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(54) **LUMINESCENCE-BASED METHODS AND PROBES FOR MEASURING CYTOCHROME P450 ACTIVITY**

AUF LUMINESZENZ BASIERENDE VERFAHREN UND SONDEN ZUR MESSUNG DER
CYTOCHROM-P450-AKTIVITÄT

PROCEDES FONDES SUR LA LUMINESCENCE ET SONDAS PERMETTANT DE MESURER
L'ACTIVITE DU CYTOCHROME P450

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- **FARACE C ET AL: "SYNTHESIS AND CHARACTERIZATION OF A NEW SUBSTRATE OF PHOTINUS-PYRALIS LUCIFERASE 4 METHYL-D-LUCIFERIN" JOURNAL OF CLINICAL CHEMISTRY AND CLINICAL BIOCHEMISTRY, vol. 28, no. 7, 1990, pages 471-474, XP008079556**
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Description**Field of the Invention**

[0001] The present invention relates to methods, substrate compounds, and kits for analyzing metabolic activity in animals, cells or in cell-free reaction formulations and for screening test compounds for their effect on metabolic activity by employing a luminogenic molecule that is a luciferin derivative or a coelenterazine derivative, as a dual cytochrome P450 substrate and bioluminescent enzyme pro-substrate. P450 metabolism of the luminogenic molecule in a first reaction generates the substrate for a bioluminescent enzyme. The bioluminescent enzyme then acts on the substrate in a second light-emitting reaction. P450 activity is then ascertained by measuring the luminescence of the reaction mixture relative to a control reaction mixture. Also described is a method and kit for relieving inhibition of luciferase by its inhibitor inorganic pyrophosphate (iPP) using a pyrophosphatase such as inorganic pyrophosphatase enzyme (iP-Pase).

Background of the Invention

[0002] The presence and activity of enzymes can be used to determine the health or metabolic state of a cell. Enzymes are also markers for the cell type since the occurrence and activity of certain enzymes is frequently characteristic of a particular cell. For instance, the activity of certain enzymes can often be used to distinguish cells of bacterial, plant or animal origin, or to distinguish the identity of tissue from which the enzyme originates.

[0003] Detection of the presence and activity of enzymes can be facilitated by substrates that are converted by the enzyme of interest to a product that has at least one property that can be measured. These reporter molecules include fluorescent and chromogenic substrates. Fluorescent substrates have been preferable because, in many cases, they have a very high sensitivity and may permit measurements in living single cells with high spatial and temporal resolution. Chromogenic substrates can be very specific but often lack a high degree of resolution. US 5,098,828 provides D-luciferin derivatives that may be converted into luciferase substrates by the action of certain enzymes.

[0004] One family of enzymes useful for measuring the activity of living cells or in extracts of cells is the Cytochrome P450 family. Cytochrome P450s (CYP450s) are a large family of heme-containing enzymes that, in addition to the endogenous role in cell proliferation and development, includes many catalysts for detoxification and activation of lipophilic xenobiotics including therapeutic drugs, chemical carcinogens and environmental toxins. In some cases the metabolite (s) is more toxic than the parent compound. However, in other cases, metabolism of a therapeutic compound reduces the bioavailability of the compound, lowering efficacy. This family of genes and the polymorphisms within the family play important roles in the interindividual variation in drug metabolism, occurrence and severity of side effects and therapeutic failures.

[0005] Hundreds of cytochrome P450s have been identified in diverse organisms including bacteria, fungi, plants, and animals (18). All CYP450s use a heme cofactor and share structural attributes. Most CYP450s are 400 to 530 amino acids in length. The secondary structure of the enzyme is about 70% alpha-helical and about 22% beta-sheet. The region around the heme-binding site in the C-terminal part of the protein is conserved among cytochrome P450s. A ten amino acid signature sequence in this heme iron ligand region has been identified which includes a conserved cysteine residue involved in binding the heme iron in the fifth coordination site. In eukaryotic CYP450s, a membrane-spanning region is usually found in the first 15-20 amino acids of the protein, generally consisting of approximately 15 hydrophobic residues followed by a positively charged residue. (18, 19.)

[0006] Some of the genes encoding CYP450s are inducible at the transcription level by the compounds they metabolize (1,2). The genes encoding CYP450s have been divided into families based on homology of deduced amino acid sequences (3). All mammals share at least 14 CYP450 families but most drug metabolism is catalyzed by only three families: CYP1, CYP2 and CYP3. Most of the P450 catalyzed drug metabolism in humans takes place in the liver and is accounted for by about 13 enzymes: CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5 and CYP3A7 (4).

[0007] Because of the central role CYP450s play in drug clearance, toxicity and drug-drug interactions, CYP450s make useful targets for narrowing the field of compounds that should be moved forward in the drug development process (5,8). Furthermore, knowledge of CYP450/drug interactions can be predictive of drug disposition in a patient. There is a need for screening assays that can be used in high throughput mode. Compounds with properties that change in an easily detectable way upon oxidation by a CYP450 are useful as probes in high throughput assays for detecting effects on CYP450 activity (6,7). There is also need for a method for analyzing metabolic activity in cells under physiological conditions, using a substrate that is specific for CYP450 isozymes and yields products that are easily detectable. The signal should be detectable in cell-free extracts of cells and in living cells and the assay should have a low background signal. WO99/60096 describes a system for indirectly measuring the activity of oxygenases, e.g. cytochrome P450, by converting the products of oxygenases into fluorescent or luminescent polymers with a coupling enzyme and measuring

the amount of fluorescence or luminescence produced.

[0008] Finally, there is a need to protect luciferase activity from its inhibitor inorganic pyrophosphate. Although the inventors do not intend to limit the source of pyrophosphate, pyrophosphate may be present as a contaminant in orthophosphate salts used in buffers containing a luciferase-based reaction or may be generated as a product of a luciferase reaction with ATP, O₂ and luciferin.

Summary of the invention

[0009] Applicants have fulfilled these needs by providing methods, substrate compounds, and kits which can identify a compound, e.g., drug candidate, affecting a cytochrome P450 enzyme in a highly specific manner.

[0010] The invention provides luminogenic molecules that are useful as P450 substrates or as dual P450 substrates and pro-substrates of bioluminescent enzymes. In one embodiment of the invention, the luminogenic molecules are derivatives of (4S)-4,5-dihydro-2-(6-hydroxy-benzothiazolyl)-4-thiazolecarboxylic acid (D-luciferin) or 2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl)-8-benzyl-3,7-dihydroimidazo [1,2-a]pyrazine-3-one (coelenterazine) which are P450 substrates and pro-substrates of luciferase. In the absence of prior P450 metabolism, these luciferin derivatives alone have limited or no capacity to interact with luciferase in light generating reactions. These compounds are selectively converted by CYP450s to light generating substrates for luciferase reactions and thereby provide the basis of assays with a luminescent readout.

[0011] The invention also provides a method for direct and indirect determination of P450 activity based on luminogenic molecules that are natural coelenterazine and coelenterazine derivatives (collectively referred to as coelenterazines).

[0012] Also disclosed are methods for using luminogenic molecules to determine whether a candidate drug (or class of candidate drugs) is a CYP450 enzyme substrate or regulator or CYP450 gene regulator, and related methods for selecting a candidate drug that will not be too efficiently metabolized by at least one CYP450 enzyme and/or that the drug will not act as an inhibitor of at least one CYP450 enzyme, and/or elicit an unfavorable drug-drug interaction.

Methods of screening a candidate drug (or libraries of drug candidates) of the present invention may be performed by a one-step or a two-step CYP450/bioluminescent enzyme method in a cell-free, cell-based, tissue-based or animal-based environment or may be part of a high or ultra high throughput screening of libraries of drug candidates.

[0013] Also described is a method and kit for relieving inhibition of luciferase by its inhibitor inorganic pyrophosphate (iPP) which may be present as a contaminant or generated as a product of a luciferase-based reaction with ATP, O₂ and luciferin.

[0014] Also described is a method and kit for enhancing or stabilizing a luminescent signal in a luciferase-based reaction that employs a reversible luciferase inhibitor.

[0015] Thus, in one embodiment of the invention, a method is provided for measuring the activity of a cytochrome P450 enzyme as claimed in claim 1.

[0016] In one aspect of this embodiment of the invention, step (a) further includes a pyrophosphatase such as an inorganic pyrophosphatase.

[0017] In another aspect of this embodiment of the invention, the luminogenic molecule, the cytochrome P450 enzyme, and the bioluminescent enzyme are contacted at about the same time.

[0018] In another aspect of this embodiment of the invention, the luminogenic molecule is contacted with at least one cytochrome P450 enzyme to form a first reaction mixture prior to contacting with the bioluminescent enzyme to form a second reaction mixture.

[0019] In another aspect of this embodiment of the invention, the second reaction mixture further comprises a detergent, preferably a non-ionic detergent.

[0020] In another embodiment of the invention, a method is provided as claimed in claim 1 for measuring cytochrome P450 enzyme activity wherein the activity is derived from a cell.

[0021] In one aspect of this embodiment of the invention, cell is recombinant and expresses the bioluminescent enzyme.

[0022] In another aspect of this embodiment of the invention, step (a) cell is further contacted with a lysis reagent.

[0023] In another aspect of this embodiment of the invention, the cell is lysed prior to step (a).

[0024] In another aspect of this embodiment of the invention, the cell is lysed prior to step (b).

[0025] In another aspect of this embodiment of the invention, the cell is contacted first with the luminogenic molecule to produce a first reaction mixture prior to contact with the bioluminescent enzyme to produce a second reaction mixture. The second reaction mixture may further comprises a detergent such as a non-ionic detergent and/or a pyrophosphatase such as an inorganic pyrophosphatase.

[0026] In another embodiment of the invention, a method is provided as claimed in claim 1 for measuring cytochrome P450 enzyme activity wherein the activity is derived from animal tissue.

[0027] In one aspect of this embodiment of the invention, the tissue is contacted first with the luminogenic molecule for a first predetermined time period prior to contact with the bioluminescent enzyme to provide a second mixture. The second reaction mixture may further comprise a detergent such as a non-ionic detergent and/or a pyrophosphatase

such as inorganic pyrophosphatase.

