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NOTICE OF ENTITLEMENT

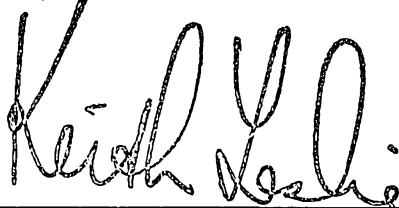
We, **Charm Sciences, Inc.** the applicant in respect of Application No. 35846/93, state the following:-

The Nominated person is entitled to the grant of the patent because the Nominated person derives title to the invention from a person who would, on the grant of a patent for the invention, be entitled to have the patent assigned to it.

The Nominated person is entitled to claim priority from the application listed in the declaration under Article 8 of the PCT because the Nominated person is the assignee of the applicant in respect of the application listed in the declaration under Article 8 of the PCT.

The application listed under Article 8 of the PCT is the first application made in a Convention country in respect of the invention.

Dated this 6th day of September, 1995



(A member of the firm of DAVIES COLLISON CAVE for
and on behalf of the applicant)



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TEST KIT AND METHOD FOR THE DETERMINATION OF PESTICIDES
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- (57) Claim

1. A bioluminescent method for the determination of the concentration of organophosphate and carbamate pesticides in a test sample, which method comprises:

- a) incubating a test sample and an insect brain material;
- b) adding a 6-substituted D luciferin ester to the incubated mixture, which D luciferin ester is inhibited in hydrolysis in the presence of the pesticide;
- c) incubating the D luciferin ester derivative admixture to liberate D luciferin;
- d) adding a mixture of adenosine triphosphate and luciferase to the D luciferin ester admixture to provide oxyluciferin and emitted luminescence;
- e) measuring the emitted luminescence; and
- f) determining the concentration of the pesticide in the test sample by comparison of the emitted measured luminescence with the luminescence of a control.

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12. A test kit when used in the bioluminescent determination of the concentration of organophosphate and carbamate pesticides in a test sample, which test comprises in combination:

- a) an insect brain material sensitive to the pesticide;
- b) a 6-substituted luciferin ester whose hydrolysis is inhibited by the pesticide to be added to an incubated test sample and the brain material; and
- c) luciferase and ATP to be added to the incubated luciferin derivative mixture.

PC ANNOUNCEMENT OF THE LATER PUBLICATION OF AMENDED CLAIMS
(AND, WHERE APPLICABLE, STATEMENT UNDER ARTICLE 19)



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US93/00436 (22) International Filing Date: 7 January 1993 (07.01.93) (30) Priority data: 07/818,782 9 January 1992 (09.01.92) US (71) Applicant: CHARM SCIENCES, INC. [US/US]; 36 Franklin Street, Malden, MA 02148 (US). (72) Inventors: ZOMER, Eliezer ; 374 Kenrick Street, Newton, MA 02158 (US). SAUL, Steven ; 11 Norkfolk Road, Arlington, MA 02174 (US). CHARM, Stanley, E. ; 10 Rowes Wharf, Boston, MA 02110 (US). (74) Agent: CROWLEY, Richard, P.; P.O. Box 901, 901 Main Street, Osterville, MA 02655-0901 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>With amended claims.</i> Date of publication of the amended claims: 19 August 1993 (19.08.93)
(54) Title: TEST KIT AND METHOD FOR THE DETERMINATION OF PESTICIDES (57) Abstract A test kit and method for a rapid and sensitive method for the multiple detection of organophosphate and carbamate pesticides. The test method employs an insect brain preparation having mixture of receptors or enzymes with sites that interact with organophosphate and carbamate pesticides. The pesticides alter or cause a significant reduction in the brain activities of the insect brain preparation which are inversely correlated with the amount of the pesticide present in the test sample. The activity is measured by employing tracer analogs or substrates which upon exposure to the insect brain preparation are altered and the changes monitored by a light emission reaction. In one embodiment, like acetyl luciferin, the hydrolysis of a luciferin derivative to D luciferin by an insect brain preparation has been found to be extremely sensitive to the organophosphates and carbamates. The luciferin liberated by the reaction is oxidized by the enzyme luciferase and adenosine triphosphate (ATP), an energy donor, and emits light measured as bioluminescence or chemiluminescence.		

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DESCRIPTION

Test Kit and Method for the Determination of Pesticides

Background of the Invention

Different kinds of organisms (arthropods, avians, mammals) are sensitive to pesticides. Pesticides are generally classified as herbicides, fungicides and insecticides. Pesticides interact with their nervous and enzymatic systems. Such toxicants may bind to binders (ion channels) located on the nerve cells, or to enzymes located around them and elsewhere. Pesticides also interact with various protective mechanisms, such as degrading enzymes and non-specific binders.

The brain and the nerve system of insects in general were the target for insecticides since their early development (1940s) for pest control in agriculture and human health. Pesticides that target the insect brain as the site of action usually display rapid action and require low dosage for good control.

