescence spectrum is also shifted from the normal blue emission (Figure 6, Curve A) to the typical fluorescein emission (Figure 6, Curve B), demonstrating the involvement of energy-transfer processes. It should be emphasized that simple reabsorption of the blue light by 3 and subsequent fluorescence cannot be the mechanism for the spectral shift as such a process would not result in enhanced efficiency.

To test the sensitivity of this chemiluminescence method for evaluating concentrations of alkaline phosphatase in solution, a series of experiments were carried out using enzyme stock solutions prepared by dilutions of the commercial sample obtained from Sigma. A conservative estimate of the concentration of phosphatase in the sample was made by assuming that the 5.1 mg of protein per mL in the sample was 100% pure alkaline phosphatase. A molecular weight of 140,000 was also used in calculating molar amounts of enzyme. The conditions were the same as described above with 2c, CTAB, and fluorescer 3

except that the final quantities of enzyme in the 3 mL solutions were:

$$A = 3.6 \times 10^{-12} \text{ moles}$$

$$B = 3.3 \times 10^{-13} \text{ moles}$$

$$5 \quad C = 3.0 \times 10^{-14} \text{ moles}$$

$$D = 2.7 \times 10^{-15} \text{ moles}$$

$$E = 2.5 \times 10^{-16} \text{ moles}$$

$$F = 2.3 \times 10^{-17} \text{ moles}$$

Under these conditions the background chemiluminescence from the non-enzymatic hydrolysis of 2c in the buffer/co-micelle environment is extremely slow and gives rise to a constant signal of only a few counts/sec (Figure 7). A typical intensity vs. time profile for enzymatic triggering with a phosphatase concentration of 2.7×10^{-15} moles in 3 mL (Experiment D) is shown in Figure 7. The light intensity increases with time over a period of 30 - 50 minutes depending on enzyme concentration. After this period the light remains constant until the dioxetane is consumed. The pre-steady state period can be eliminated if the sample containing the dioxetane and enzyme is incubated at 37 - 45°C for several minutes before analysis with the luminometer.

Plots of either the integrated light intensity or intensities at a specific time point vs. the quantity of enzyme give excellent correlations. For example, Figure 8 shows a plot of the log (total enzymatic luminescence from time zero to 3 minutes) vs. log (moles of alkaline phosphatase). The reproducibility of each run was better than 4% and plots such as shown in Figure 8 gave correlation coefficients of >0.99 .

Enzymatic triggering experiments such as those described above were also carried out in Immulon[™] microtitre wells from Dynatech, Inc. made of transparent polystyrene. The wells were used individually and placed in a light-tight holder which could be thermostatted. The chemiluminescence was detected at the bottom of the well using a fiber optic connected to the photon-counting

luminometer described previously. This experimental set-up allowed much smaller reaction volumes to be used. For example, a series of experiments using dioxetane 2c, CTAB, and 3 in 200 μ L of 221 buffer with amounts of alkaline phosphatase ranging from 5×10^{-15} to 2×10^{-18} moles (or 2 attomoles) were carried out. Figure 9 shows the intensity vs. time profile for an experiment with 2.3×10^{-17} moles of enzyme. A more realistic assumption for the purity of the enzyme sample might be 10%. Under those conditions it is seen from Figure 10 that this chemiluminescent technique with dioxetane 2c permits the detection of less than 0.2 attomoles of alkaline phosphatase.

The chemiluminescence generated by the enzymatic triggering of dioxetane 2c can also be detected photographically using X-ray film and instant film. For example Figures 11 and 12 show the chemiluminescence recorded on ASA 3000 Polaroid™ Type 57 film. Solutions of 221 buffer (100 μ L, 0.75 M, pH 9.1) containing dioxetane 2c (4.3×10^{-5} M), $\text{Mg}(\text{OAc})_2$ (8.0×10^{-4} M), CTAB (1.13×10^{-3} M), and fluorescein surfactant 3 (5.6×10^{-5} M) were incubated in Dynatech Immulon wells in the presence of varying amounts of alkaline phosphatase using the same enzyme stock solutions listed above. The wells were incubated for 1 hour at 37°C and then photographed at that temperature for 15 minutes by placing the wells directly on the film in a light-tight incubator. The light intensity recorded on the film clearly provides a measure of the enzyme concentration.

4. Chemiluminescent Enzyme-Linked Assays.

Enzymatic triggering of appropriately substituted dioxetanes provides an ultrasensitive detection method for enzyme-linked biological assays. For example, phosphate-substituted dioxetane 2c can be used with enzyme linked immunoassays and DNA probes that utilize alkaline phosphatase as the marker for detection. Previous detection methods make use of substrates which develop a color or become fluorescent upon reaction with this enzyme.

The sensitivity of the chemiluminescent technique with dioxetane 2c is illustrated by an enzyme-linked immunosorbant assay (ELISA) for the retinal protein, S-antigen. Using procedures of L. A. Donoso (L. A. Donoso, C. F. Merryman, K. E. Edelberg, R. Naidis, and C. Kalsow, Investigative Ophthalmology & Visual Science, 26, 561 (1985)), a series of 7 Immulon™ wells were coated with varying amounts of S-antigen (112, 56, 28, 14, 7, 3, 1.3 ng), reacted with a monoclonal antibody (MAbA9-C5) developed in mouse, and finally reacted with anti-mouse IgG coupled to alkaline phosphatase. A chemiluminescence assay of each well was then conducted individually by adding 100 μ L of 221 buffer (0.75 M, pH 9.1) containing $\text{Mg}(\text{OAc})_2$ (8.0×10^{-4} M), CTAB (1.13×10^{-3} M), and fluorescein surfactant 3 (5.6×10^{-5} M). The well was placed in the micro-luminometer and equilibrated to 37°C for 3 minutes and 10 μ L of a stock solution of dioxetane 2c in 221 buffer was injected to give a final concentration of 2c of 1.36×10^{-4} M. A typical chemiluminescence intensity vs. time profile is shown in Figure 13. The reagent background luminescence is very low and constant at 15 - 20 counts/sec (Figure 13). As shown in Figure 14, the integrated light intensity correlates with the amount of antigen coated on the well. Following the experiments with the luminometer, the wells containing the same solution were subsequently used for a photographic detection experiment (Figure 15). The 7 wells were placed in a holder and incubated for 30 minutes at 37°C and then photographed at that temperature with ASA 3000 Polaroid™ Type 57 film for 15 min in a light-tight incubator. As shown in Figure 15, the light intensity recorded on the film also correlates with the amount of S-antigen coated on the well. The reproducibility of the photographic assay is illustrated in Figure 16. Four wells coated with 50 ng of antigen were treated with buffer and dioxetane as above, incubated for 1 hour at 45°C, and then photographed with the film for 30 sec at that temperature. It should be noted that in both

photographic assays the control well containing only buffer solution and dioxetane is not visible, again demonstrating the extremely low background produced by non-enzymatic hydrolysis of the dioxetane.

5 5. Enzymatic Triggering of Hydroxy-Substituted Dioxetane 2a with Urease in the Presence of Fluorescent Micelles. A solution was prepared using 160 mg of CTAB and 12.4 mg of fluorescein surfactant 3 in 200 mL of distilled water. A urea solution was made by dissolving 200 mg of
10 urea in 100 mL of distilled water with EDTA added to give a final concentration of 0.4 mM. The substrate solution for the urease experiments was obtained by mixing 10mL of each stock solution.

Experiments were carried out with urease (Sigma)
15 by incubation at room temperature for periods of 0.5 to 2 hours. Subsequent injection of 10 μ L of 3×10^{-3} M dioxetane 2a produced chemiluminescence which was monitored by the luminometer and by instant film. The intensity of the luminescence provided a measure of the concentration of
20 urease.

In the preferred method and compositions, the dioxetane is used in an amount between about 10^{-2} and 10^{-6} M; the surfactant in an amount greater than about 10^{-4} M and the co-surfactant fluorescein surfactant in an amount
25 between about 10^{-3} and 10^{-6} M. The co-surfactant quenches itself when used alone in solution. In general the molar ratio of the dioxetane to fluorescent compound is between about 1,000 to 1 and 1 to 1 whether in solution or in a solid composition.