[0028] In another embodiment of the invention, a method is provided for measuring cytochrome P450 enzyme activity in an animal as claimed in claim 16.

[0029] In one aspect of this embodiment of the invention, the reaction mixture further comprises a detergent such as a non-ionic detergent and/or a pyrophosphatase such as an inorganic pyrophosphatase.

[0030] In one aspect of this embodiment of the invention, the animal is a transgenic animal having a bioluminescent enzyme transgene.

[0031] In another aspect of this embodiment of the invention, the bioluminescent enzyme transgene is a luciferase transgene.

[0032] Also disclosed is a method for screening a compound for its effect on cytochrome P450 activity comprising:

- (a) providing a compound for screening,
- (b) providing a luminogenic molecule wherein the molecule is a cytochrome P450 substrate and a pro-substrate of bioluminescent enzyme;
- (c) contacting the compound, the luminogenic molecule, at least one cytochrome P450 enzyme, and a bioluminescent enzyme to produce a reaction mixture; and
- (d) determining cytochrome P450 activity, if any, resulting from the interaction of the compound with the cytochrome P450 enzyme by measuring luminescence of the reaction mixture.

[0033] In one aspect of this embodiment of the invention, the compound, luminogenic molecule, the cytochrome P450 enzyme, and the bioluminescent enzyme are contacted at about the same time.

[0034] In another aspect of this embodiment of the invention, the compound, luminogenic molecule and at least one cytochrome P450 enzyme are contacted first to form a first reaction mixture prior to contacting with the bioluminescent enzyme to form a second reaction mixture. The second reaction mixture may further include a detergent such as a non-ionic detergent and/or a pyrophosphatase such as an inorganic pyrophosphatase.

[0035] In another aspect of this embodiment of the invention, the compound is contacted first with the one or more cytochrome P450 enzymes to form a first reaction mixture, the first reaction mixture are then contacted with the luminogenic molecule to form a second reaction mixture, and the second reaction mixture is then contacted with a bioluminescent enzyme to form a third reaction mixture. The third reaction mixture may further include a detergent such as a non-ionic detergent and/or a pyrophosphatase such as an inorganic pyrophosphatase.

[0036] In another embodiment of the invention, a method is provided as claimed in claim 1 for determining the effect of a compound on cytochrome P450 enzyme activity derived from a cell, the method further comprising the steps of:

- (a) providing a compound for testing
- (b) contacting a cell with a test compound, the luminogenic molecule and the bioluminescent enzyme; and preferably
- (c) determining cytochrome P450 enzyme activity of the cell, if any, resulting from the exposure of the cell to the test compound by measuring and comparing luminescence from said cell with a second cell not exposed to the test compound

[0037] In one aspect of this embodiment of the invention, the cell is recombinant and expresses the bioluminescent enzyme.

[0038] In another aspect of this embodiment of the invention, the cell is contacted first with the compound to produce a first reaction mixture prior to contact with the luminogenic molecule to produce a second reaction mixture. The second mixture may further comprise a bioluminescent enzyme. The bioluminescent enzyme may be added to the second reaction mixture after a predetermined time period. The second reaction mixture may further include a detergent such as a non-ionic detergent and/or a pyrophosphate such as inorganic pyrophosphate.

[0039] In another aspect of this embodiment of the invention, step (b) further includes a pyrophosphatase such as an inorganic pyrophosphatase.

[0040] In another embodiment of the invention, a method is provided as claimed in claim 1 for determining the effect of a compound on cytochrome P450 enzyme activity derived from animal tissue, the method further comprising the steps of:

- (a) providing a test compound;
- (b) contacting an animal tissue with the test compound, the luminogenic molecule and the bioluminescent enzyme; and preferably
- (c) determining cytochrome P450 enzyme activity of the tissue, if any, resulting from the exposure of the tissue to the test compound by measuring and comparing luminescence from said tissue with a control tissue not exposed to the test compound.

[0041] In one aspect of this embodiment of the invention, the animal tissue expresses the bioluminescent enzyme.

[0042] In another aspect of this embodiment of the invention, the tissue is contacted with the test compound to produce a first mixture prior to contact with the luminogenic molecule to produce a second mixture. The second mixture may further comprise a bioluminescent enzyme. The bioluminescent enzyme may be added to the second reaction mixture after a predetermined time period. The second reaction mixture may further include a detergent such as a non-ionic detergent.

[0043] In another aspect of this embodiment of the invention, step (b) may further include a pyrophosphatase such as an inorganic pyrophosphatase.

[0044] In another embodiment of the invention, a method is provided as claimed in claim 43 for determining the effect of a compound on cytochrome P450 enzyme activity in an animal wherein:

a compound for testing has been provided;
the test compound has been administered to an animal
the luminogenic molecule has been administered to the animal;
a biological sample has been obtained from said animal; the method comprising

(a) contacting the biological sample with the bioluminescent enzyme; and

(b) determining cytochrome P450 enzyme activity of said animal after exposure of said animal to the test compound by measuring and comparing luminescence from said biological sample with a second biological sample taken from an animal not exposed to said test compound.

[0045] In one aspect of the invention, the luminogenic compound has been administered after the test compound has been administered, after a predetermined time period has elapsed.

[0046] In another aspect of this embodiment of the invention, the biological sample was taken from the animal just prior to exposure to the test compound.

[0047] In another aspect of this embodiment of the invention, the biological sample comprises blood, serum, bile, urine, feces, or tissue.

[0048] In another embodiment of the invention, a method is provided as claimed in claim 16 for determining the effect of a compound on cytochrome P450 enzyme activity in a transgenic animal having a bioluminescent enzyme transgene, said method further comprising:

(a) providing a compound for testing,
(b) administering the test compound to a transgenic animal having a bioluminescent enzyme transgene;
(c) administering the luminogenic molecule to the animal; and
(d) determining cytochrome P450 enzyme activity of said animal after exposure of said animal to the test compound by measuring and comparing bioluminescence from tissue from said transgenic animal with a second biological sample taken from another transgenic animal not exposed to said test compound.

[0049] In one aspect of this embodiment of the invention, step (c) is performed after step (b) after a predetermined time period has elapsed.

[0050] In another aspect of this embodiment of the invention, the bioluminescent enzyme transgene is a luciferase transgene.

[0051] In another embodiment of the invention, a high throughput method is provided as claimed in claim 48 for rapidly screening a plurality of compounds to determine their effect on cytochrome P450 activity, said method comprising:

(a) providing compounds for screening;
(b) contacting the compounds to be screened with (i) the luminogenic molecule; (ii) one or more cytochrome P450 enzymes; and (iii) one or more bioluminescent enzymes to form reaction mixtures, each reaction mixture having one or more compounds; and
(c) determining cytochrome P450 enzyme activity, if any, resulting from the interaction of one or more compounds with one or more cytochrome P450 enzymes by measuring bioluminescence of the reaction mixtures.

[0052] In one aspect of this embodiment of the invention, the compounds are contacted first with the one or more cytochrome P450 enzymes to form first reaction mixtures, the first reaction mixtures are then contacted with the luminogenic molecule to form second reaction mixtures, and the second reaction mixtures are then contacted with a bioluminescent enzyme to form third reaction mixtures. The third reaction mixture may further include a detergent such as a non-ionic detergent.

[0053] In another aspect of this embodiment of the invention, the compounds are contacted first with one or more cytochrome P450 enzymes and the luminogenic molecule to form first reaction mixtures prior to contact with one or more bioluminescent enzymes to form a second reaction mixture. The second reaction mixture may further comprise a detergent such as a non-ionic detergent.

[0054] In another aspect of this embodiment of the invention, the compounds are contacted simultaneously or contemporaneously with the one or more cytochrome P450 enzymes and the luminogenic molecule to form first reaction mixtures prior to contacting with one or more bioluminescent enzymes to form second reaction mixtures.

[0055] In another aspect of this embodiment of the invention, step (b) further comprises a pyrophosphatase such as an inorganic pyrophosphatase.

[0056] In another embodiment of the invention, a high throughput method is provided as claimed in claim 48 for rapidly screening a plurality of compounds to determine their effect on cytochrome P450 activity derived from a cell, said method comprising:

(a) providing compounds for screening;

(b) contacting cells with the compounds to be screened, the luminogenic molecule, and the one or more bioluminescent enzymes to form reaction mixtures, each reaction mixture having one or more compounds;

(c) determining cytochrome P450 enzyme activity, if any, resulting from the interaction of one or more compounds with one or more cytochrome P450 enzymes by measuring bioluminescence of the reaction mixtures.

[0057] In one aspect of this embodiment of the invention, the cells are recombinant and express bioluminescent enzyme.

[0058] In another aspect of this embodiment of the invention, a bioluminescent enzyme from an exogenous source is used.

[0059] In another aspect of this embodiment of the invention, steps (b) and/or (c) further comprises a pyrophosphatase such as an inorganic pyrophosphatase.

[0060] In another aspect of this embodiment of the invention, the cells are first contacted with the compounds and luminogenic molecule for a first predetermined time period, then contacted with the bioluminescent enzyme for a second predetermined time period. Detergent such as non-ionic detergent may be present during the second predetermined time period.

[0061] In another aspect of this embodiment of the invention, the cells are first contacted with the compounds for a first predetermined time period, then contacted with the luminogenic molecule for a second predetermined time period, then contacted with the bioluminescent enzyme for a third predetermined time period. Detergent such as non-ionic detergent may be present in the mixture during the third predetermined time period.

[0062] In another aspect of this embodiment of the invention, the cells are first contacted with the compounds for a first predetermined time period, then contacted with the luminogenic molecule and bioluminescent enzyme for a second predetermined time period.

[0063] In another aspect of this embodiment of the invention, the cells, compounds, luminogenic molecule, and bioluminescent enzyme are contacted simultaneously.

[0064] In another embodiment of the invention, a high throughput method is provided as claimed in claim 48 for rapidly screening a plurality of compounds to determine their effect on cytochrome P450 activity derived from animal tissue, said method comprising:

(a) providing compounds for screening;

(b) contacting animal tissue with the compounds to be screened, the luminogenic molecule, and the one or more bioluminescent enzymes to form reaction mixtures, each reaction mixture having one or more compounds;

(c) determining cytochrome P450 enzyme activity, if any, resulting from the interaction of one or more compounds with one or more cytochrome P450 enzymes by measuring bioluminescence of the reaction mixtures.