Since the early 1970s, attempts have been made to use in vitro brain preparation to study the mode of action of pesticides. Only limited studies were reported on using this method for monitoring pesticides. Lack of stability and sensitivity as compared with the traditional gas chromatography (GC) or mass spectrometry made this early attempt useless. The use of color reaction requires high enzyme and substrate concentration to monitor brain preparation activity and the results were insensitive assays. The enzymes used were taken through laborious purifications, and it was found that only a limited spectrum of pesticides could be detected. Apparently, the source and the purified preparation were of limited sensitivity and for only a few pesticides which made it unsuitable as a monitoring and screening assay.

In recent years, bioluminescence or chemiluminescence has been used to detect extremely small quantities of material. Assays using these signals have been developed to measure substrates, such as ATP or NADH, and enzymes, such as alkaline phosphatase. Advantages to using bioluminescence as an assay system is the sensitivity of the reaction and the speed in which that reaction can be measured. For example, the bioluminescence reaction with the enzyme luciferase catalyzes the reaction between luciferin and ATP to produce light in milliseconds.

It is desirable to test for the concentration of pesticides in various materials, such as soil and water for health and safety purposes. In particular, it is desirable to provide an effective test kit and method to determine the concentration level of organophosphate and carbamate pesticides at low levels, such as below 50 ppb and even as low as 5 ppb. Specific organophosphate insecticides may be tested employing antibodies, but these have limited use as broad spectrum screening methods. Herbicides may be tested on a specific basis by chromogenic enzyme-based test methods, but such tests do not provide accurate results at low concentration levels and are susceptible to color interpretation.

Therefore, a new, accurate, effective test kit and method for the determination of pesticides, such as organophosphate and carbamate pesticides, are desirable.

Summary of the Invention

The invention relates to a test kit and method for the determination of pesticides. In particular, the invention concerns a chemiluminescence or bioluminescence test method and test kit for the determination of organophosphate and carbamate-type pesticides at levels below about 50 ppb.

~~A test kit and method has been developed for the rapid, generally 10 to 15 minutes, and sensitive, less than 50 ppb or lower, method for the multiple detection~~



According to one aspect of the present invention there is provided a bioluminescent method for the determination of the concentration of organophosphate and carbamate pesticides in a test sample, which method comprises:

- a) incubating a test sample and an insect brain material;
- 5 b) adding a 6-substituted D luciferin ester to the incubated mixture, which D luciferin ester is inhibited in hydrolysis in the presence of the pesticide;
- c) incubating the D luciferin ester derivative admixture to liberate D luciferin;
- d) adding a mixture of adenosine triphosphate and luciferase to the D luciferin ester admixture to provide oxyluciferin and emitted luminescence;
- 10 e) measuring the emitted luminescence; and
- f) determining the concentration of the pesticide in the test sample by comparison of the emitted measured luminescence with the luminescence of a control.

According to another aspect of the present invention there is provided a method for the determination of the concentration of organophosphate and carbamate pesticides
15 in a test sample, which method comprises:

- a) incubating a test sample and an insect brain homogenate material selected from the group consisting of: bees; flies; and silkworms;
- b) adding D luciferin acetate to the incubated mixture;
- c) incubating the D luciferin acetate mixture to liberate D luciferin;
- 20 d) adding a mixture of adenosine triphosphate and luciferase to the D luciferin ester admixture to provide oxyluciferin and emitted luminescence;
- e) measuring the emitted luminescence during a defined time period; and
- f) determining the concentration of the pesticide in the test sample by comparison of the emitted, measured luminescence with the luminescence of a control
25 sample or standard.

According to a further aspect of the present invention there is provided a test kit when used in the bioluminescent determination of the concentration of organophosphate and carbamate pesticides in a test sample, which test comprises in combination:

- a) an insect brain material sensitive to the pesticide;
- 30 b) a 6-substituted luciferin ester whose hydrolysis is inhibited by the pesticide to be added to an incubated test sample and the brain material; and
- c) luciferase and ATP to be added to the incubated luciferin derivative mixture.



According to a still further aspect of the present invention there is provided a method of preparing a 6-substituted D luciferin ester when used in the bioluminescent method or the test kit defined above, which method comprises:

- 5 reacting in a liquid solvent a substituted N-acylated imidazole compound with D luciferin to provide a substituted N-acylated derivative luciferin compound and imidazole.

A test kit and method has been developed for the rapid, generally 10 to 15 minutes, and sensitive, less than 50 ppb or lower, method for the multiple detection



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of organophosphate and carbamate pesticides in water, soil, food (e.g. meat, fish, fruits and vegetables) and other materials. The test method employs an insect brain preparation having a mixture of receptors or enzymes with sites that interact with the pesticides and particularly that react or interact with organophosphate and carbamate pesticides. The pesticides alter or cause a significant reduction in the brain activities of the insect brain preparation which are inversely correlated with the amount of the pesticide present in the test sample. The activity is measured by employing tracer analogs or substrates which upon exposure to the insect brain preparation are altered chemically and/or physically and the changes monitored by a light emission reaction. The activities can be chemical as in an enzymatic reaction or physical as in receptor binding.