30 Buffers are used to adjust the pH so as to optimize enzyme activity. With the phosphate (as the X-oxy group) substituted dioxetanes the pH is between 9 and 10 so that non-enzymatic hydrolysis of the phosphate group is minimized resulting in low background luminescence.

35 2-Methyl 2-amino-1-propanal (221) is a preferred buffer. Other buffers are tris(hydroxymethyl)aminomethane and carbonate. Inorganic salts such as magnesium acetate are

also used to activate the enzyme. The buffer system is chosen to provide maximal catalytic activity for the enzyme and an acceptor for the X-oxy group cleaved from the dioxetane, such as the phosphate.

5 The present invention incorporates a stable dioxetane and fluorescent energy acceptor, preferably in organized molecular assemblies such as micelles affording efficient energy transfer. These procedures are applicable with other types of organized assemblies including reversed
10 micelles, liposomes, microemulsions, films, monolayers and polymers.

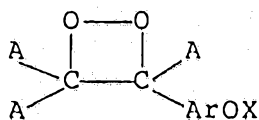
 It will be appreciated that the dioxetane and acceptor can be in solution in a solvent or in a solid form such as on a film. The solid phase provides ease of
15 positioning these molecules together.

 It is intended that the foregoing description be only illustrative and that the present invention be limited only by the hereinafter appended claims.

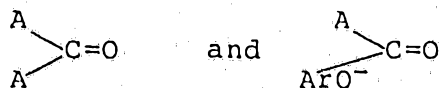
THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for generating light which comprises:

(a) providing in a setting where the light is to be produced a fluorescent compound dispersed in or on one of a surfactant, a micelle, liposome, reversed micelle, microemulsion, film, including a monolayer or a polymer which brings the fluorescent compound into a closely spaced relationship with and in admixture with a 1,2-dioxetane which is stable in the setting where light is to be produced of the formula



wherein ArOX represents an aryl group substituted with an X-oxy group which forms an oxide intermediate 1,2-dioxetane compound when triggered by removing with an activating agent so that the intermediate 1,2-dioxetane compound decomposes and releases electronic energy to form light and two carbonyl containing compounds of the formula



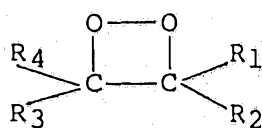
wherein X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate 1,2-dioxetane and wherein A are passive organic groups which allow the light to be produced by the 1,2-dioxetane when X is removed; and

(b) triggering the stable 1,2-dioxetane with the activating agent wherein the fluorescent compound accepts the electronic energy generated upon decomposition of the oxide intermediate 1,2-dioxetane compound and produces a more intense light than is produced by the triggering of the dioxetane alone.

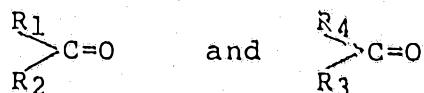


2. A method for generating light which comprises:

(a) providing in a setting where the light is to be produced a fluorescent compound dispersed in or on one of a surfactant, a micelle, liposome, reversed micelle, microemulsion, film, including a monolayer or a polymer which brings the fluorescent compound into a closely spaced relationship with and in admixture with a 1,2-dioxetane compound which is stable in the setting where the light is to be produced of the formula



wherein R₁ and R₂ together and R₃ and R₄ together can be joined as spirofused cycloalkylene which can contain hetero atoms selected from the group consisting of N, S, O and P in addition to carbon and aryl rings, wherein at least one of R₁ and R₂ or R₃ and R₄ is an aryl group substituted with an X-oxy group which forms an oxide intermediate 1,2-dioxetane compound when triggered by removing X with an activating agent selected from acids, bases, salts, enzymes, inorganic and organic catalysts and other electron donors so that the oxide intermediate 1,2-dioxetane compound decomposes and releases electronic energy to form light and two carbonyl containing compounds of the formula:



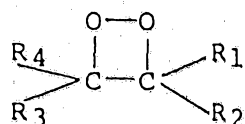
wherein those of R₁, R₂, R₃ or R₄ which are unsubstituted by an X-oxy group are carbon and hetero atom containing organic groups which provide stability for the stable 1,2-dioxetane compound and wherein X is a chemically labile group which is removed by the activating agent to form the oxide intermediate 1,2-dioxetane; and

(b) triggering the stable 1,2-dioxetane with an activating agent to remove X wherein the fluorescent compound accepts the electronic energy generated upon decomposition of the oxide intermediate 1,2-dioxetane

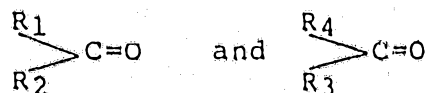


compound and produces a more intense light than is produced by the triggering of the dioxetane alone.

3. A method for generating light which comprises:
 (a) providing in a setting where the light is to be produced a fluorescent compound dispersed in or on one of a surfactant, a micelle, liposome, reversed micelle, microemulsion, film, including a monolayer or a polymer which brings the fluorescent compound and the dioxetane into a closely spaced relationship with and in admixture with a dioxetane compound which is stable in the setting where the light is to be produced of the formula:



wherein R₁ is selected from alkyl, alkoxy, aryloxy dialkylamino, trialkyl or aryl silyloxy and aryl groups including spirofused aryl groups joined with R₂, wherein R₂ is an aryl group which can include R₁ and is substituted with an X-oxy group which forms an oxide intermediate 1,2-dioxetane compound when triggered by removing X with an activating agent selected from acids, bases, salts, enzymes, inorganic and organic catalysts and other electron donors so that the oxide intermediate 1,2-dioxetane compound decomposes and releases electronic energy to form light and two carbonyl containing compounds of the formula:



wherein X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate 1,2-dioxetane compound and wherein R₃ and R₄ are selected from aryl, heteroalkyl and alkyl groups which can be joined together as spirofused polycyclic alkyl and polycyclic aryl groups; and

- (b) triggering the stable 1,2-dioxetane with the activating agent to remove X wherein the fluorescent compound accepts the electronic energy generated upon



decomposition of the oxide intermediate 1,2-dioxetane compound and produces a more intense light than is produced by the triggering of the dioxetane alone.

4. The method of Claim 1 wherein the 1,2-dioxetane compound is decomposed by an enzyme from a biological source as the activating agent which removes X.

5. The method of Claim 4 wherein the 1,2-dioxetane has a phosphate group as the X-oxy group and the enzyme is alkaline phosphatase.

6. The method of Claim 4 wherein the 1,2-dioxetane has an acetoxy group as the X-oxy group and the enzyme is acetylcholinesterase.

7. The method of Claim 4 wherein the 1,2-dioxetane has an acetoxy group as the X-oxy group and wherein the enzyme is arylesterase.

8. The method of Claim 1 as a step in an immunoassay or a nucleic acid probe assay as the setting where the light is produced.

9. The method of Claim 2 wherein R₃ and R₄ are combined together to form a polycyclic polyalkylene group containing 6 to 30 carbon or hetero atoms selected from the group consisting of N, O, S and P atoms.



10. The method of Claim 9 wherein R_2 is selected from naphthyl and phenyl groups containing the X-oxy group and wherein R_1 is a methoxy group.
11. The method of Claim 10 wherein the X-oxy group is selected from hydroxyl, trialkyl or aryl silyloxy, inorganic oxyacid salt, oxygen-pyranoside, arylcarboxyl ester and alkylcarboxyl ester radicals and wherein X is removed by the activating agent.
12. The method of Claim 11 wherein the X-oxy group is a phosphate group as the inorganic oxyacid salt and wherein the phosphate radical is removed with a phosphatase as the activating agent.
13. The method of Claim 11 wherein the X-oxy group is selected from alkyl and aryl carboxyl ester radicals and wherein the ester radical is removed with an esterase.
14. The method of Claim 2 wherein the 1,2-dioxetane compound is decomposed by an enzyme from a biological source as the activating agent which removes X.
15. The method of Claim 14 wherein the 1,2-dioxetane has a phosphate group as the X-oxy group and the enzyme is alkaline phosphatase.
16. The method of Claim 14 wherein the 1,2-dioxetane has an acetoxyl group as the X-oxy group and the enzyme is acetylcholinesterase.