[0065] In one aspect of this embodiment of the invention, the tissue expresses at least one bioluminescent enzyme.

[0066] In another aspect of this embodiment of the invention, the tissue is first contacted with the compounds and luminogenic molecule for a first predetermined time period prior to contact with the bioluminescent enzyme. Detergent such as non-ionic detergent may be added after the first predetermined time period. Detergent and bioluminescent enzyme may be added at the same time.

[0067] In another aspect of this embodiment of the invention, detergent is added prior to addition of the bioluminescent enzyme.

[0068] In another aspect of this embodiment of the invention, the tissue is first contacted with the compounds for a first predetermined time period, then contacted with the luminogenic molecule for a second predetermined time period, then contacted with the bioluminescent enzyme for a third predetermined time period. Detergent such as non-ionic

detergent may be added after the second predetermined time period. Detergent and bioluminescent enzyme may be added at the same time.

[0069] In another aspect of this embodiment of the invention, the tissue is first contacted with the compounds for a first predetermined time period, then contacted with the luminogenic molecule and bioluminescent enzyme for a second predetermined time period.

[0070] In another aspect of this embodiment of the invention, the tissue, compounds, luminogenic molecule, and bioluminescent enzyme are contacted simultaneously. In another aspect of this embodiment of the invention, steps (b) or (c) further comprise (iv) a pyrophosphatase such as an inorganic pyrophosphatase.

[0071] In another embodiment of the invention, a high throughput method is provided as claimed in claim 48 for rapidly screening a plurality of compounds to determine their effect on cytochrome P450 activity of animal, said method comprising:

(a) providing compounds for screening;

(b) contacting a living teleost with the compounds to be screened, the luminogenic molecule, and the bioluminescent enzyme to form reaction mixtures and each reaction mixture having one or more compounds;

(c) determining cytochrome P450 enzyme activity, if any, resulting from the interaction of one or more compounds with one or more cytochrome P450 enzymes by measuring bioluminescence of the reaction mixtures that include test compounds in comparison to control mixtures without test compounds.

[0072] In one aspect of this embodiment of the invention, the teleost is transgeneic and expresses bioluminescent enzyme.

[0073] In another aspect of this embodiment of the invention, the teleosts are first contacted with the compounds and luminogenic molecule for a first predetermined time period prior to contact with the bioluminescent enzyme.

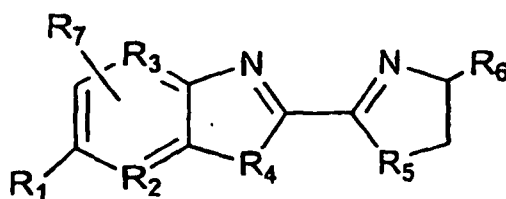
[0074] In another aspect of this embodiment of the invention, the teleosts are first contacted with the compounds for a first predetermined time period, then contacted with the luminogenic molecule for a second predetermined time period, then contacted with the bioluminescent enzyme for a third predetermined time period.

[0075] In another aspect of this embodiment of the invention, the teleosts are first contacted with the compounds for a first predetermined time period, then contacted with the luminogenic molecule and bioluminescent enzyme for a second predetermined time period.

[0076] In another aspect of this embodiment of the invention, the teleosts, compounds, luminogenic molecule, and bioluminescent enzyme are contacted simultaneously.

[0077] In another aspect of this embodiment of the invention, steps (b) or (c) further comprise: (iv) a pyrophosphatase such as an inorganic pyrophosphatase.

[0078] In another embodiment of the invention, in any of the above methods, the bioluminescent enzyme is a luciferase. The luciferin derivative has a formula:



wherein

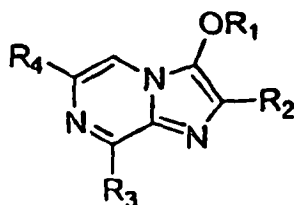
R_1 represents hydrogen, hydroxyl, amino, C_{1-20} alkoxy, substituted C_{1-20} alkoxy, C_{2-20} alkenyloxy, substituted C_{2-20} alkenyloxy, halogenated C_{2-20} alkoxy, substituted halogenated C_{2-20} alkoxy, C_{3-20} alkynyloxy, substituted C_{3-20} alkynyloxy, C_{3-20} cycloalkoxy, substituted C_{3-20} cycloalkoxy, C_{3-20} cycloalkylamino, substituted C_{3-20} cycloalkylamino, C_{1-20} alkylamino, substituted C_{1-20} alkylamino, di C_{1-20} alkylamino, substituted di C_{1-20} alkylamino, C_{2-20} alkenylamino, substituted C_{2-20} alkenylamino, di C_{2-20} alkenylamino, substituted di C_{2-20} alkenylamino, C_{2-20} alkenyl C_{1-20} alkylamino, substituted C_{2-20} alkenyl C_{1-20} alkylamino, C_{3-20} alkynylamino, substituted C_{3-20} alkynylamino, di C_{3-20} alkynylamino, substituted di C_{3-20} alkynylamino, C_{3-20} alkynyl C_{1-20} alkylamino, substituted C_{3-20} alkynyl C_{1-20} alkylamino, C_{3-20} alkynyl C_{2-20} alkenylamino, or substituted C_{3-20} alkynyl C_{2-20} alkenylamino;

R_2 and R_3 independently represents C or N;

R_4 and R_5 independently represents S, O, NR_8 wherein NR_8 represents hydrogen or C_{1-20} alkyl, CR_9R_{10} wherein R_9

and R_{10} independently represent H, C_{1-20} alkyl, or fluorine;
 R_6 represents CH_2OH ; COR_{11} wherein R_{11} represents H, OH, C_{1-20} alkoxide, C_{2-20} alkenyl, or $NR_{12}R_{13}$ wherein R_{12} and R_{13} are independently H or C_{1-20} alkyl; or $-OM^+$ wherein M^+ is an alkali metal or a pharmaceutically acceptable salt; and
 R_7 represents H, C_{1-6} alkyl, C_{1-20} alkenyl, halogen, or C_{1-6} alkoxide,
 with the proviso that R_1 is not OH or NH_2 , if R_3 and R_2 are both carbon, R_4 and R_5 are both S, R_7 is H, R_6 is COR_{11} and R_{11} is OH, such that the luminogenic molecule is not luciferin or aminoluciferin.

[0079] The coelenterazine derivative has the formula:



wherein

R_1 is C_{1-20} alkyl, branched C_{3-20} alkyl, C_{3-20} cycloalkyl, aralkyl, C_{1-20} alkyl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or di C_{1-20} alkylamino, aralkyl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or di C_{1-20} alkylamino; and

R_2 , R_3 , and R_4 are independently hydrogen, C_{1-20} alkyl, C_{3-20} cycloalkyl, branched C_{3-20} alkyl, aryl, aralkyl, C_{1-20} alkyl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or di C_{1-20} alkylamino, aralkyl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or di C_{1-20} alkylamino, aryl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or di C_{1-20} alkylamino. Preferably R_4 is aryl or aryl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or C_{1-20} dialkylamino.

[0080] In another embodiment of the invention, a kit is provided as claimed in claim 87.

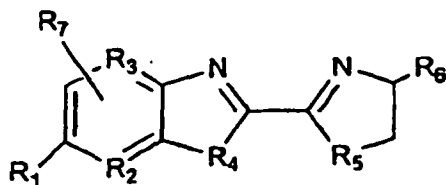
[0081] In one aspect of this embodiment of the invention the kit further comprises one or more bioluminescent enzymes such as a luciferase. Examples of luciferase include, without limitation, firefly luciferase or Renilla luciferase.

[0082] In another aspect of this embodiment of the invention, the kit further comprises ATP and magnesium ions.

[0083] In another aspect of this embodiment of the invention, the kit further comprises a detergent such as a non-ionic detergent.

[0084] In another aspect of this embodiment of the invention, the kit further comprising a pyrophosphatase such as an inorganic pyrophosphatase.

[0085] The luciferin derivative in the kit has a formula:

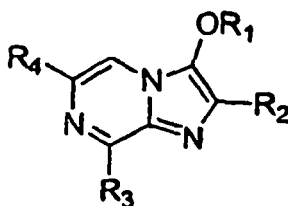


wherein

R_1 represents amino, halogenated C_{2-20} alkoxy, substituted halogenated C_{2-20} alkoxy, C_{3-20} alkynoxy, substituted C_{3-20} alkynoxy, C_{3-20} cycloalkoxy, substituted C_{3-20} cycloalkoxy, C_{3-20} cycloalkylamino, substituted C_{3-20} cycloalkylamino; C_{1-20} alkylamino, substituted C_{1-20} alkylamino, di C_{1-20} alkylamino, substituted di C_{1-20} alkylamino, C_{2-20} alkenylamino, substituted C_{2-20} alkenylamino, di C_{2-20} alkenylamino, substituted di C_{2-20} alkenylamino, C_{2-20} alkenyl C_{1-20} alkylamino, substituted C_{2-20} alkenyl C_{1-20} alkylamino, C_{3-20} alkynylamino, substituted C_{3-20} alkynylamino, di C_{3-20} alkynylamino, substituted di C_{3-20} alkynylamino, C_{3-20} alkynyl C_{1-20} alkylamino, substituted C_{3-20} alkynyl C_{1-20} alkylamino, C_{3-20} alkynyl C_{2-20} alkenylamino,

or substituted C₃₋₂₀ alkynyl C₂₋₂₀ alkenylamino;
 R₂ and R₃ independently represents C or N;
 R₄ and R₅ independently represents S, O, or CR₉R₁₀ wherein R₉ and R₁₀ independently represent H, C₁₋₂₀ alkyl, or fluorine;
 5 R₆ represents CH₂OH; COR₁₁ wherein R₁₁ represents H, OH, C₂₋₂₀ alkenyl, or NR₁₂R₁₃ wherein R₁₂ and R₁₃ are independently H or C₁₋₂₀ alkyl; or -OM⁺ wherein M⁺ is an alkali metal or a pharmaceutically acceptable salt; and
 R₇ represents H, C₁₋₂₀ alkenyl, halogen, or C₁₋₆ alkoxide,
 with the proviso that R₁ is not OH or NH₂, if R₃ and R₂ are both carbon, R₄ and R₃ are both S, R₇ is H, R₆ is
 10 COR₁₁ and R₁₁ is OH such that luminogenic molecule is not luciferin and aminoluciferin.