For example, in one embodiment in an enzymatic reaction, the hydrolysis of a luciferin derivative to luciferin by a brain preparation has been found to be extremely sensitive to organophosphates and carbamates. The luciferin liberated by the reaction ~~is~~^{may be} oxidized by the enzyme luciferase and adenosine triphosphate (ATP), an energy donor, and emits light measured as bioluminescence or chemiluminescence. The bioluminescence or chemiluminescence emitted is measured and monitored at a low level and high speed employing a luminometer or scintillation counter. The assay is carried out in three simple steps of a short incubation period, e.g. 2 to 10 minutes, of the test sample and the brain preparation; addition of a substrate tracer or pesticide analog tracer, such as a luciferin derivative with additional incubation, e.g. 2 to 5 minutes; and the measurement directly or after a separation step to obtain liquid fraction for light emission by activation with the enzyme (luciferin-luciferase). The general assay preparation is illustrated in Table 1.

Therefore, a ^{preferred} test method has been found for the determination of pesticides which are sensitive to insect

brain preparation and particularly organophosphate and carbamate pesticides. The test method comprises incubating a mixture of a test sample and an insect brain preparation; adding to the incubated mixture a D luciferin derivative, such as, but not limited to: D luciferin acetate, a novel compound whose hydrolysis is inhibited in the presence of the pesticide; incubating the test sample, brain preparation and D luciferin derivative mixture to liberate luciferin; admixing a portion of the incubated D luciferin derivative-containing admixture with ATP and luciferase as a reaction mixture to provide an oxidized luciferin (oxyluciferin) and emitted luminescence; measuring the emitted luminescence, for example, with a luminometer; and determining the concentration of the pesticide in the test sample by comparison of the emitted, measured luminescence of a standard or of a control sample.

The test kit for the determination of pesticides comprises in combination an insect brain preparation which is sensitive to the pesticide, e.g. bee brain homogenate, a D luciferin derivative which is inhibited in hydrolysis in the presence of the pesticide, such as D luciferin acetate; the enzyme luciferase and ATP to form a reaction mixture when added together to the incubated test sample, brain preparation and D luciferin derivative. The test kit may include those standard articles of laboratory equipment and chemicals, like buffers, needed to carry out and measure the results to include, but not be limited to: incubation dishes or plates and an incubation water bath, etc.; buffers; a luminometer to measure emitted luminescence; a standard control chart or graph of luminescence vs. pesticide concentration for comparison and determination of the pesticide concentration; and separating equipment, such as a chromatographic column or ultrafiltration membrane to obtain a liquid fraction of the incubated mixture.

A wide variety of insect brain tissue material may be used in the test method of the invention, such

as the crude, stabilized brain tissue, particularly the homogenate of arthropods, for example, but not limited to: bees; beetles; aphids; mosquitoes; silkworms; mites; blow flies; and houseflies (Musca domestica) alone or
5 in combination.

Brain preparations from various sources have differences in specificity for pesticides. Therefore, it is important to obtain one or more brain preparations which will react with a broad spectrum of pesticides
10 for monitoring purposes. Insects as the target for pesticides are one of the best sources for brain preparations. Specificity and sensitivity can vary from one insect to another. Bees were chosen as a preferred choice for their known sensitivity to a variety
15 of insecticides. It was found that bee brain preparation was approximately 2 to 4 logs more sensitive in the bioluminescence assay system than any previously reported assay system.

One D luciferin derivative suitable for use in the pesticide test method comprises 6-acetyl D luciferin or luciferin acetate. Suitable luciferin derivatives are those 6-substituted D luciferin compounds which in the presence of insect brain preparation react to cleave the substituted ester group at the 6-position
20 of the D luciferin derivative to provide D luciferin for further reaction with ATP and luciferase to produce luminescence which can be measured. The luciferin derivative 6-acetyl D luciferin is prepared by reacting D luciferin with excess acetyl imidazole in
25 a solvent, e.g. water-solvent solution which acetylates D luciferin under mild conditions with about 100% efficiency to provide the 6-acetyl D luciferin.

It has been found that both organophosphate and carbamate pesticides inhibit the hydrolysis of
35 D acetyl luciferin by insect brain preparations. There are significant advantages in the use of a D luciferin derivative, like D luciferin acetate, as a substrate



in the test method. There is an unusual, unexpected, high specificity of the bee or silkworm brain preparations toward D luciferin acetate and only small quantities of the D luciferin acetate are required for the assay.

5 For example, the concentration of D luciferin acetate in the incubation mixture may be as low as 1×10^{-11} moles or less and the assay sensitivity for luciferin is 1 to 5×10^{-13} moles. The assay time is about 10 minute in total. While D luciferin acetate has been

10 found to be a preferred D luciferin derivative for use in the test method, other D luciferin derivatives with similar reactions may be employed. Those luciferin derivatives disclosed in the publication of the Journal of Clinical Chemistry and Chemical Biochemistry entitled

15 "Synthesis and Characterization of Luciferin Derivatives for Use in Bioluminescence-Enhanced Enzyme Immunoassay New Ultrasensitive Detection Systems for Enzyme Immunoassay", Miska et al, J. Clin Chem Clin Biochem 25(1) 1987, pp. 23-30, are not suitable for use in the

20 test method and include specifically D-luciferin methyl ester, D-luciferyl-L-phenylalanine, D luciferyl-L-Na-arginine, D-luciferin-O-sulphate and D-luciferin-O-phosphate.