17. The method of Claim 14 wherein the 1,2-dioxetane has an acetoxy group as the X-oxy group and the enzyme is arylesterase.

18. The method of Claim 2 wherein the setting where the light to be produced is in an immunoassay or nucleic acid probe assay where the admixture is triggered to produce light which is detected.

19. The method of Claim 2 wherein R₁ is an alkoxy group.

20. The method of Claim 2 wherein R₁ is a methoxy group and R₂ is a phenyl group substituted with the X-oxy group and wherein R₃ and R₄ are joined together as an adamantyl group.

21. The method of Claim 20 wherein the X-oxy group is selected from hydroxyl, acetoxy, phosphate and oxygen-pyranoside.

22. The method of Claim 1 wherein the fluorescent compound is a fluorescein compound.

23. The method of Claim 1 wherein a cationic surfactant are provided in the admixture with the 1,2-dioxetane and fluorescent compound.

24. The method of Claim 23 wherein the cationic surfactant is a cetyltrimethylammonium salt.

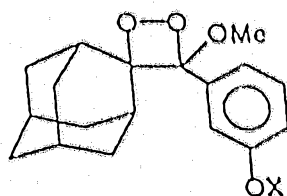
25. The method of Claim 24 wherein the fluorescent compound is a fluorescein compound.

26. The method of Claim 1 wherein the fluorescent compound is 5-(N-tetradeca-nonylamino-fluorescein) which also acts as a co-surfactant with a cetyltrimethylammonium salt as a surfactant.

27. The method of Claim 1 wherein the fluorescent compound is 1-hexadecyl-6-hydroxy-2-benzotriazamide which also acts as a co-surfactant with a cetyltrimethylammonium salt as a surfactant.

28. In a method for detecting DNA by hybridization of the DNA with a DNA probe having an enzyme label and then generating a detectable signal by means of the enzyme label, the improvement which comprises:

(a) providing a 1,2-dioxetane of the formula:



wherein X is a group reactive with the enzyme label to produce light;

(b) providing a fluorescent compound which enhances the light from the 1,2-dioxetane in admixture with the hybrid of the probe and the DNA; and

(c) detecting the light from the hybrid produced by the reaction of the enzyme label with the dioxetane.



²⁹
~~44.~~

The method of Claim ²⁸~~43~~ wherein OX is a phosphate group which reacts with alkaline phosphatase as the enzyme label.

³⁰
~~45.~~

The method of Claim ²⁹~~44~~ wherein the phosphate group is PO_3Na_2 .

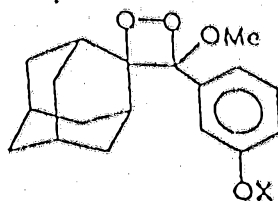
³¹
~~46.~~

The method of Claim ²⁸~~43~~ wherein OX is an acetoxy group which reacts with an esterase as the enzyme label.

³²
~~47.~~

In a method for generating light by reacting a 1,2-dioxetane with an activating agent, the improvement which comprises:

(a) providing in admixture in a setting where light is to be produced a 1,2-dioxetane of the formula:



wherein OX is a phosphate group reactive with alkaline phosphatase to produce light and a fluorescent compound which enhances the light from the 1,2-dioxetane in admixture with the dioxetane; and

(b) triggering the 1,2-dioxetane with alkaline phosphatase to generate the light.

³³
~~48.~~

The method of Claim ³²~~47~~ wherein the phosphate group is PO_3Na_2 .

³⁴
~~49~~. The method of Claim ³²~~47~~ wherein a cationic
surfactant is admixed with the fluorescent compound.

³⁵
~~50~~. The method of Claim ³¹~~49~~ wherein the fluorescent
compound is 5-(N-tetradecanonylamino)fluorescein.

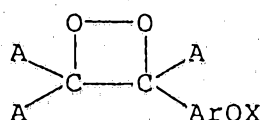
³⁶
~~51~~. The method of Claim ³² wherein the fluorescent
compound is 5-(N-tetradecanoylamino)fluorescein which acts
as a co-surfactant with a cetyltrimethylammonium salt as a
surfactant.



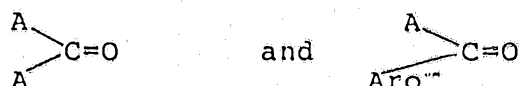
37
52.

A composition which generates light upon triggering which comprises in admixture:

- (a) a fluorescent compound; and
- (b) a stable 1,2-dioxetane of the formula:



wherein ArOX represents an aryl group substituted with an X-oxy group which forms an unstable oxide intermediate 1,2-dioxetane compound when triggered to remove X by an activating agent so that the unstable 1,2-dioxetane compound decomposes and releases electronic energy to form light and two carbonyl containing compounds of the formula:



wherein X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate 1,2-dioxetane and wherein A are passive organic groups which allow the light to be produced wherein the stable 1,2-dioxetane is decomposed with the activating agent, wherein the dioxetane and the fluorescent compound are provided in a closely spaced relationship in the presence of a surfactant, micelle, liposome, reversed micelle, microemulsion, film, including a monolayer, or polymer and wherein the fluorescent compound accepts the electronic energy generated upon decomposition of the unstable oxide intermediate and produces a more intense light than is produced by the triggering of the dioxetane alone.

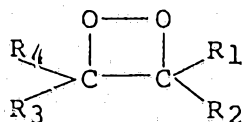


38
53.

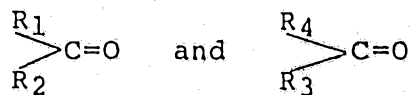
A composition which generates light upon triggering which comprises in admixture:

- (a) a fluorescent compound; and
- (b) a stable 1,2-dioxetane compound of the

formula:



wherein one of R₁ and R₂ together and R₃ and R₄ together can be joined as spirofused alkylene which can contain hetero atoms selected from the group consisting of N, S, O and P in addition to carbon and aryl rings, wherein at least one of R₁ and R₂ or R₃ and R₄ is an aryl group substituted with an X-oxy group which forms an unstable oxide intermediate 1,2 dioxetane compound when triggered to remove X by an activating agent selected from acids, bases, salts, enzymes, inorganic and organic catalysts and electron donors so that the unstable 1,2-dioxetane compound decomposes and releases electronic energy to form light and two carbonyl containing compounds of the formula:



wherein those of R₁, R₂, R₃ or R₄ which are unsubstituted by an X-oxy group are organic groups which provide stability for the stable 1,2-dioxetane compound and wherein X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate wherein the stable 1,2-dioxetane is decomposed with the activating agent, wherein the dioxetane and the fluorescent compound are provided in a closely spaced relationship in

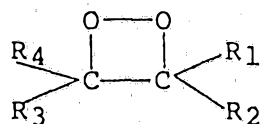


the presence of a surfactant, micelle, liposome, reversed micelle, microemulsion, film, including a monolayer, or polymer and wherein the fluorescent compound accepts the electronic energy generated upon decomposition of the unstable oxide intermediate and produces a more intense light than is produced by the triggering of the dioxetane alone.

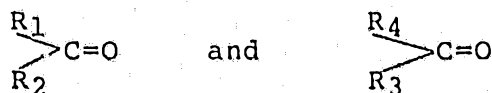
39
54.

A composition which generates light upon triggering which comprises in admixture:

- (a) a fluorescent compound; and
- (b) a stable dioxetane compound of the formula:



wherein R₁ is selected from alkyl, alkoxy, aryloxy dialkylamino, trialkyl or aryl silyloxy and aryl groups including spirofused aryl groups with R₂, wherein R₂ is an aryl group which can include R₁ and is substituted with an X-oxy group which forms an unstable oxide intermediate 1,2-dioxetane compound when triggered to remove X by an activating agent selected from acids, bases, salts, enzymes, inorganic and organic catalysts and electron donors so that the unstable 1,2-dioxetane compound decomposes and releases electronic energy to form light and two carbonyl containing compounds of the formula:



wherein X is a chemically labile group which is removed by the activating agent to form the unstable oxide intermediate and wherein R₃ and R₄ are selected from aryl, heteroalkyl and alkyl groups which can be joined together as spirofused polycyclic alkyl and polycyclic aryl groups wherein the stable 1,2-dioxetane is decomposed with the activating agent, wherein the dioxetane and the fluorescent compound are provided in a closely spaced relationship in the presence of a surfactant, micelle, liposome, reversed micelle, microemulsion, film, including a monolayer, or polymer and wherein the fluorescent compound accepts the electronic energy generated upon decomposition of the unstable oxide intermediate and produces a more intense light than is produced by the triggering of the dioxetane alone.