[0086] In another embodiment of the invention, a kit is provided as claimed in claim 96, wherein the coelenterazine derivative has a formula:



wherein

R₁ is C₁₋₂₀ alkyl branched C₃₋₂₀ alkyl, C₃₋₂₀ cycloalkyl, aralkyl, C₁₋₂₀ alkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or diC₁₋₂₀ alkylamino, aralkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or diC₁₋₂₀ alkylamino; and
 25 R₂, R₃, and R₄ are independently hydrogen, C₁₋₂₀ alkyl, C₃₋₂₀ cycloalkyl, branched C₃₋₂₀ alkyl, aryl, aralkyl, C₁₋₂₀ alkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or diC₁₋₂₀ alkylamino, aralkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or diC₁₋₂₀ alkylamino, aryl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or di C₁₋₂₀ alkylamino. Preferably R₄ is aryl or aryl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or C₁₋₂₀ dialkylamino.

[0087] In another aspect of this embodiment of the invention, the kit further comprises a reversible luciferase inhibitor. Preferably, the reversible luciferase inhibitor is 2-(4-aminophenyl)-6-methylbenzothiazole (APMBT) or 2-amino-6-methylbenzothiazole (AMBT).

[0088] In another embodiment of the invention, a D-luciferin derivative is provided as claimed in claim 100 that is a substrate of a cytochrome P450 enzyme and a pro-substrate of luciferase enzyme.

[0089] In another embodiment of the invention, a composition is provided which comprises the D-luciferin derivative as claimed in claim 100.

[0090] In one aspect of this embodiment of the invention, the composition further comprises a pyrophosphatase such as an inorganic pyrophosphatase.

[0091] Preferred D-luciferin derivatives include:

luciferin 6' 2-chloroethyl ether;

luciferin 6' 4-picolinyl ether;

luciferin 6' 4-trifluoromethylbenzyl ether;

luciferin 6' 2-picolinyl ether;

luciferin 6' 3-picolinyl ether;

luciferin 6' benzyl ether;

luciferin 6' 4-trifluoromethylbenzyl ether;

luciferin 6' phenylethyl ether;

luciferin 6' geranyl ether; or

luciferin 6' prenyl ether.

[0092] The invention comprises

a method for measuring cytochrome P450 activity in an animal which has a bioluminescent enzyme transgene and to which a luminogenic molecule as claimed in Claim 1, or a D-Luciferin derivative as claimed in Claim 100, has been administered, the method comprising determining cytochrome P450 activity by measuring bioluminescence in the living, intact animal.

[0093] A method for measuring P450 enzyme activity is also described, said method comprising

- (a) providing a coelentraine or a coelenterazine derivative that is a P450 substrate and is chemiluminescent;
- (b) contacting a coelentraine or coelenterazine derivative with at least one cytochrome P450 enzyme to form a reaction mixture; and
- (c) determining cytochrome P450 activity by measuring chemoluminescence of the reaction mixture.

[0094] A method for measuring cytochrome P450 enzyme activity in a cell is also described, said method comprising:

- (a) providing a coelentraine or a coelenterazine derivative that is a P450 substrate and is chemiluminescent;
- (b) contacting a cell with the coelentraine or coelenterazine derivative to form a reaction mixture; and
- (c) determining cytochrome P450 activity of the cell by measuring the chemiluminescence of the reaction mixture.

[0095] In one aspect of this method, step (b) cell is further contacted with a lysis agent.

[0096] In another aspect of this method, the cell is lysed prior to step (b).

[0097] In another aspect of this method, the cell is lysed prior to step (c).

[0098] A method for measuring cytochrome P450 enzyme activity in animal tissue is also described, said method comprising:

- (a) providing coelentraine or a coelenterazine derivative that is a P450 substrate and is chemiluminescent;
- (b) contacting an animal tissue with the coelentraine or a coelenterazine derivative and a bioluminescent enzyme to provide a mixture; and
- (c) determining cytochrome P450 activity of the tissue by measuring luminescence of the mixture.

[0099] A method for measuring cytochrome P450 enzyme activity in an animal is also described, said method comprising:

- (a) providing coelentraine or a coelenterazine derivative that is a P450 substrate and is chemiluminescent;
- (b) administering the coelentraine or a coelenterazine derivative to an animal;
- (c) obtaining a biological sample from the animal; and
- (d) determining cytochrome P450 activity of the animal by measuring chemiluminescence of the sample.

[0100] A method for screening a compound for its effect on cytochrome P450 activity is also described, said method comprising:

- (a) providing a compound for screening;
- (b) providing coelentraine or a coelenterazine derivative that is a substrate of cytochrome P450 and is chemiluminescent;
- (c) contacting the compound, coelentraine or a coelenterazine derivative, and a cytochrome P450 enzyme to produce a reaction mixture; and
- (d) determining cytochrome P450 activity, if any, resulting from the interaction of the compound with the cytochrome P450 enzyme by measuring chemiluminescence of the reaction mixture.

[0101] A method for determining the effect of a compound on cytochrome P450 enzyme activity of a cell is also described, said method comprising the steps of:

- (a) providing a compound for testing;

(b) contacting a cell with a test compound and coelentraine or a coelentraine derivative that is a substrate of cytochrome P450 and that is chemiluminescent; and
 (c) determining cytochrome P450 enzyme activity of the cell, if any, resulting from the exposure of the cell to the test compound by measuring and comparing chemiluminescence from said cell with a second cell not exposed to the test compound.

[0102] A method for determining the effect of a compound on cytochrome P450 enzyme activity of animal tissue is also described, said method comprising the steps of

(a) providing a test compound;
 (b) contacting an animal tissue with a test compound and coelentraine or a coelentraine derivative that is a substrate of cytochrome P450 and that is chemiluminescent; and
 (c) determining cytochrome P450 enzyme activity of the tissue, if any, resulting from the exposure of the tissue to the test compound by measuring and comparing chemiluminescence from said tissue with a control tissue not exposed to the test compound.

[0103] A method for determining the effect of a compound on cytochrome P450 enzyme activity in an animal is also described, said method comprising

(a) providing a compound for testing;
 (b) administering the test compound to an animal;
 (c) administering coelentraine or a coelentraine derivative that is a substrate of cytochrome P450 and that is chemiluminescent;
 (d) obtaining a biological sample from said animal;
 (e) determining cytochrome P450 enzyme activity of said animal after exposure of said animal to the test compound by measuring and comparing chemiluminescence from said biological sample with a second biological sample taken from an animal not exposed to said test compound.

[0104] A high throughput method for rapidly screening a plurality of compounds to determine their effect on cytochrome P450 activity is also described, said method comprising:

(a) providing compounds for screening;
 (b) contacting the compounds to be screened with (i) coelentraine or a coelentraine derivative that is a substrate of cytochrome P450 and that is chemiluminescent; and (ii) one or more cytochrome P450 enzymes, each reaction mixture having one or more compounds; and
 (c) determining cytochrome P450 enzyme activity, if any, resulting from the interaction of one or more compounds with one or more cytochrome P450 enzymes by measuring chemiluminescence of the reaction mixtures.

[0105] A high throughput method for rapidly screening a plurality of compounds to determine their effect on cytochrome P450 activity of a cell is also described, said method comprising:

(a) providing compounds for screening
 (b) contacting cells with the compounds to be screened and coelentraine or a coelentraine derivative that is a substrate of cytochrome P450 and that is chemiluminescent to form reaction mixtures, each reaction mixture having one or more compounds;
 (c) determining cytochrome P450 enzyme activity, if any, resulting from the interaction of one or more compounds with one or more cytochrome P450 enzymes by measuring chemiluminescence of the reaction mixtures.

[0106] A high throughput method for rapidly screening a plurality of compounds to determine their effect on cytochrome P450 activity of animal tissue is described, said method comprising: (a) providing an animal tissue with CYP450 activity

(a) providing compounds for screening;
 (b) contacting animal tissue with the compounds to be screened and coelentraine or a coelentraine derivative that is a substrate of cytochrome P450 and that is chemiluminescent to form reaction mixtures, each reaction mixture having one or more compounds;
 (c) determining cytochrome P450 enzyme activity, if any, resulting from the interaction of one or more compounds with one or more cytochrome P450 enzymes by measuring chemiluminescence of the reaction mixtures.

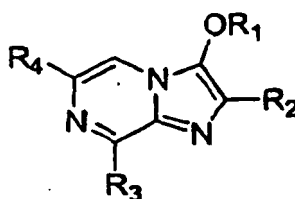
[0107] A high throughput method for rapidly screening a plurality of compounds to determine their effect on cytochrome P450 activity of animal is described, said method comprising:

(a) providing compounds for screening;

(b) contracting a living teleost with the compounds to be screened and coelenterazine or a coelenterazine derivative that is a substrate of cytochrome P450 and that is chemiluminescent to form reaction mixtures, each reaction mixture having one or more compounds;

(c) determining cytochrome P450 enzyme activity, if any, resulting from the interaction of one or more compounds with one or more cytochrome P450 enzymes by measuring chemiluminescence of the reaction mixtures that include test compounds in comparison to control mixtures without test compounds.

[0108] In any of the above methods involving coelenterazine or derivative that is a substrate of cytochrome P450 and that is chemiluminescent, preferably the coelenterazine derivative has a formula:



wherein

R_1 is C_{1-20} alkyl, branched C_{3-20} alkyl, C_{3-20} cycloalkyl, aralkyl, C_{1-20} alkyl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or di- C_{1-20} alkylamino, aralkyl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or di- C_{1-20} alkylamino; and

R_2 , R_3 , and R_4 are independently hydrogen, C_{1-20} alkyl, C_{3-20} cycloalkyl, branched C_{3-20} alkyl, aryl, aralkyl, C_{1-20} alkyl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or di- C_{1-20} alkylamino, aralkyl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or di- C_{1-20} alkylamino, aryl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or di- C_{1-20} alkylamino.

[0109] In one aspect of these methods, preferably R_4 is aryl or aryl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or C_{1-20} dialkylamino.