To test the ability of the assay system to monitor

25 for the presence of pesticides in food, six apples from different sources were tested. Of the six apples, four apples were positive and two apples were negative. The four positive apples were obtained from local orchards, while the negative apples were purchased from

30 local supermarkets. Local water was also tested and was found marginal positive. Table 3 gives detection levels expected in water for 15 different organophosphate and carbamate pesticides.

Test Procedure Used for Organophosphate and Carbamate

35 Pesticides

1. Sample is preincubated with a predetermined quantity (dilution 1:200) of brain preparation for 5 minutes.

2. Luciferin acetate (200⁷ pmoles) is added to the incubation mixture for additional 5 minutes.
 3. A portion of this incubation mixture (100 µl) is withdrawn and added to the luciferase and ATP reaction mixture (1 ml).
 4. Bioluminescence reading for 2-5 seconds is monitored.
 5. The reading of the pesticide sample is compared to the control sample to quantitate the percent inhibition of the sample (see Tables 3, 4, 5 and 6).
- 10 The invention will be described for the purposes of illustration only in connection with certain embodiments; however, it is recognized that those persons skilled in the art may make various improvements, additions, changes and modifications to the illustrated
- 15 embodiments all falling within the spirit and scope of the invention.

Description of the Embodiments

A. Synthesis of luciferin acetate (6-acetyl D luciferin)

- Dissolve 2 mg sodium D-luciferin (purified from firefly) into 2 ml water, or dissolve 2 mg synthetic D-luciferin into 2 ml methanol as stock 3.3 mM luciferin solutions in an amber vial. Take 25 µl of either stock solution and add it to 1 ml distilled water in an amber microcentrifuge tube to get an 82.5 µM solution of luciferin. Dissolve 60 mg of N-acetylimidazole into 1.0 ml acetone to get a 545 mM solution of N-acetylimidazole. Add 30 µl of 545 mM solution of N-acetylimidazole to the 82.5 µM aqueous solution of luciferin. Mix several times and monitor the decrease of bioluminescence as the reaction continues to completion. The reaction is complete when bioluminescence can no longer be observed when using the above reaction mixture as a source for luciferin. Keep solution on ice. The concentration of N-acetylimidazole in this reaction mixture is 16.35 mM or approximately 200 times the concentration of luciferin.

D-Luciferin from firefly was purchased from Sigma. Synthetic D-luciferin was purchased from Boehringer Mannheim. Also, a D-luciferin is available from Bio-Orbit. Luciferin stock solution from Sigma was dissolved in water while D-luciferin stock solution from Boehringer or Bio-Orbit was dissolved in methanol. N-acetylimidazole was purchased from Sigma. The reaction described above was performed in 1 ml and 1 concentration range, but it could be scaled up using a larger volume size. Other derivatives of luciferin, such as luciferin phosphate, luciferin sulfate, luciferin arginine have been described in the literature, but to our knowledge, luciferin acetate has not been described, nor to our knowledge has it been used in a coupled reaction with brain extract to monitor pesticides.

B. Preparation of Insect Brain Extract

Insects are stored from at -20°C or below. Insect heads are collected by dissection with a scalpel and placed in a 50 ml beaker containing approximately 15 ml ice cold 0.07 M phosphate buffer, pH 7.0, containing 1 mM EDTA (ethylenediaminetetra acetic acid) and 1 μM phenylthiourea. The insect heads are gently homogenized at low speed in a Tekmar Tissumizer. Aliquots of this crude extract are transferred to microcentrifuge tubes and centrifuged. The supernatant is retained and applied to a sephadex G-25 column equilibrated with 0.07 M phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 μM phenylthiourea. Fractions are collected every 2 minutes and monitored for esterase activity using luciferin acetate as a substrate and measuring bioluminescence. Fractions with esterase activity are pooled and used as the insect head extract for use in the pesticide assay.

Brain extracts have been prepared primarily from honey bees, but also has been extracted from silkworm and blowflies. Protein concentration of the bee head extract is between 2 to 5 mg/ml. For the assay, 5 to 10 μl is used per assay.

C. Bioluminescence Reaction

1. Reaction buffer for bioluminescence: Weigh out 4.48 g tricine, 0.6 g magnesium sulfate, 0.146 g EDTA, 100 mg bovine serum albumin, and 77 mg dithiothreitol into 600 ml water. Adjust pH to 7.8 with 10% sodium hydroxide and add distilled water to 1 liter.

2. Luciferase preparation: For luciferase (1 mg) from firefly purchased from Boehringer Mannheim dissolve in 1 ml 0.5 M Tris-acetate buffer, pH 7.5, and let stand 30 minutes. Portion out 40 μ l of this solution into glass test tubes and freeze at -20°C . Redissolve frozen stock with 1 ml bioluminescence buffer. For luciferase purchased from Bio-Orbit take 3 mg and dissolve in 1 ml bioluminescence buffer. Each luciferase solution is kept on ice. For each bioluminescent assay, a 30 μ l aliquot from either luciferase solution described above is used. For the Boehringer luciferase, approximately 1 μ g enzyme is used for each assay.

3. ATP Preparation: ATP (adenosine 5'-triphosphate) is preweighed from Sigma and contains 1 mg ATP and 40 mg magnesium sulfate. To this vial add 10 ml water and 100 μ l 1.0 M HCl and vortex. ATP solution is kept on ice. The stock solution is 1.8 mM and for each bioluminescent assay, 30 μ l is used for a final concentration of 54 μ M.