⁴⁰
~~55~~. The composition of Claim ³⁶~~51~~ wherein the mole ratio of dioxetane to fluorescent compound is between about 1,000 to 1 and 1 to 1.

⁴¹
~~56~~. The composition of Claim ³⁷~~52~~ wherein the fluorescent compound is a fluorescein compound.

⁴²
~~57~~. The composition of Claim ³⁷~~52~~ wherein in addition a surfactant is provided in the composition.



⁴³
~~58.~~ The composition of Claim ³⁸~~53~~ wherein R₁ is a methoxy group and R₂ is a phenyl group substituted with an X-oxy group and wherein R₃ and R₄ are joined together as an adamantyl group.

⁴⁴
~~59.~~ The composition of Claim ⁴³~~58~~ wherein the X-oxy group is selected from hydroxyl, trialkyl or aryl silyloxy, inorganic oxyacid salt, oxygen-pyranoside, arylcarboxyl esters and alkylcarboxyl esters and wherein X is removed by the activating agent.

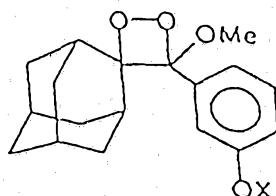
⁴⁵
~~60.~~ The composition of Claim ⁴⁴~~59~~ wherein the fluorescent compound is a fluorescein compound.

⁴⁶
~~61.~~ The composition of Claim ³⁷~~52~~ wherein a cationic surfactant is provided in the composition, wherein the fluorescent compound is chemically linked with a hydrocarbon chain to provide a fluorescent hydrocarbon which acts as a co-surfactant with a cationic surfactant to increase the light from the dioxetane and fluorescent compound and wherein the dioxetane is present in an amount between about 10⁻² to 10⁻⁶ M, the surfactant is present in an amount greater than 10⁻⁴ M and the co-surfactant is present in an amount between 10⁻³ to 10⁻⁶ M.

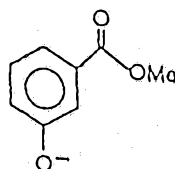
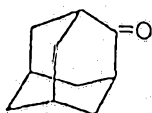
⁴⁷
~~62.~~ The composition of Claim ⁴⁶~~61~~ wherein the cationic surfactant is a cetyltrimethylammonium salt.

⁴⁸
~~63.~~ The composition of Claim ³⁷~~52~~ wherein the dioxetane and fluorescent compound are in a closely spaced relationship in a micelle, liposome, reversed micelle, microemulsion, film, monolayer or polymer.

⁴⁹
~~64~~. The composition of Claim ~~52~~³⁷ wherein the 1,2-dioxetane has the formula:



wherein X is a group reactive with the activating agent to produce the two carbonyl containing compounds



and light.

⁵⁰
~~65~~. The composition of Claim ~~64~~⁴⁹ wherein OX is a phosphate group and the activating agent is alkaline phosphatase.

⁵¹
~~66~~. The composition of Claim ~~64~~⁴⁹ wherein in addition a surfactant is admixed in the composition.

⁵²
~~67~~. The composition of Claim ~~66~~⁵¹ wherein the surfactant is a cetyltrimethylammonium salt.

⁵³
~~68~~. The composition of Claim ~~64~~⁴⁹ wherein the fluorescent compound is a fluorescein compound.

⁵⁴
~~69~~. The composition of Claim ⁵³~~68~~ wherein in addition a surfactant is admixed in the composition.

⁵⁵
~~70~~. The composition of Claim ⁵⁴~~69~~ wherein the surfactant is a cetyltrimethylammonium salt.

⁵⁶
~~71~~. The composition of Claim ⁵³~~68~~ wherein the fluorescein compound is fluorescein chemically linked to a hydrocarbon to provide a fluorescein surfactant.

⁵⁷
~~72~~. The composition of Claim ⁴⁹~~64~~ wherein the fluorescent compound is selected from the group consisting of 5-N-tetradecanoylamino fluorescein and 1-hexadecyl-6-hydroxy-2-benzothiazamide.

⁵⁸
~~73~~. The composition of Claim ⁵⁷~~72~~ wherein in addition a non-fluorescent surfactant is admixed in the composition.

⁵⁹
~~74~~. The composition of Claim ⁵⁸~~73~~ wherein the surfactant is a cetyltrimethylammonium salt.

⁶⁰
~~75~~. The composition of Claim ³⁷~~52~~ wherein the fluorescent compound is fluorescein chemically linked to a hydrocarbon to provide a fluorescein surfactant which increases the light from the fluorescein compound and the dioxetane.



⁶¹
~~76~~. The composition of Claim ³⁷~~32~~ wherein the fluorescent compound is selected from the group consisting of 5-N-tetradecanoylamino fluorescein and 1-hexadecyl-6-hydroxy-2-benzothiazamide.

⁶²
~~77~~. The composition of Claim ⁶¹~~76~~ wherein in addition a surfactant is admixed in the composition.

⁶³
~~78~~. The composition of Claim ⁶²~~77~~ wherein the surfactant is a cetyltrimethylammonium salt.

⁶⁴
~~79~~. The composition of Claim ³⁹~~54~~ wherein the fluorescent compound is fluorescein chemically linked to a hydrocarbon to provide a fluorescein surfactant.

⁶⁵
~~80~~. The composition of Claim ³⁹~~54~~ wherein the fluorescent compound is selected from the group consisting of 5-N-tetradecanoylamino fluorescein and 1-hexadecyl-6-hydroxy-2-benzothiazamide.

⁶⁶
~~81~~. The composition of Claim ⁶⁵~~80~~ wherein in addition a surfactant is admixed in the composition.

⁶⁷
~~82~~. The composition of Claim ⁶⁶~~81~~ wherein the surfactant is a cetyltrimethylammonium salt.

⁶⁸
~~83~~. The composition of Claim ³⁷~~52~~ wherein the dioxetane and the fluorescent compound are provided in a closely spaced relationship in a solid or liquid medium.



⁶⁹
84. The composition of Claim ³⁸~~53~~ wherein the dioxetane and the fluorescent compound are provided in a closely spaced relationship in a solid or liquid medium.

⁷⁰
85. The composition of Claim ³⁹~~54~~ wherein the dioxetane and the fluorescent compound are provided in a closely spaced relationship in a solid or liquid medium.

⁷¹
86. The composition of Claim ³⁹~~54~~ wherein in addition a surfactant is provided in the composition.

⁷²
87. A method of any one of claims 1 to 3 ~~claim~~ substantially as herein described with reference to any one of the examples or figures.

⁷³
88. A composition of any one of claims ³⁷~~52~~ to ³⁷~~54~~ substantially as herein described with reference to any one of the examples or figures.