[0110] In another aspect of these methods, the coelenterazine derivative is coelenterazine HH, methoxycoelenterazine HH or coelenterazine.

[0111] A method for enhancing the stability of a luminescent signal generated by a luciferase-based reaction mixture is described, said method comprising contacting a luciferase with a reversible luciferase inhibitor in an amount effective to enhance the stability and prolong the lifetime of the luminescent signal relative to the luminescent signal generated in a comparable luciferase-based reaction mixture in the absence of the inhibitor.

[0112] In one aspect of this method, the reversible luciferase inhibitor is a competitive inhibitor.

[0113] In another aspect of this method, the reversible luciferase inhibitor comprises 2-(4-aminophenyl)-6-methylbenzothiazole (APMBT) or 2-amino-6-methylbenzothiazole (AMBT).

[0114] In another aspect of this method, the effective amount of the inhibitors ranges from about 1 micromolar to about 1 millimolar in the reaction mixture.

[0115] In another aspect of this method, the effective amount of the inhibitor ranges from about 1 micromolar and about 500 micromolar in the reaction mixture.

[0116] In another aspect of this method, the effective amount of the inhibitor ranges from about 10 micromolar to about 200 micromolar in the reaction mixture.

[0117] In another aspect of this method, the effective amount of the inhibitor ranges is about 100 micromolar in the reaction mixture.

Brief description of the drawings

[0118]

Figure 1. Luminescent CYP450 reaction scheme.

Figure 2. Structures: D-luciferin ((4S)-4,5-dihydro-2-(6-hydroxy-benzothiazolyl)-4-thiazolecarboxylic acid) and D-

luciferin derivatives.

Figure 3. *Two-step luminescent CYP450 reactions.* D-luciferin derivatives were incubated in a CYP450 reaction mix for 60 minutes at 37°C before combining with a luciferase reaction mixture. In -CYP450 controls, CYP450 Sf9 cell microsomes were replaced with control (no CYP450) Sf9 cell membranes (panels B, C, D and E) or H₂O (panel A) or both (panel F, G, H, and I). Luminescence was read within 12 minutes of combining the reactions on a Turner Reporter (panels A, B, C and E) or Berthold Orion (panels D, F, G, and H) luminometer.

Figure 4. *Time-dependence of CYP450/substrate incubation in two-step luminescent CYP450 reactions.* D-luciferin derivatives were incubated in a CYP450 reaction mix for the indicated times at 37°C before combining with a luciferase reaction mixture. For -CYP450 controls CYP450 Sf9 cell microsomes were replaced with H₂O. Luminescence was read within 12 minutes of combining the reactions on a Turner Reporter (panels A and C) or Berthold Orion (panel B) luminometer.

Figure 5. *Time course of light output from two-step luminescent CYP450 reactions.* Luc ME was incubated in CYP450 reaction mixes for 60 minutes at 37°C before combining with a luciferase reaction mixture. In CYP450 controls CYP450 Sf9 cell microsomes were replaced with H₂O. Luminescence was read on a Turner Reporter luminometer beginning 3 minutes after combining the reactions and at successive intervals as indicated for 284 minutes.

Figure 6. *One-step luminescent CYP450 assays at room temperature.* Luc ME was incubated in combined CYP450 and luciferase reaction mixes at room temperature (~22°C). For -CYP450 controls CYP450 Sf9 cell microsomes were replaced with H₂O. CYP450 and a luciferase reaction mix were added simultaneously to a CYP450 reaction mix and light output was read immediately (time=0). Readings were then taken every 4.25 minutes for 15.5 hours on a Turner Reporter luminometer.

Figure 7. *One-step luminescent CYP450 assays at 37°C.* D-luciferin derivatives were incubated in combined CYP450 and luciferase reaction mixes at 37°C. For -CYP450 controls CYP450 Sf9 cell microsomes were replaced with H₂O. CYP450 and a luciferase reaction mix were added simultaneously to a CYP450 reaction mix and light output was read immediately (time=0). Readings were then taken every 10 minutes for 3 hours on a Turner 20/20 luminometer.

Figure 8. *Pooled human liver microsomes in two-step luminescent CYP450 reactions.* D-luciferin derivatives were incubated in a CYP450 reaction mix with pooled human liver microsomes for 60 minutes at 37°C before combining with a luciferase reaction mixture. For controls liver microsomes were replaced with control (no CYP450) Sf9 cell membranes. Luminescence was read within 12 minutes of combining the reactions on a Berthold Orion luminometer. Vehicle for sulfaphenazole, ketoconazole and alpha-naphthoflavone was 1% acetonitrile and 1 mg/mL bovine serum albumin in H₂O. Values labeled "nt" are vehicle controls. Concentrations of sulfaphenazole, ketoconazole and alpha-naphthoflavone in the reactions were 100micromolar, 100 micromolar and 10 micromolar, respectively.

Figure 9. *Two-step detection of CYP450 de picolinylase activity.* D-luciferin derivatives luc2PE, luc3PE, and luc4PE were incubated in a CYP450 reaction mix for 60 minutes at 37°C before combining with a luciferase reaction mixture. In this Figure, the bars labeled "Sf9" are the controls. These are Sf9 cell membranes without CYP450 expression. Luminescence was read within 12 minutes of combining the reactions on a Berthold Orion luminometer.

Figure 10. *CYP450-catalyzed conversion of luciferin derivatives to luciferin.* Luciferin derivatives (100 micromolar) were incubated in a CYP450 reaction mix for the indicated time intervals. At the end of each time interval, the reaction mixture was quenched with Tergitol to 0.1% (v/v), then frozen in liquid nitrogen. 95 microliter aliquots of the reaction mixture were analyzed by HPLC and luciferin was detected by fluorescence with excitation at 330 nm and emission at 520 nm. The zero time points represent the luciferin content of the derivatives from controls (no enzyme).

Figure 11. *Detection of Cyp450 inhibition by known CYP450 substrates.*

Luciferin derivatives as substrates for luminescent CYP450 assays were evaluated as probes for detecting other CYP450 substrates. CYP450 substrates tested were diclofenac for CYP2C9 and phenacetin for CYP1A1 and CYP1A2. The reactions were performed as described in Example 1 except the first step (CYP450 reaction) was in a 50 microliter reaction volume with 1 picomole of CYP450. In the second step a 50 microliter luciferase reaction was added to give final concentrations of 50 micrograms/mL Ultra Glo luciferase, 200 micromolar ATP, 0.1% Tergitol (v/v), 4.0 mM MgSO₄ and 100 mM Tricine pH 8.4. Panel A illustrates inhibition of CYP1A2 by phenacetin using Luc ME as substrate. Panel B illustrates inhibition of CYP1A1 by phenacetin using Luc CEE as substrate. Panel C illustrates inhibition of CYP2C9 by diclofenac using HLuc as substrate.

Figure 12: *P450 action on methoxy-coelenterazine HH, coelenterazine HH and coelenterazine by chemiluminescent and bioluminescent detection.* Panel A shows bioluminescence from methoxy coelenterazine-HH in relative light units (RLU) generated in a Renilla luciferase containing reaction following incubation of methoxy-coelenterazine HH with (+) or without (-) various P450 isozymes. Panel B shows the fold increase in bioluminescence from reactions containing methoxy-coelenterazine HH and P450 (+P450 RLU/-P450 RLU). Panel C shows chemiluminescence from methoxy-coelenterazine HH in RLU generated following incubation of methoxy-coelenterazine HH with (+) or without (-) various P450 isozymes. Panel D shows the fold increase in chemiluminescence from reactions containing methoxy-coelenterazine HH and P450 (+P450 RLU/-P450 RLU). Panel E shows bioluminescence from coelenter-

azine HH in RLU generated in a Renilla luciferase containing reaction following incubation of coelenterazine HH with (+) or without (-) various P450 isozymes. Panel F shows the fold decrease in bioluminescence from reactions containing coelenterazine HH and P450 (+P450 RLU/-P450 RLU). Panel G shows chemiluminescence from coelenterazine HH in RLU generated following incubation of coelenterazine HH with (+) or without (-) various P450 isozymes. Panel H shows the decrease in chemiluminescence from reactions containing coelenterazine HH and P450 (+P450 RLU/-P450 RLU). Panel I shows bioluminescence from coelenterazine in RLU generated in a Renilla luciferase containing reaction following incubation of coelenterazine with (+) or without (-) various P450 isozymes. Panel J shows the decrease in bioluminescence from reactions containing coelenterazine and P450 (+P450 RLU/-P450 RLU). Panel K shows chemiluminescence from coelenterazine in RLU generated following incubation of coelenterazine with (+) or without (-) various P450 isozymes. Panel L shows the decrease in chemiluminescence from reactions containing coelenterazine and P450 (+P450 RLU/-P450 RLU).

Figure 13. *Protection of luciferase from inhibitory buffer using yeast iPPase*. Yeast inorganic pyrophosphatase was found to be effective in reversing iPP inhibition of luciferase when inhibitory KPO_4 buffer used.

Figure 14. *Inorganic pyrophosphatases protect luciferase from pyrophosphatase contamination*. Inorganic pyrophosphatases from different sources were found to reverse iPP inhibition of luciferase when inhibitory KPO_4 buffer is used.

Figure 15. *Protection of luciferase from added iPP using iPPase*. Inorganic pyrophosphatase was found to be effective in reversing iPP inhibition of a luciferase-based reaction when iPP is added to the reaction.

Figure 16. *Cell-based Luminescent CYP450 Assays*. Primary rat hepatocytes were treated for 2 days with inducers of CYP450 gene expression: 5 micromolar 3-methylcholanthrene (MC), 50 micromolar dexamthasone (Dex) or 50 micromolar rifampicin (Rif) and their vehicle controls, 0.05, 0.1 and 0.1% DMSO, respectively (uninduced); and an inhibitor of CYP450: 100 micromolar troleandomycin (Tro). The induction medium was then replaced with 300 microliters of 100 micromolar luciferin-CEE (panels A and B), 200 micromolar luciferin-BE or 200 micromolar luciferin-BE plus Tro dissolved in hepatocyte culture medium and allowed to incubate for 4 hours. 100 microliters of medium was then removed from wells and combined with luciferin detection reagent (see example 15) and 200 microliters of luciferin detection reagent was added to the remaining 200 microliters of medium on cells. Luminescence from 200 microliters of culture medium reactions (panels A & C) and cell lysate reactions (panels B & D) was quantified.