4. Luciferin Acetate: Take 100 μ l 82.5 μ M solution of luciferin acetate prepared as described above in section A and 900 μ l distilled water and add to amber microcentrifuge tube. This solution is 8.25 μ M. For pesticide assays, a 25 μ l portion of the 8.25 μ M solution is added to 2 ml 0.07 M phosphate buffer, pH 7.0, to give a 0.1 μ M concentration of luciferin acetate. A 100 μ l portion of this solution is withdrawn and incubated with 1 ml of the bioluminescent buffer. Final concentration is approximately 10 nM luciferin acetate or 10 pmoles luciferin acetate.

5. Bioluminescence Measurement: A 1 ml portion of the bioluminescent buffer is added to a 13 x 100 mm test tube. A 100 μ l portion of the luciferin acetate solution (0.1 μ M) is withdrawn and added. Then 30 μ l aliquots of the luciferase and ATP solutions are added to the test tube. The test tube is vortexed and the bioluminescence measured after 2 to 5 seconds using a luminometer. The bioluminescence reading with luciferin acetate will be negative. If luciferin is measured at 10 pmoles then the reading in the luminometer will be approximately 100,000. If the concentration of any of the reagents described above is increased, then the light reading will be increased. Although the present concentrations give acceptable values, the concentration of the reagents may be altered to give optimal performance. The above liquid reagents may also be immobilized in individual tablet form to provide stability and consistency to the reagents, such as in compressed tablet form with inert cellulosic fillers.

D. Assay of Pesticides Using Bioluminescence

1. To 13 x 100 mm test tubes add 2 ml 0.07 M phosphate buffer, pH 7.0. Add 25 μ l positive pesticide control (organophosphate and/or carbamate pesticide), 25 μ l water to negative control tube and 25 μ l of test sample to another tube. If test sample contains solvent other than water, then use this solvent as the negative control tube.

Although 25 μ l is indicated above, the volume size of sample could be less or greater as long as brain activity is not affected by the solvent.

2. Add insect brain extract (5 or 10 μ l depending on activity) to the 2 ml incubation mixture in step 1, vortex and incubate at 35°C in temperature block for 10 minutes. Use timer with alarm to monitor time.

3. At 10 minutes, add 25 μ l 8.25 μ M luciferin acetate solution to 2 ml incubation mixture in step 2. Reset timer to zero and restart timer.

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4. At 5 minutes, take 100 μ l portion from each sample. Use separate pipette tip for each sample and add the 100 μ l portion to separate 13 x 100 mm test tubes containing 1 ml of bioluminescence buffer.

5 In this reaction, the sample is withdrawn after 5 minutes. Since the reaction is kinetic, the concentration of luciferin formed in the reaction will increase with time. Therefore, the time an aliquot can be withdrawn can vary as long as it is in the linear section of the curve, and aliquots from samples are withdrawn at the same time.

10 5. Add 30 μ l ATP solution followed by 30 μ l luciferase solution to one of the assay tubes in step 4. Vortex and measure bioluminescence for 2 to 5 seconds. 15 Record value and proceed to the next tubes as above.

6. Zero control bioluminescence reaction will be uninhibited by pesticide and will have a high bioluminescence reading. In the positive samples the esterolytic activity of the bee extract will be inhibited 20 and less luciferin will form giving a low bioluminescent reading. Divide sample reading or positive control by zero control and multiply to 100 to get percent inhibition.

25 The source for some of the chemicals used has been set forth; however, other suppliers can supply these chemicals and therefore, the procedure is not reliant on any one source for a particular reagent except for the preparation of luciferin acetate.

30 Tables 5-11 represent test data showing the increase in bioluminescence as a function respectively of time, luciferase, ATP, luciferin acetate and bee head brain preparation in the test assay.