DATED: 19 July 1990

THE BOARD OF GOVERNORS OF WAYNE STATE UNIVERSITY

By their Patent Attorneys:

PHILLIPS ORMONDE & FITZPATRICK

David B Fitzpatrick



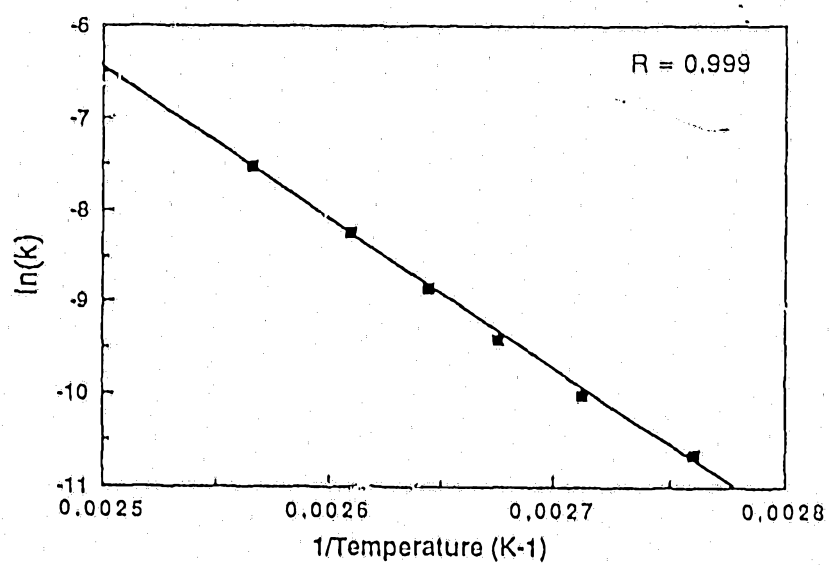


FIGURE 1

36645/89

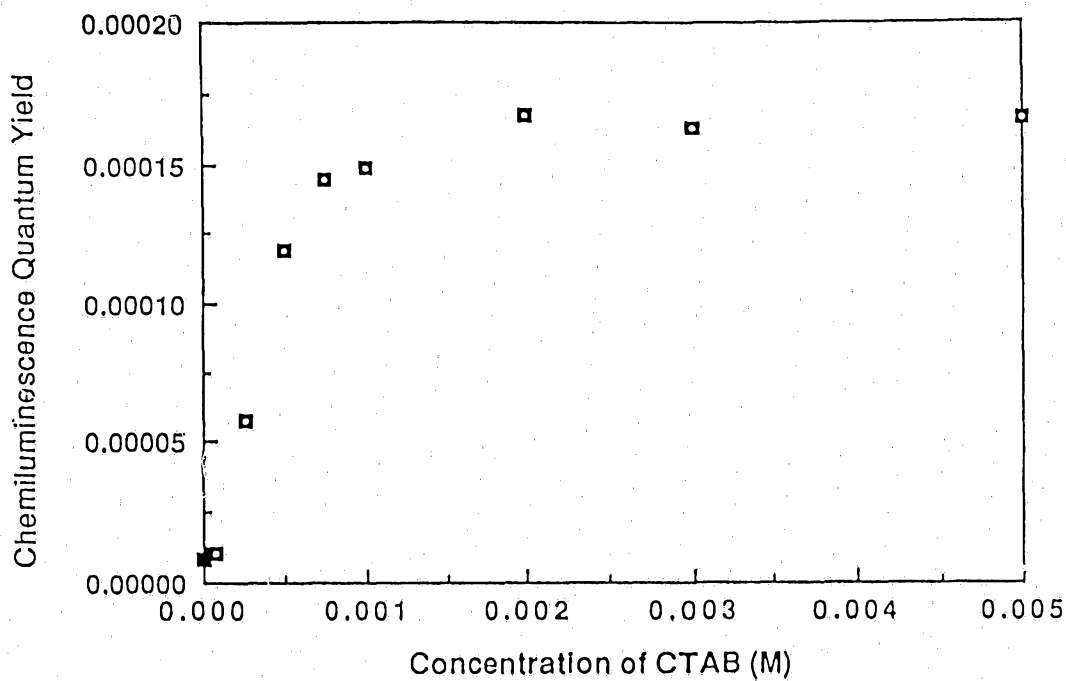


FIGURE 2