Figure 17. *Stabilization of luminescent signals using luciferase inhibitors*. Inhibition of luciferase by an inhibitor 2-(4-anzinophenyl)-6-methylbenzothiazole (APMBT) or 2-amino-6-methyl benzothiazole (AMBT) stabilizes the luminescent signal in a luminescent CYP450 assay. 50 microliter CYP1A1 reactions (0.5 pmol recombinant CYP1A1 enzyme, 30 μ M Luciferin chloroethyl ether, 100 mM KPO_4 , 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 0.02 unit glucose-6-phosphate dehydrogenase) were incubated at 37°C for 20 min. After the incubation, 50 microliters of a luciferin detection reagent (100 micrograms/mL thermostable luciferase (from *Photuris pennsylvanica*), 400 micromolar ATP, 0.6% Prionex, 2 units/mL iPPase, 200 mM Tricine pH 8.4, 20 mM $MgSO_4$, 2% Tergitol) containing either 100 micromolar APMBT, 100 micromolar AMBT, or no inhibitor were added to each aliquot of CYP1A1 reaction. Luminescence was read immediately and at subsequent 5 minute intervals for 1 hour.

Detailed description of the invention

[0119] As defined herein, the term "cytochrome P450" or "CYP450" or "P450 enzyme," unless specified otherwise, refers to a family of heme-based oxidase enzymes involved in the metabolism of hydrophobic drugs, carcinogens, and other potentially toxic compounds and metabolites circulating in blood. It is known that the liver is the major organ for xenobiotic metabolism, containing high levels of the most important CYP450 mixed-function oxidases. These mixed-function oxidases are divided into subfamilies, which include CYP1A, 2A, 2C, 2D, 2E, and 3A. Within these subfamilies, there are numerous human P450 enzymes, often termed "isozymes" or "isoforms." The human CYP3A, CYP2D6, CYP2C, and CYP1A isoforms are known to be important in drug metabolism. See, e.g., Murray, M., 23 Clin. Pharmacokinetics 132-46 (1992). CYP3A4 is by far the major isoform in human liver and the small intestines, comprising 30% and 70% respectively of the total CYP450 protein in those tissues. Based primarily on *in vitro* studies, it has been estimated that the metabolism of 40% to 50% of all drugs used in humans involve CYP3A4 catalyzed oxidations. See Thummel, K. E. & Wilkinson, G. R., In Vitro and In Vivo Drug Interactions Involving Human CYP 3A, 38 Ann. Rev. Pharmacol. Toxicol., 389-430 (1998).

[0120] The term "luminescent", as used herein, includes bio-luminescence (i.e. light produced by a living organism), or chemi-luminescence (light produced when a chemical reaction proceeds). When the enzyme involved has evolved in an organism by natural selection for the purpose of generating light, or the enzyme involved is a mutated derivative of such an enzyme, the luminescent reactions are also called "bioluminescent reactions" and the enzyme involved is also called a "bioluminescent enzyme." Examples of bioluminescent enzymes include, without limitation, firefly luciferase, *Renilla* luciferase, *Cypridina* luciferase, *Aequorin* photoprotein, *Obelin* photoprotein, and the like.

[0121] The term "luminogenic molecule" as used herein refers to a molecule capable of creating light via a chemical

or biochemical reaction (e.g. beetle luciferin (or D-luciferin), coelenterazine, or a functional analog thereof). The luminogenic molecule could be either a P450 substrate or a P450 substrate/bioluminescent enzyme pro-substrate. Generally, a luminogenic molecule is either a high-energy molecular species (e.g. a stabilized dioxetane), or it is transformed into a high-energy molecular species by a chemical reaction. The chemical reaction is usually oxidation by oxygen, superoxide, or peroxide. In each case, the energy within the luminogenic molecule is released by the chemical reaction. Although at least some of this energy is released as photons of light, the energy can also be released in other forms, such as heat. The luminogenic molecules that do not yield light disperse their energy through alternative modes, often termed "dark pathways".

[0122] The term "luciferin derivative" as used herein refers to a type of luminogenic molecule or compound having a substantial structure of D-luciferin and maybe a substrate of one or more cytochrome P450 enzymes and a pro-substrate of luciferase. In the presence of cytochrome P450, the compound is metabolized into luciferin, a substrate of luciferase. In the absence of prior P450 metabolism, some of the the compound(s) may bind to luciferase as evidenced by their capacity to inhibit a reaction with luciferin (data not shown), however, they are not turned over as substrate in light-generating reactions. Without being bound by any theory of operation, it is believed that these compounds are most likely competitive inhibitors of luciferase.

[0123] The term "coelenterazines" as used herein refers to natural coelenterazines and coelenterazine derivatives. Coelenterazines are known to luminesce when acted upon by a wide variety of bioluminescent proteins, specifically marine luciferases. Examples of marine luciferases include *Renilla* luciferase, aequorin, *Gaussia* luciferase, *Oplophorus* luciferase, and *Cypridina* luciferase. Useful, but non-limiting, coelenterazines are disclosed in U.S. patent application no. 10/053,482, filed 11/2/01.

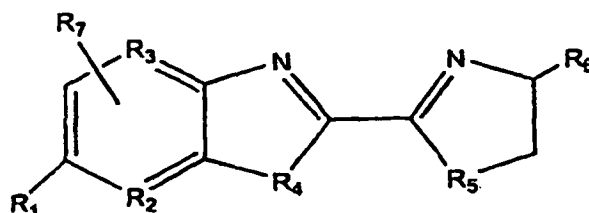
[0124] Coelenterazines are available from Promega Corporation, Madison, WI and from Molecular Probes, Inc., Eugene, OR. Coelenterazines may also be synthesized as described for example in Shimomura et al., *Biochem. J.* 261: 913-20, 1989; Inouye et al., *Biochem. Biophys. Res. Comm.* 233: 349-53, 1997; and Teranishi et al., *Anal. Biochem.* 249: 37-43, 1997.

[0125] The term "luciferase," unless specified otherwise, refers to a naturally occurring or mutant luciferase. The luciferase, if naturally occurring, may be obtained easily by the skilled from an organism. If the luciferase is one that occurs naturally or is a mutant, which retains activity in the luciferase-luciferin reaction, of a naturally occurring luciferase, it can be obtained readily from a culture of bacteria, yeast, mammalian cells, insect cells, plant cells, or the like, transformed to express a cDNA encoding the luciferase, or from an *in vitro* cell-free system for making the luciferase from a nucleic acid encoding same. Luciferases are available from Promega Corporation, Madison, WI

[0126] The term "pyrophosphatase," unless specified otherwise, refers to any agent such as an enzyme (naturally occurring or mutant) that is capable of breaking down or hydrolyzing pyrophosphate that is generated during the course of a reaction, already present in the reaction mixture, or introduced into a reaction mixture. The agent should be added at a concentration sufficient to either catalyze the hydrolysis of pyrophosphate in the reaction mixture at a rate that will prevent accumulation of pyrophosphate or prevent accumulation of pyrophosphate in any other manner. The amount of agent needed is readily determined by standard procedures. While inorganic pyrophosphatases (hydrolyases) are preferred agents in practicing this invention, there are many enzyme types (e.g., transferases, kinases, and synthetases) that may also be used in the practice of the invention. See, for example, U.S. Patent no. 6,291,164 which is incorporated by reference in its entirety. A number of such enzymes have been cloned and expressed in a recombinant host. See, for example, Lador, U.S. et al., *J. Biol. Chem.* 266:16550-16555 (1991) (Pyrophosphate: fructose-6-phosphate 1-phosphotransferase); Leyh, T. S. et al., *J. Biol. Chem.* 263:2409-2416 (1988) (ATP: sulfate adenyllyltransferase); Leyh, T. S. et al., *J. Biol. Chem.* 267:10405-10410 (1992) (ATP: sulfate adenyllyltransferase); Weissborn, A. C., et al., *J. Bacteriology* 176:2611-2618 (1994) (UTP:glucose-1-phosphate uridylyltransferase); Allen, T. et al., *Mol. Biochem. Parasitol.* 74:99 (1995) (Adenine phosphoribosyltransferase); Vonstein, V. et al., *J. Bacteriol.* 177:4540 (1995) (Orotate phosphotibosyltransferase); Charnig, Y. Y. et al., *Plant Mol. Biol.* 20:37 (1992) (Glucose-1-phosphate adenyllyltransferase); Kim, D. J. and Smith, S. M., *Plant Mol. Biol.* 26:423 (1994) (Phosphoenolpyruvate carboxyldnase); Jiang, Y. et al., *Exp. Parasitol.* 82:73 (1996) (Hypoxanthine-guanine phosphoribosyltransferase); Pla, J. et al., *Gene* 165:115 (1995) (ATP phosphoribosyltransferase); Feldman, R. C. et al., *Infect. Immun.* 60:166 (1992) (Uracil phosphoribosyltransferase); Vinitzky, A., *J. Bacteriol.* 173:536 (1991) (Micotinate phosphoribosyltransferase); Ludin, K. M. et al., *Curr. Genet.* 25:465 (1994) (Amidophosphoribosyltransferase); Rose, A. B. et al., *Plant Physiol.* 100:582 (1992) (Anthranilate phosphoribosyltransferase); Hughes, K. T. et al., *J. Bacteriol.* 175:479 (1993) (Quinolinate phosphoribosyltransferase); Jagadeeswaran, P. et al., *Gene* 31:309 (1984) (Xanthine-guanine phosphoribosyltransferase); Nakagawa, S., *Biosci. Biotech. Biochem.* 59: 694 (1995) (FMN adenyllyltransferase); Marolda, C. L. and Valvano, M. A., *J. Bacteriol.* 175:148 (1993) (Mannose-1-phosphate guanylyltransferase); Kalmar, G. B., *Proc. Natl. Acad. Sci. USA* 87:6029 (1990) (Choline phosphate cytidyltransferase); Muller-Rober, B. et al., *Plant Mol. Biol.* 27:191 (1995) (Glucose-1-phosphate adenyllyltransferase); Shanmugam, K. et al., *Plant Mol. Biol.* 30:281 (1996) (tRNA nucleotidyltransferase); Zapata, G. A. et al., *J. Biol. Chem.* 264: 14769 (1989) (Acylneuraminate cytidyltransferase); and Vakylenko, S. B. et al., *Antibiot. Khimioter.* 38:25 (1993) (Gentamycin 2'-nucleotidyltransferase). If such enzymes are used, it may be necessary to also employ a substrate which

is capable of either accepting a phosphate radical to give a phosphorylated product from pyrophosphate or effecting transfer of a pyrophosphate radical when in the presence of the enzyme.