TABLE 1

Schematic of Pesticide Assay

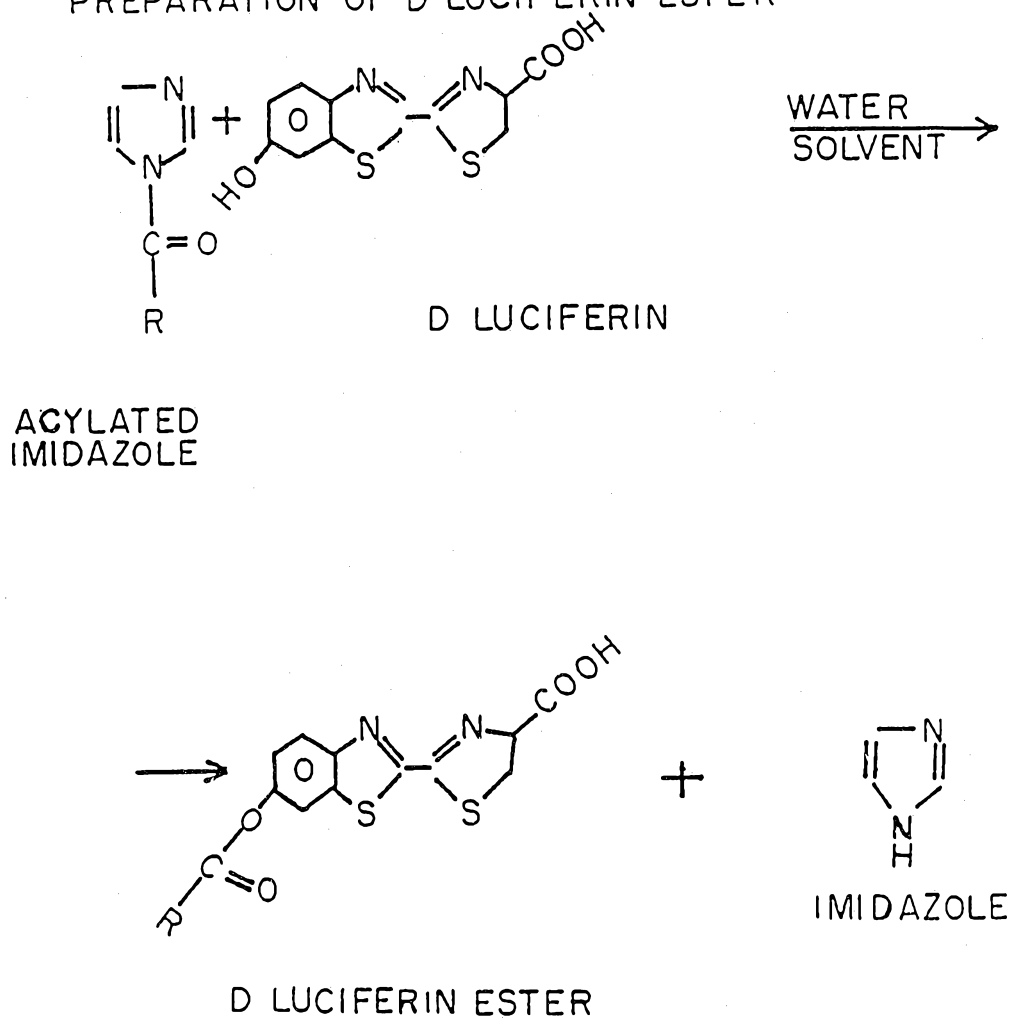
1. BE ^{a)} (brain extract)	+	pesticide ^{b)}	35°C	BE - pesticide + BE
2. D Luciferin derivative ^{c)}			BE	D luciferin + luciferin derivative
3. ATP + luciferin			Luciferase	oxyluciferin + pyrophosphate + AMP (adenosine monophosphate) + luminescence (to be measured) ^{d)}

Legend

- a) for example, insect bee brain homogenate
- b) organophosphate and carbamate pesticides
- c) for example, D luciferin acetate
- f) luminometer

TABLE 2

PREPARATION OF D LUCIFERIN ESTER



LEGEND

R IS AN ALKYL GROUP, e.g. C₁-C₈,
OR PHENYL GROUP

14
TABLE 3

Charm Pesticide Assay for
Organophosphate and Carbamate Inhibition
of Bee Brain Activity for Ac-luciferin

<u>Pesticide</u>	<u>I₅₀^{a)}</u> <u>(ppb)</u>
Carbamates	
Methomyl	4
Propoxur	10
Carbofuran	8
Bendocarb	12
Organophosphates	
Mevinphos	2
Ethion	2
Chlorpyrifos	1
Phorate	2
Malathion	6
Oxydemeton-methyl	1
Disulfoton	5
Methyl parathion	1
DDVP	0.004
Naled	0.05
Diazinon	15

a) I₅₀ is the concentration of inhibitor which gives a 50% decrease in enzymatic activity as measured by bioluminescence.

TABLE 4

Inhibition of Silkworm Brain Activities by
Various Organophosphate Pesticides at 25 PPB

<u>Pesticide</u>	<u>%</u> <u>Inhibition</u>
Phorate	50.5
Naled	98.0
Methyl parathion	-0-
Ethion	77.0
Oxydemeton-methyl	-0-
Diazinon	-0-
DDVP	100.0
Disulfoton	25.0
Mevinphos	50.0

TABLE 5

Inhibition of Bee Brain Extract by Organophosphates as a Function of Pesticide Concentration

<u>Methyl Parathion</u>		<u>Phorate</u>		<u>Chlorpyrifos</u>		<u>Ethion</u>	
ppb	% activity	ppb	% activity	ppb	% activity	ppb	% activity
0	100	0	100	0	100	0	100
0.625	62	1.25	70	0.625	68	0.3125	84
1.25	40	2.5	29	1.25	34	0.625	71
2.5	23	3.125	23	2.50	24	1.25	65
5.0	15	6.25	8	3.125	13	2.5	37
		12.5	3	6.25	10		
<u>Diazinon</u>		<u>Oxydemeton-methyl</u>		<u>Disulfoton</u>		<u>Mevinphos</u>	
ppb	% activity	ppb	% activity	ppb	% activity	ppb	% activity
0	100	0	100	0	100	0	100
25	40	0.3125	90	0.3125	66	0.3125	64
50	45	0.625	43	6.25	47	0.625	52
100	28	1.25	47	12.5	35	1.25	48
		2.5	37	25.0	19	2.5	24
		25.0	21			3.125	12
						6.25	9
<u>Malathion</u>		<u>DDVP</u>		<u>Naled</u>			
ppb	% activity	ppb	% activity	ppb	% activity		
0	100	0	100	0	100		
6.25	53	0.0013	74	0.031	50		
12.5	50	0.0023	49	0.625	29		
25.0	27	0.0028	60	0.125	30		
50.0	27	0.0057	52	0.250	25		
		0.011	25				