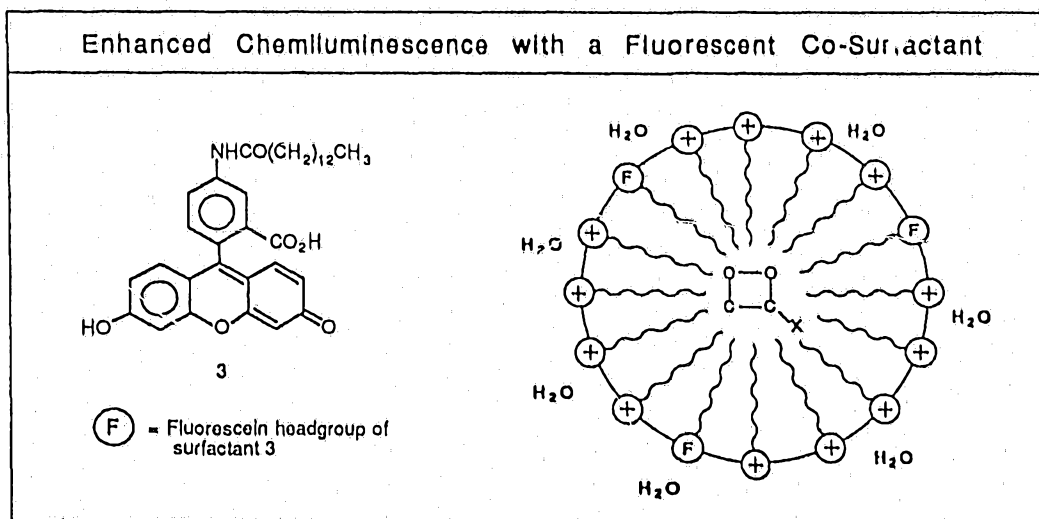


FIGURE 2a

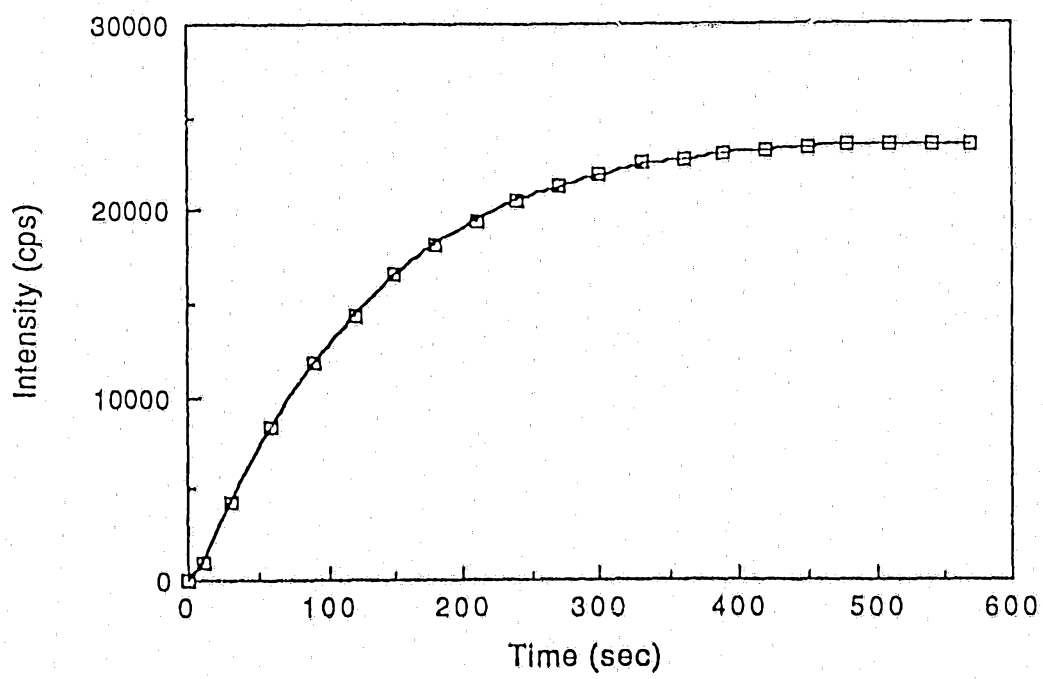


FIGURE 3

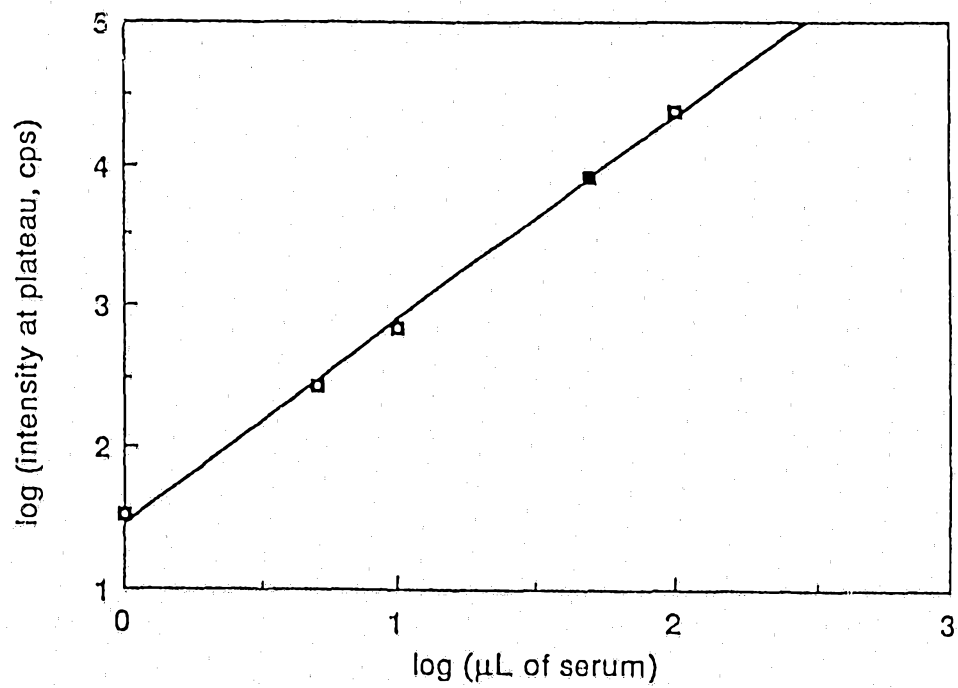


FIGURE 4

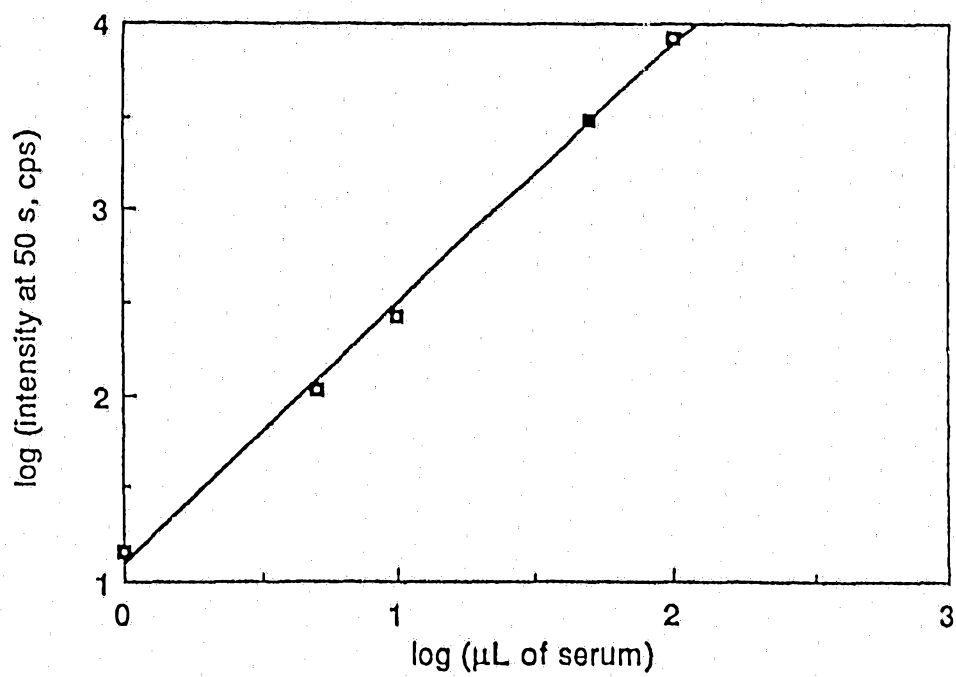


FIGURE 5

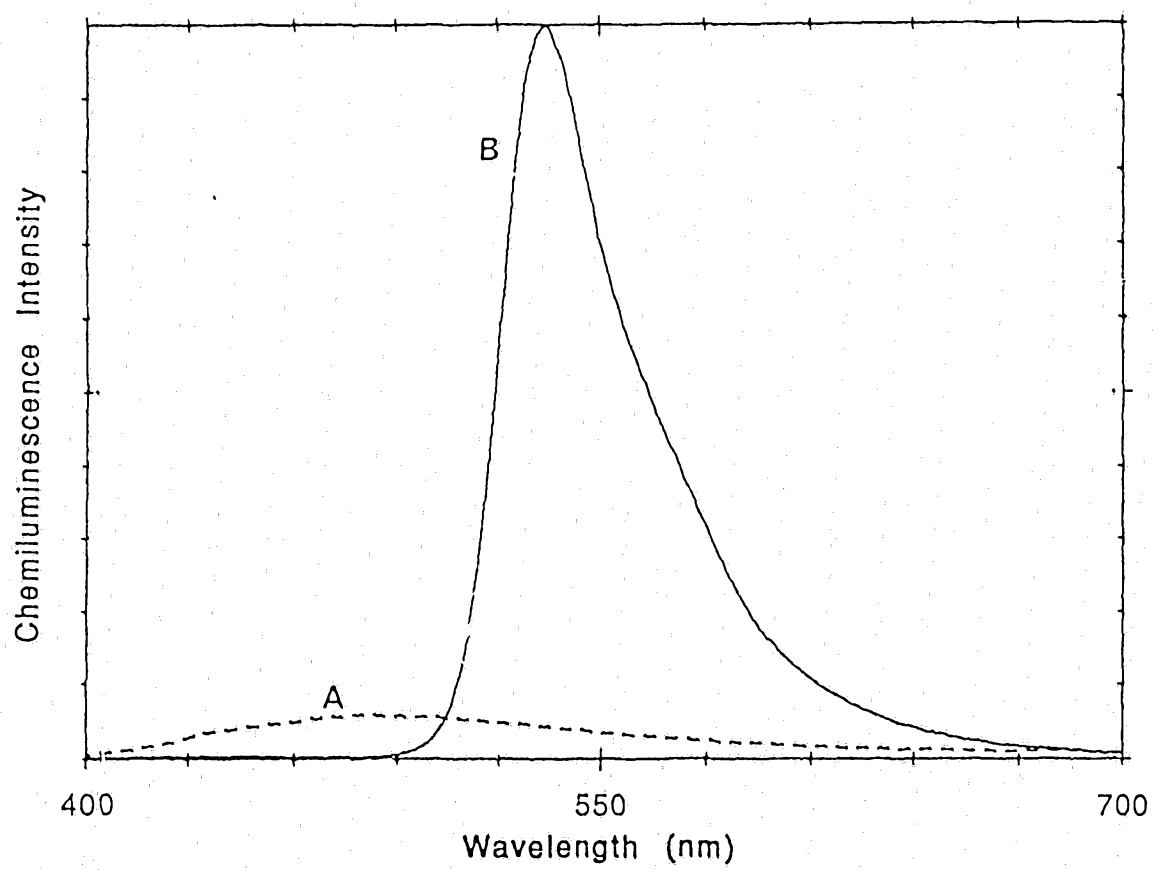


FIGURE 6