[0127] Luciferin derivatives are provided that are substrates of CYP450 and are pro-substrates of luciferase. When these luciferin derivatives are exposed to certain CYP450 isoforms, these isoforms metabolize the derivatives into compounds that can be readily detected in a light-emitting reaction in the presence of the enzyme luciferase. In the absence of CYP450, the luciferin derivatives may bind to luciferase, however they are not turned over as substrate in light-generating reactions. In practising the methods of the invention, the luciferin derivatives of the invention have the following formula:



R_1 represents hydrogen, hydroxyl, amino, C_{1-20} alkoxy, substituted C_{1-20} alkoxy, C_{2-20} alkenyloxy, substituted C_{2-20} alkenyloxy, halogenated C_{2-20} alkoxy, substituted halogenated C_{2-20} alkoxy, C_{3-20} alkynyloxy, substituted C_{3-20} alkynyloxy, C_{3-20} cycloalkoxy, substituted C_{3-20} cycloalkoxy, C_{3-20} cycloalkylamino, substituted C_{3-20} cycloalkylamino, C_{1-20} alkylamino, substituted C_{1-20} alkylamino, di C_{1-20} alkylamino, substituted di C_{1-20} alkylamino, C_{2-20} alkenylamino, substituted C_{2-20} alkenylamino, di C_{2-20} alkenylamino, substituted di C_{2-20} alkenylamino, C_{2-20} alkenyl C_{1-20} alkylamino, substituted C_{2-20} alkenyl C_{1-20} alkylamino, C_{3-20} alkynylamino, substituted C_{3-20} alkynylamino, di C_{3-20} alkynylamino, substituted di C_{3-20} alkynylamino, C_{3-20} alkynyl C_{1-20} alkylamino, substituted C_{3-20} alkynyl C_{1-20} alkylamino, C_{3-20} alkynyl C_{2-20} alkenylamino, or substituted C_{3-20} alkynyl C_{2-20} alkenylamino;

R_2 and R_3 independently represents C or N;

R_4 and R_5 independently represents S, O, NR_8 wherein NR_8 represents hydrogen or C_{1-20} alkyl, CR_9R_{10} wherein R_9 and R_{10} independently represent H, C_{1-20} alkyl, or fluorine;

R_6 represents CH_2OH ; COR_{11} wherein R_{11} represents H, OH, C_{1-20} alkoxide, C_{2-20} alkenyl, or $NR_{12}R_{13}$ wherein R_{12} and R_{13} are independently H or C_{1-20} alkyl; or $-OM^+$ wherein M^+ is an alkali metal or a pharmaceutically acceptable salt; and

R_7 represents H, C_{1-6} alkyl, C_{1-20} alkenyl, halogen, or C_{1-6} alkoxide,

with the proviso that R_1 is not OH or NH_2 , if R_3 and R_2 are both carbon, R_4 and R_5 are both S, R_7 is H, R_6 is COR_{11} and R_{11} is OH, such that the luminogenic molecule is not luciferin or aminoluciferin.

[0128] In practising the methods of this invention, particularly preferred luciferin derivatives include luciferin 6' methyl ether (Luc ME), luciferin 6' ethyl ether (Luc EE), luciferin 6' chloroethyl ether (Luc CEE), luciferin 6' benzyl ether (Luc BE), luciferin 6' 3-picolinyl ether (Luc 3PE) and 6' deoxyluciferin (H Luc).

[0129] In another embodiment of the invention, a method is provided for measuring the activity of a cytochrome P450 enzyme. A luminogenic molecule as defined in claim 1 that is a P450 substrate and a bioluminescent enzyme pro-substrate is contacted with one or more cytochrome P450 enzymes and bioluminescent enzyme, either simultaneously or in a stepwise manner, for a predetermined time. In the presence of P450, the luminogenic molecule is metabolized into a substrate for the bioluminescent enzyme in a first reaction. The bioluminescent enzyme then acts on the substrate in a second light emitting reaction. Cytochrome P450 activity is determined by measuring the amount of luminescence that is generated from reaction mixture relative to a control (e.g., no P450 enzyme). For the P450 reaction to occur, P450 reductase, NADPH and Mg^{+2} are generally present in the system. Similarly, the presence of ATP and Mg^{+2} is generally necessary for firefly luciferase activity but not for Renilla luciferase activity. Any suitable concentration of luminogenic molecule may be employed in the reaction mixture. In practicing this invention, the concentration of the luminogenic molecule generally ranges between about 10 nM to 1 mM, preferably in the linear range of the substrate dose response by a particular P450 isoform, most preferably at the K_m for the particular substrate/P450 isoform reaction or at V_{max} for that reaction.

[0130] The invention also provides a method for determining P450 activity based on luminogenic molecules as defined in claim 1 that are natural coelenterazine and coelenterazine derivatives (collectively referred to as coelenterazines). The P450 acts on these luminogenic molecules in one of two ways. In one reaction pathway, the luminogenic molecules are P450 substrates and bioluminescent enzyme pro-substrates and do not exhibit the characteristic coelenterazine chemiluminescent (luminescence in the absence of a bioluminescent enzyme, e.g. Renilla-type luciferase). P450 me-

tabolism of the luminogenic molecule in a first reaction generates the substrate for the Renilla luciferase. The Renilla luciferase then acts on the substrate in a second light-emitting reaction. P450 activity is then ascertained by measuring the luminescence of the reaction mixture relative to a control reaction mixture. In the second reaction pathway, coelenterazine or coelenterazine derivatives exhibit chemiluminescence and are substrates for Renilla-type luciferase. P450 metabolism of such a luminogenic molecule results in the loss of chemiluminescence and activity with Renilla-type luciferase. In both types of reaction pathways, P450 activity may be detected either directly by a change in chemiluminescence by the action of the P450 and indirectly by a change in bioluminescence from a Renilla-type luciferase. Useful coelenterazines are disclosed in U.S. patent application no. 10/053,482, filed 11/2/01.

[0131] Luciferases differ somewhat in the ranges of conditions, of pH, ionic strength, temperature, ATP concentration, magnesium ion concentration, luciferin concentration, and the like, over which they are active in the luciferase-luciferin reaction. Likewise, cytochrome P450 enzymes differ somewhat in the ranges of conditions, of pH, ionic strength, temperature, cofactor requirements, substrate concentration, and the like, over which they are active in. It is, however, a simple matter for a skilled artisan to ascertain such ranges, and even the optimum ranges, for any particular luciferase and any particular cytochrome P450 enzyme. In practicing this invention, the amount of luciferase enzyme that may be employed in a reaction mixture generally ranges between about 0.1 microgram/mL to about 200 microgram/mL, preferably about 0.5 microgram/mL to about 100 microgram/mL. The amount of P450 enzyme that may be employed in a reaction mixture generally ranges between about 0.1 picomoles to about 200 picomoles, preferably about 0.4 to about 80.0 picomoles.

[0132] The skilled artisan is also aware that compositions other than those specifically recited above will be or may be present in any assay reaction mixture, in order to, for example, maintain or enhance the activity of the enzyme or as a consequence of the procedures used to obtain the aliquot of sample being subjected to the assay procedures. Thus, typically buffering agents, such as Tricine, HEPPS, HEPES, MOPS, Tris, glycylglycine, a phosphate salt, or the like, will be present to maintain pH and ionic strength; a proteinaceous material, such as a mammalian serum albumin (preferably bovine serum albumin) or lactalbumin or an ovalbumin, that enhances the activity of luciferases in the luciferase-luciferin reaction, may be present; EDTA or CDTA (cyclohexylenediaminetetraacetate) or the like, may be present, to suppress the activity of metal-containing proteases or phosphatases that might be present in systems (e.g., cells) from which luciferase to be assayed is extracted and that could adversely affect the luciferase or the ATP. Glycerol or ethylene glycol, which stabilize luciferases, might be present. Similarly, detergents or surfactants, particularly non-ionic detergents, such as those of octoxynol (e.g., sold under the trademark "Triton X" of Rohm & Haas, such as Triton X-100) might be included, typically as remnants, carried into a solution used in an assay according to the invention, of a solution used to lyse cells from which luciferase is extracted for the assay. Counterions to the magnesium will, of course, be present; as the skilled will understand, the chemical identities and concentrations of these counterions can vary widely, depending on the magnesium salt used to provide the magnesium ion, the buffer employed, the pH of the solution, the substance (acid or base) used to adjust the pH, and the anions present in the solution from sources other than the magnesium salt, buffer, and acid or base used to adjust pH. In practicing this invention, MgSO_4 or MgCl_2 are the preferred sources of magnesium ion. In one procedure, the magnesium ion can be supplied as the carbonate salt, to provide the desired magnesium ion concentration, in a solution with the buffer to be used (e.g., Tricine) and then the pH of the buffered solution can be adjusted by addition of a strong acid, such as sulfuric, which will result in loss of most of the carbonate (and bicarbonate) as carbon dioxide and replacement of these anions with sulfate, bisulfate, Tricine anion, and possibly also other types of anions (depending on other substances (e.g., phosphate salts) that provide anions and might be present in the solution). Oxygen-diffusion from the air into the solution in which the assay method is carried out is sufficient to provide the molecular oxygen required in the P450 and luciferase-luciferin reactions. In any case, it is well within the skill of the ordinarily skilled to readily ascertain the concentrations of the various components in an assay reaction mixture, including the components specifically recited above in the description of the method, that are effective for activity of the P450 enzyme in the P450 reaction and luciferase in the luciferase-luciferin reaction.

[0133] The luminescence of the luciferin-luciferase reaction may be measured using a commercially available luminometer, a scintillation counter, a photomultiplier photometer or a photoemulsion film. In one aspect of this invention, a one-step method for measuring P450 activity is provided.