15

¹⁶
TABLE 7

Increase in Bioluminescence as a Function
of Time Using Pesticide Assay in Control Reaction

<u>Time (min)</u>	<u>Light Reading</u>
0	0
0.75	390
1.75	1230
2.75	3030
3.75	3510
5.0	5550
7.0	9360
9.0	11460
11.0	13200
14.0	20640
16.0	27300

TABLE 8

Increase in Bioluminescence as a Function of
Luciferase Using the Pesticide Assay in Control Reaction

<u>Luciferase (μg)</u>	<u>Light Reading</u>
0	0
0.17	390
0.34	1200
0.5	1470
0.67	4830
0.83	7740
1.0	8580
1.17	17280
1.34	20700
1.51	31868
1.68	40140

TABLE 9

Increase in Bioluminescence as a Function
of ATP Using the Pesticide Assay in Control Reaction

<u>ATP (μM)</u>	<u>Light Reading</u>
0	0
0.9	390
1.8	1289
3.6	4110
5.4	8220
9.0	20760
10.8	24060

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TABLE 10

Increase in Bioluminescence as Function of
Luciferin Acetate Concentrate
Using the Pesticide Assay in Control Reaction

<u>Luciferin Acetate (μM)</u>	<u>Light Reading (at 3 min)</u>
0	0
0.2	8520
0.4	15120
0.8	61680
1.6	180240
3.2	467280
6.4	724080

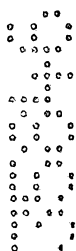
TABLE 11

Increase in Bioluminescence as a Function of
Bee Extract Concentration
Using Pesticide Assay in Control Reaction

<u>Bee Extract (μl)</u>	<u>Light Reading</u>
0	0
10	1920
20	6484
40	22260
80	28880

- 17a -

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.



THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

2 **Claim 1.** A bioluminescent method for the determination
of the concentration of organophosphate and carbamate
pesticides in a test sample, which method comprises:
4 a) incubating a test sample and an insect brain
material;
6 b) adding a 6-substituted D luciferin ester to the
incubated mixture, which D luciferin ester is inhibited
8 in hydrolysis in the presence of the pesticide;
c) incubating the D luciferin ester derivative
10 admixture to liberate D luciferin;
d) adding a mixture of adenosine triphosphate and
12 luciferase to the D luciferin ester admixture to
provide oxyluciferin and emitted luminescence;
14 e) measuring the emitted luminescence; and
f) determining the concentration of the pesticide in
16 the test sample by comparison of the emitted measured
luminescence with the luminescence of a control.

2 **Claim 2.** The method of claim 1 wherein the luciferin
ester is D luciferin acetate.

2 **Claim 3.** The method of claim 1 wherein the insect
brain material is selected from the group consisting of:
bees, blow flies, house flies, silkworms or other
4 arthropods.

2 **Claim 4.** The method of claim 1 wherein the insect
brain material comprises a brain homogenate of honey bee
heads.

2 **Claim 5.** The method of claim 1 which includes
determining the concentration of pesticides at 50 ppb or
lower.

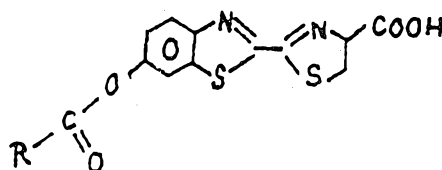
2 **Claim 6.** The method of claim 1 which includes
incubating the test sample and insect brain material for a
time period of about 2 to 10 minutes.

2 **Claim 7.** The method of claim 1 which includes
incubating the D luciferin ester admixture for a time period
of about 2 to 5 minutes.

2 **Claim 8.** The method of claim 2 wherein the
concentration of the D luciferin acetate in the admixture is
about 1×10^{-11} M or less.

2 **Claim 9.** The method of claim 1 wherein the pesticide
is selected from the group consisting of: methomyl;
propoxur; carbofuran; bendocarb; mevinphos; ethion;
4 chlorpyrifos; phorate; malathion; oxydemeton-methyl;
disulfoton; methyl parathion; DDVP; naled; and diazanon.

Claim 10. The method of claim 1 wherein the luciferin
ester has the structure:



wherein R is a monovalent organic radical selected from the
group of C_1 - C_6 alkyl and benzyl.

2 **Claim 11.** A method for the determination of the
concentration of organophosphate and carbamate pesticides in
a test sample, which method comprises:

- 4 a) incubating a test sample and an insect brain
homogenate material selected from the group consisting
6 of: bees; flies; and silkworms;
8 b) adding D luciferin acetate to the incubated
mixture;
10 c) incubating the D luciferin acetate mixture to
liberate D luciferin;
12 d) adding a mixture of adenosine triphosphate and
luciferase to the D luciferin ester admixture to
provide oxyluciferin and emitted luminescence;
14 e) measuring the emitted luminescence during a
defined time period; and
16 f) determining the concentration of the pesticide in
the test sample by comparison of the emitted, measured
18 luminescence with the luminescence of a control sample
or standard.