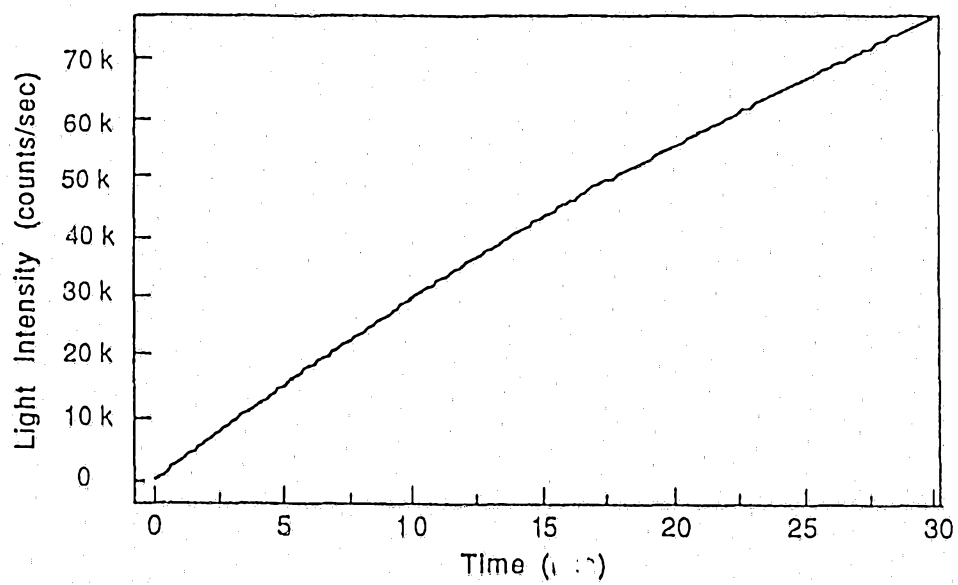


FIGURE 7

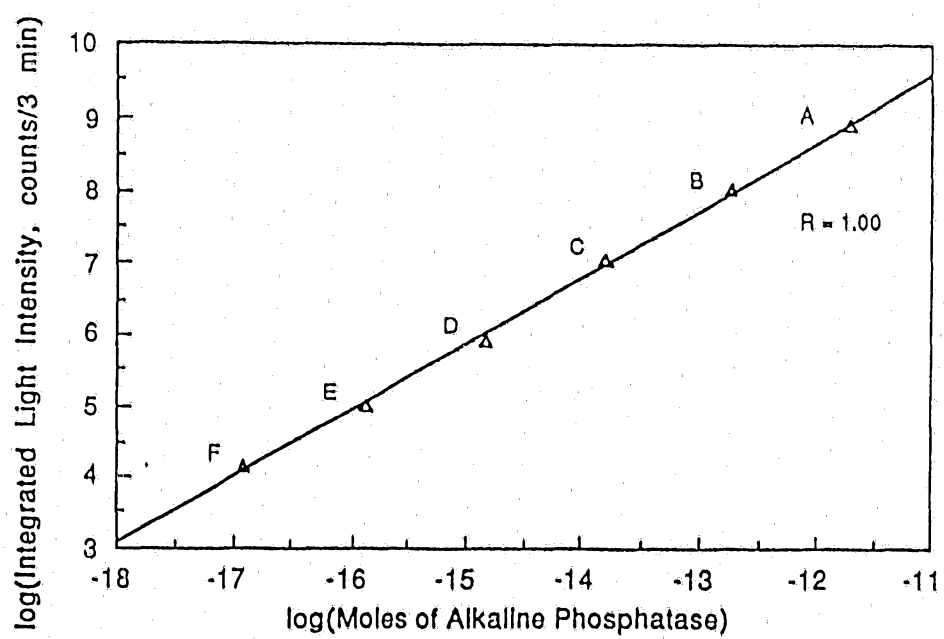


FIGURE 8

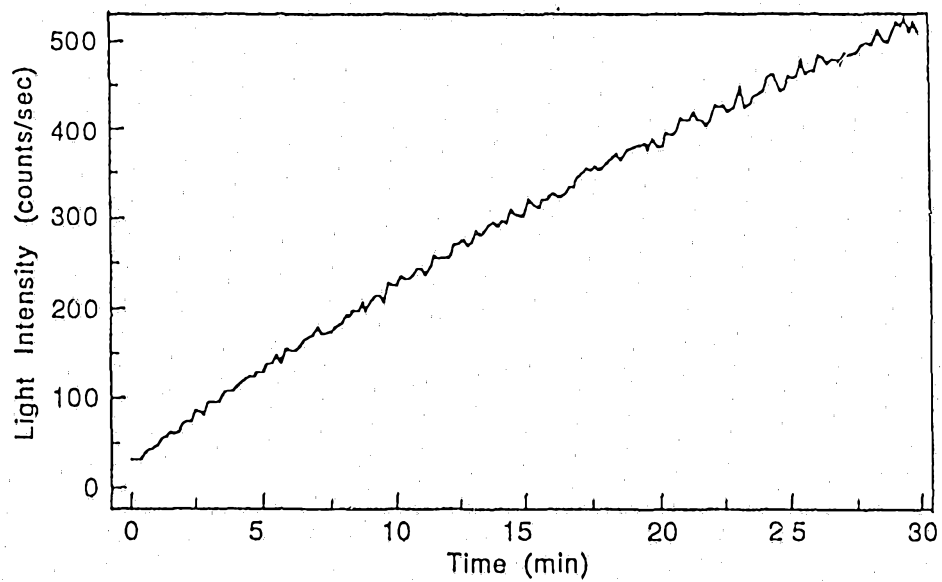


FIGURE 9

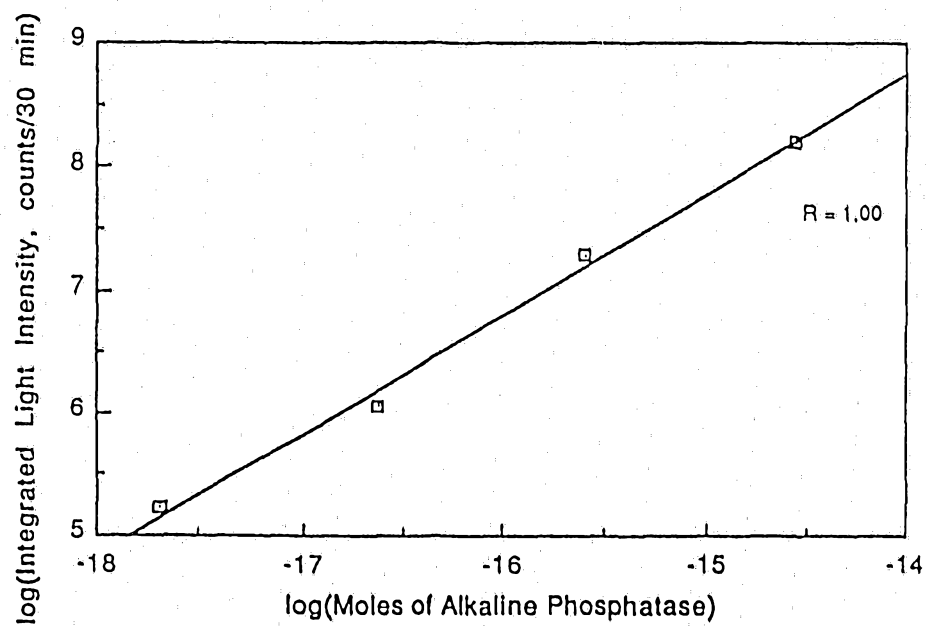
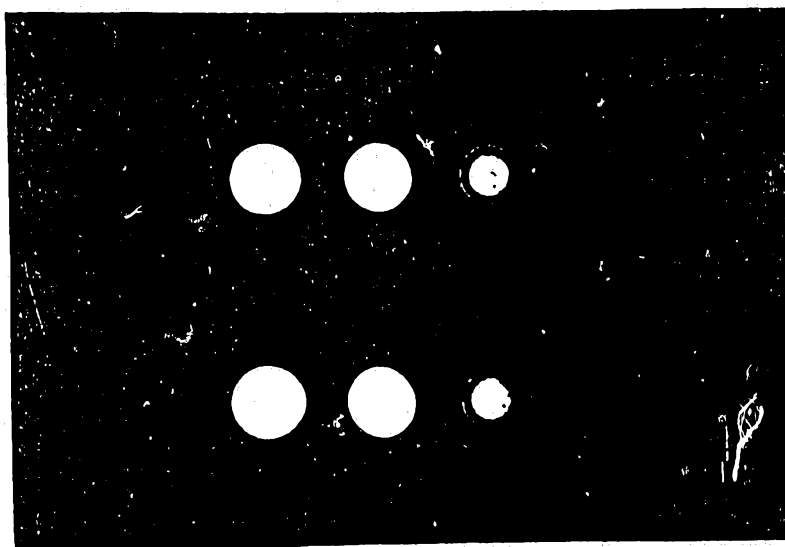
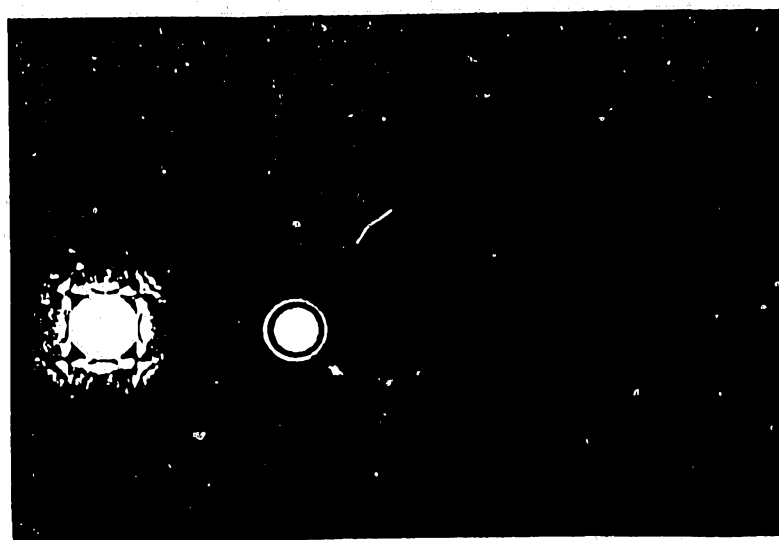