2 **Claim 12.** A test kit when used in the bioluminescent determination of the
concentration of organophosphate and carbamate pesticides in a test sample, which test
comprises in combination:



- a) an insect brain material sensitive to the pesticide;
- b) a 6-substituted luciferin ester whose hydrolysis is inhibited by the pesticide to be added to an incubated test sample and the brain material; and
- c) luciferase and ATP to be added to the incubated luciferin derivative mixture.

5 **Claim 13.** The test kit of claim 12 which includes:

- a) incubation means to incubate first the test sample with the brain material and later incubate the test sample and brain material with the 6-substituted luciferin ester;
 - b) means to measure the emitted luminescence; and
 - c) control means to compare with the emitted, measured luminescence to determine
- 10 the concentration of the pesticide in the test sample."

Claim 14. The test kit of claim 12 wherein the luciferin derivative is D luciferin acetate.

Claim 15. The test kit of claim 12 wherein the brain material is a brain homogenate selected from the group consisting of: bees, blow flies, house flies, silkworms

15 or other arthropods.

Claim 16. A method of preparing a 6-substituted D luciferin ester when used in the bioluminescent method of any one of claims 1 to 11 or the test kit of any one of claims 12 to 15, which method comprises:

 reacting in a liquid solvent a substituted N-acylated imidazole compound with D

20 luciferin to provide a 6-acylated derivative of D-luciferin and imidazole.

Claim 17. The method of claim 16 which includes monitoring the decrease in bioluminescence as a measure of the completeness of the reaction.

Claim 18. The method of claim 16 wherein the substituted group of the imidazole compound is selected from the group consisting of: alkyl, benzyl and phenyl

25 radicals.

Claim 19. The method of claim 16 wherein the 6-acylated derivative of D-luciferin is D-luciferin acetate.

30 DATED this 31st day of January, 1996

Charm Sciences, Inc.

By Its Patent Attorneys

DAVIES COLLISON CAVE



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 93/00436

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12Q1/66; C07D277/68		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12Q ; C07D	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	DATABASE WPIL Section Ch, Week 8229, Derwent Publications Ltd., London, GB; Class D04, AN 82-61256E & SU,A,865 904 (AS SIBE PHYS INST) 25 September 1981 see abstract --- -/--	1-14
¹⁰ Special categories of cited documents : ¹⁰ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
18 MAY 1993		10. 06. 93
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		GRIFFITH G.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CHEMICAL ABSTRACTS, vol. 82, no. 3, 20 January 1975, Columbus, Ohio, US; abstract no. 11930, D. G. RUSNESS ET AL. 'Firefly luciferase inhibition by isopropyl-3-chlorocarbanilate and isopropyl-3-chlorohydroxycarbanilate analogs.' page 97 ;column 1 ; see abstract & PESTIC. BIOCHEM. PHYSIOL. vol. 4, no. 2, 1974, pages 109 - 119	1-14
A	--- CHEMICAL ABSTRACTS, vol. 89, no. 5, 31 July 1978, Columbus, Ohio, US; abstract no. 37448, E. SLAVA 'Modeling as a possible principle for studying the toxicity of herbicides.' page 121 ;column 1 ; see abstract & EKSP. VODN. TOKSIKOL. vol. 6, pages 240 - 247	1-14
A	--- BIOLOGICAL ABSTRACTS vol. 71, no. 8 , 1981, Philadelphia, PA, US; abstract no. 56384, V. A. KRATASYUK ET AL. 'Determination of microquantities of 2,4-dinitrofluorobenzene by a bioluminescent method.' page 5909 ; see abstract & BIOL. NAUKI (MOSC) vol. 0, no. 11, 1979, pages 106 - 112	1-14
A	--- TRAC, TRENDS IN ANALYTICAL CHEMISTRY vol. 2, no. 1, January 1983, CAMBRIDGE GB pages 7 - 9 J. C. WANNLUND 'Analytical applications of firefly luciferase'	
A	--- WO,A,9 004 037 (GENLUX FORSCHUNGSGESELLSCHAFT FÜR BIOLOGISCHE VERFAHREN MBH) 19 April 1990	
X	--- WO,A,8 702 667 (R. GEIGER ET AL.) 7 May 1987 see examples 18,19,21	15
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claims No.
P,X	BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN vol. 65, no. 10, October 1992, TOKYO JP pages 2604 - 2610 Y. TOYA ET AL. 'Improved synthetic methods of firefly luciferin derivatives for use in bioluminescent analysis of hydrolytic enzymes; carboxylic esterase and alkaline phosphatase.' see the whole document ---	15,16
A	CHEMICAL AND PHARMACEUTICAL BULLETIN vol. 30, no. 10, October 1982, TOKYO JP pages 4242 - 4244 T. KAMIJO ET AL. '1-acyl-3-substituted imidazolium salts as highly reactive acylating agents.' see page 4242 -----	17,18

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9300436
SA 69811

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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18/05/93

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		EP-A, B 0243429	04-11-87
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		US-A- 5098828	24-03-92