FIGURE 10



A B C D

FIGURE 11



A B C D

FIGURE 12

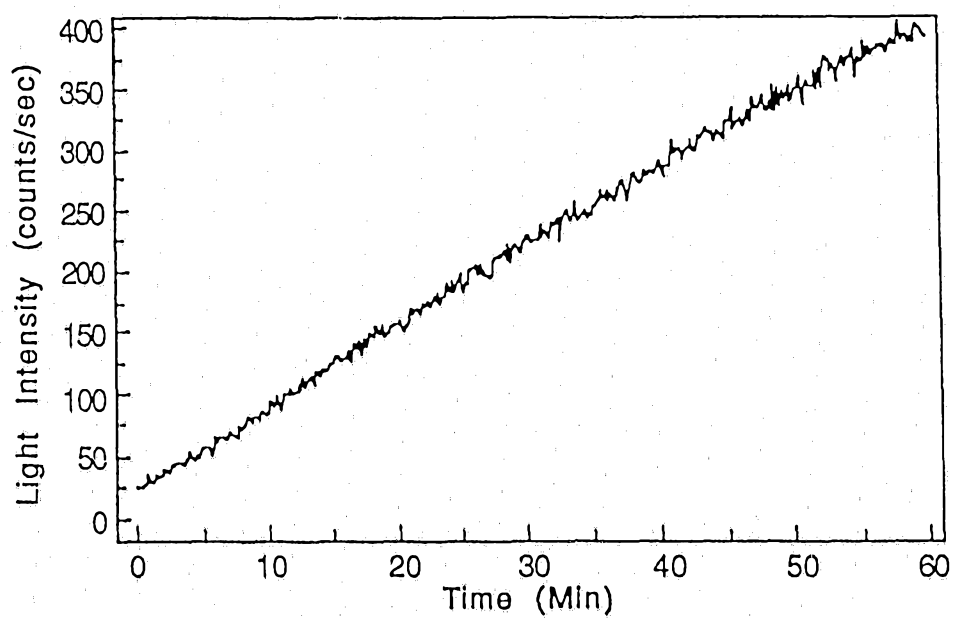


FIGURE 13

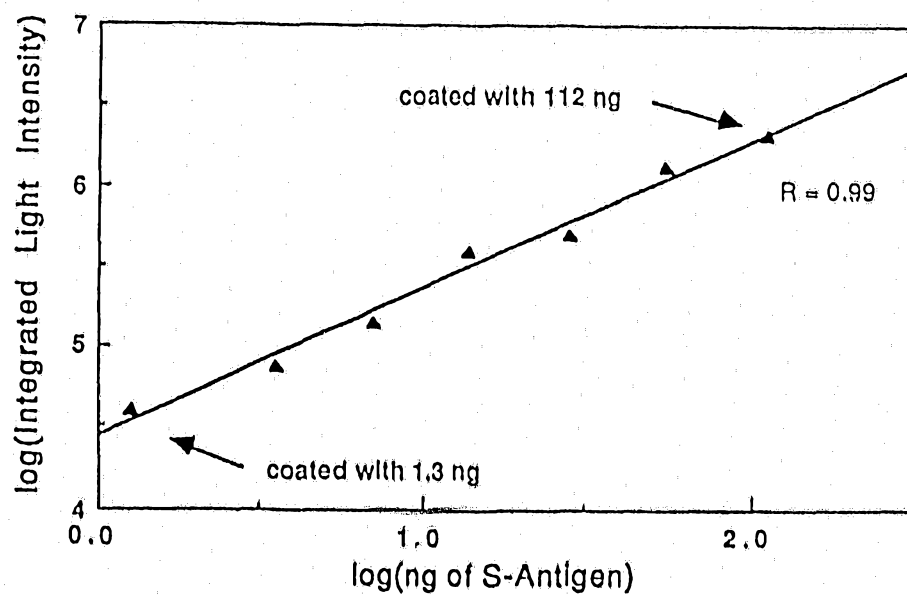


FIGURE 14

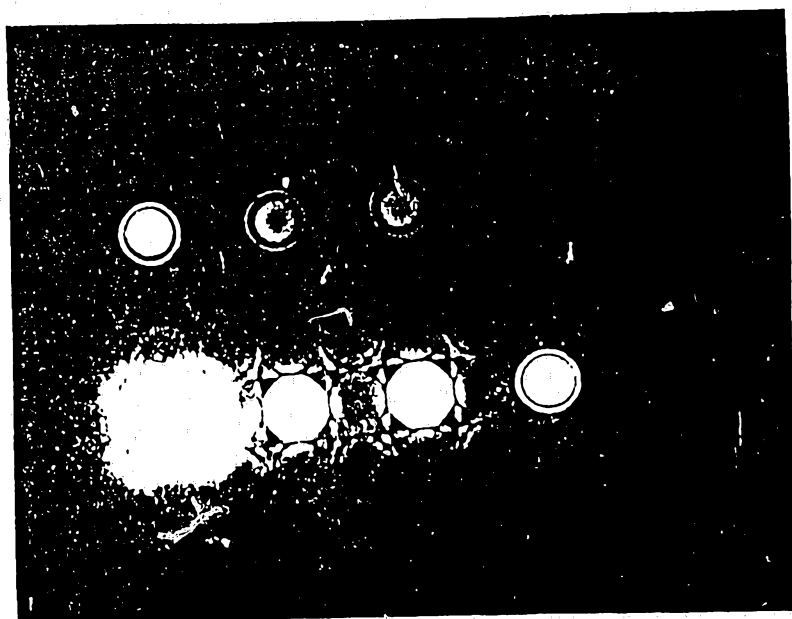


FIGURE 15

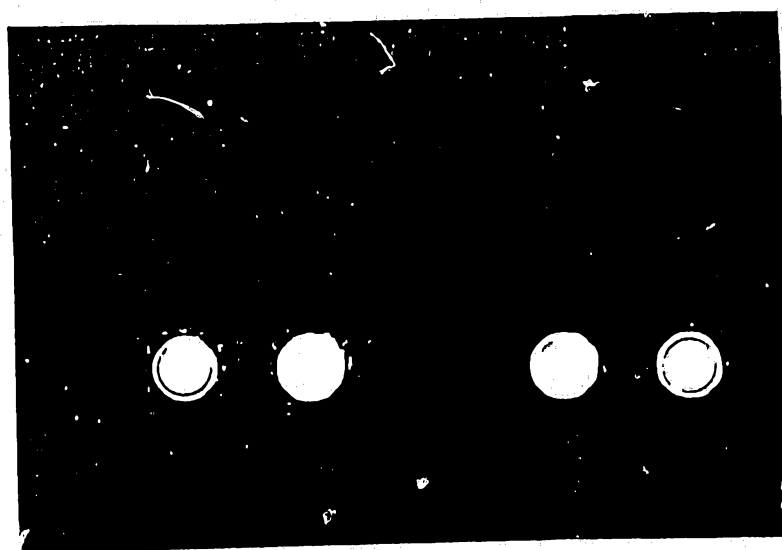


FIGURE 16