[0134] In the one-step method, the luminogenic molecule as defined in claim 1 is contacted with both the cytochrome P450 enzyme and the bioluminescent enzyme simultaneously or contemporaneously and the mixture is allowed to incubate for a predetermined time period. The cytochrome P450 metabolizes the luminogenic molecule into a substrate for the bioluminescent enzyme in a first reaction. The bioluminescent enzyme then acts on the substrate in a second light emitting reaction. Cytochrome P450 activity is indirectly determined by measuring the amount of luminescence that is generated from the assay mixture relative to a control mixture. Controls may involve replacement of P450 enzyme with water or the P450 buffer, replacement of recombinant P450 membrane preparation with a similar preparation that lacks P450 enzyme, elimination of NADPH, or heat denaturation of P450 enzyme prior to addition of the luciferin substrate. Luminescence can be measured after a predetermined incubation time period or continuously from the time the reaction is initiated. For instance, the assay results shown in Figures 6 and 7 were read continuously from the time the reaction

was initiated.

[0135] In another and preferred aspect of this invention, a two-step method for measuring P450 activity is provided. In the two-step method, the luminogenic molecule as defined in claim 1 is first incubated with the cytochrome P450 enzyme for a first predetermined time period. The P450 enzyme metabolizes the luminogenic molecule and converts it into a substrate for the bioluminescent enzyme. Thereafter, the reaction mixture containing the P450 enzyme and substrate is contacted with a bioluminescent, e.g. luciferase, enzyme for a second predetermined time period. The bioluminescent enzyme acts on the substrate in a second light emitting reaction. Cytochrome P450 activity is then indirectly determined by measuring the amount of luminescence that is generated from the reaction mixture relative to a control (e.g., no active P450 enzyme).

[0136] In practicing this aspect of the invention, a detergent, preferably non-ionic, is preferably added to the P450/luminogenic molecule mixture just prior to or at the same time as contact with the bioluminescent, e.g., luciferase, enzyme in the second step of the two-step reaction system. The detergent quenches the P450 enzyme without interfering with the luciferase enzyme, thus allowing the analyst to measure luciferin (or luciferin derivative metabolite(s)) concentration dependent luminescence at the time of stopping the reaction without the complexity of having luciferin continuously added to the pool by an active P450 enzyme. Moreover, the non-ionic detergent stimulates the luciferase and results in a somewhat brighter reaction. In the case where a test compound such as a drug is a luciferase inhibitor, the non-ionic detergent diminishes the inhibitory effect on the luciferase and thus offers an advantage to the analyst who is interested only in the effects of the test compound on P450 activity. Suitable, but non-limiting, detergents include Tergitol (non-ionic); Brij 35 (non-ionic); Brij 58 (non-ionic); Polymixin B; Triton X-100 (non-ionic); Triton X-305 (non-ionic); Triton N101 (non-ionic); Chaps (zwitterionic); Chapso (zwitterionic); Bigchap (non-ionic); Thesit (non-ionic); Pluronic L64 (non-ionic); Rhodasurf 870; Chemoal LA-9; Sulfonol 465; Deoxycholate (anionic); and CTAB (cationic). In practicing this invention, Tergitol type NP-9, a polyglycol ether non-ionic surfactant, is preferred. The amount of detergent present in the assay mixture generally ranges between about 0.03% to about 2.0%, preferably between about 0.1% to about 1.0%.

[0137] For the one-step system and the first step of the two-step reaction systems, the reaction mixture generally included the following components: 25 mM KPO_4 at pH 7.4 for CYP2C9, 100 mM KPO_4 at pH 7.4 for CYP1A2 etc. Other components were 1.3 mM NADP^+ , 3.3 mM glucose-6-phosphate, 0.4 units/mL glucose-6-phosphate dehydrogenase, 3.7 mM MgCl_2 . For the one-step system, 200 micromolar ATP is also used. For the second step (luciferase reaction) of the two-step system, 5 mM to 200 mM Tricine buffer, preferably 20 mM to 200 mM Tricine buffer, is used although HEPES, HEPPS, MOPS, Phosphate, and Bicine buffers are also useful. Other components include 0.7 mM to 7.1 mM MgSO_4 (or MgCl_2), and 0.1 micromolar to 1 mM, preferably 10 micromolar to 250 micromolar, ATP. For the one-step system and the first step of the two-step reaction system, the reactions are generally carried out at about 20° to 42°C, preferably about 22° to 37°C. For the second step of the two-step reaction system, the reactions are generally carried out at a temperature of about 4° to 60°C, preferably about 20° to 42°C. While any suitable predetermined time may be used for the one-step or two-step reaction systems, the predetermined time for the one-step reaction system and the first step of the two-step reaction system generally ranges between about 1 minute to about 18 hours, usually about 1 minute to about 4 hours (for the one step reaction system) or about 10 minutes to about 1 hour (for the first step of the two-step reaction system). For the second step of the two-step reaction system, luminescence is determined immediately to about 18 hours, preferably immediately to about 3 hours after initiation of the luciferase reaction.

[0138] It has been discovered that the use of certain luciferase stabilizing molecules such as reversible inhibitors of luciferase may provide a protective effect against the known self-catalyzed auto-degradation of the luciferase enzyme, thus prolonging the luminescence signal and facilitating batch processing of reaction mixtures. As defined herein, a luciferase stabilizing agent is any molecule or group of molecules that substantially stabilizes luciferase against self-catalyzed auto-degradation of luciferase by slowing down the bioluminescence reaction. Without the stabilizing agent, the luminescent signal half-life may be as low as a few minutes, e.g., about three minutes. When stabilizing agent is used, the signal half life of the luciferase based reaction can be extended by as long as two hours or longer including overnight, depending on the choice of stabilizing agent and the concentration used. Examples of luciferase stabilizing molecules include, without limitation, reversible inhibitors of luciferase. Bioluminescent signals may decay rapidly, especially in the case where a firefly luciferase is used. These enzymes are known to inactivate at a rate that is positively correlated with the luminogenic reaction rate. While bright signals resulting from rapid luciferase reaction rates may be desirable, the rapid inactivation at high rates imposes a practical limitation on the assay. A stable luminescent signal would facilitate reading multiple samples in sequence without a significant time-dependent decay between samples. To achieve a stable luminescent signal, it is possible to slow the reaction rate and thereby diminish rate of signal decay. One method for slowing the luciferase reaction rate is to include a luciferase inhibitor that is competitive with luciferin. In this invention, 2-amino-6-methylbenzothiazole (AMBT) or 2-(4-aminophenyl)-6-methylbenzothiazole (APMBT), both competitive inhibitors of firefly luciferase, were found to be particularly useful in achieving a stable luminescent signal. Generally, the luciferase stabilizing agent is introduced after the completion of the P450 reaction and may be introduced prior to or at the start of the second step of two-step luminescent CYP450 assays.

[0139] In practising the invention, any suitable amount of luciferase inhibitor may be used that is effective to enhance the stability and prolong the lifetime of the luminescent signal relative to the luminescent signal generated in a comparable luciferase-based reaction mixture in the absence of the inhibitor. Preferably the amount of inhibitor should be such that the signal half-life is at least about two hours or more to allow for sample batch processing. Generally, the amount of the inhibitor ranges from about 1 micromolar to about 1 millimolar in the reaction mixture, preferably from about 1 micromolar and about 500 micromolar in the reaction mixture, more preferably from about 10 micromolar to about 200 micromolar in the reaction mixture, and most preferably about 100 micromolar in the reaction mixture:

[0140] In another embodiment of the invention, test compounds such as candidate drugs can be screened and evaluated for their activities as substrates of or regulators, either inhibitors or activators of a cytochrome P450 enzyme by using the luminogenic molecules, e.g. luciferin derivatives, of the present invention as defined in claim 1. A candidate drug may be determined to be regulator or a substrate of a cytochrome P450 enzyme by contacting a cytochrome P450 enzyme with the candidate drug, under conditions suitable for interaction therebetween, providing at least one luciferin derivative, under conditions that would, in the absence of an inhibitor or substrate of the cytochrome P450 enzyme, be suitable for interaction with the cytochrome P450 enzyme, and detecting the presence of luminescent signal of luciferin and/or a luciferin derivative metabolite in the presence of the luciferase, wherein luciferin and/or a luciferin; derivative metabolite would be, in the absence of an inhibitor of the cytochrome P450 enzyme, the product of the reaction between the cytochrome P450 enzyme and the luciferin derivative. Such efficient P450 substrates and regulators, as deemed appropriate by those of skill in the art, may be removed from a screening library where such efficient cytochrome P450 substrates and regulators are not desired in the remainder of the screening for a candidate drug.

[0141] In one aspect of the invention, a method is provided to distinguish between a substrate and an inhibitor of cytochrome P450 enzymes. Typically, the candidate compound is incubated with at least one cytochrome P450 enzyme under conditions, which allow for metabolism of the candidate compound prior to providing the luminogenic molecule, e.g., luciferin derivative, as defined in claim 1 under conditions that would, in the absence of an inhibitor or substrate of the cytochrome P450 enzyme, be suitable for interaction between the luciferin derivative and the cytochrome P450 enzyme. Any luciferin produced by the P450 metabolism of the luciferin derivative would then interact with luciferase in a light-emitting second reaction. The resulting light emitting reaction is compared to the one obtained from contacting a cytochrome P450 enzyme with the candidate drug, under conditions suitable for interaction therebetween, providing at least one luciferin derivative, under conditions that would, in the absence of an inhibitor of the cytochrome P450 enzyme, be suitable for interaction between the luciferin derivative and the cytochrome P450 enzyme. Metabolism of the candidate drug by a cytochrome P450 enzyme reduces its concentration in the assay medium and may lead to an apparent loss of cytochrome P450 inhibitory activity compared to conditions without metabolism of the compound which would indicate it was a substrate for the enzyme. An inhibitory compound that was not metabolized would show equal potency, irrespective of the time of addition of the optical cytochrome p450 enzyme substrate.

[0142] In another aspect of the invention, the drug candidate is preferably contacted first with the P450 enzyme for a first predetermined time period. Thereafter, the mixture is contacted with the luminogenic molecule, e.g., luciferin derivative, as defined in claim 1 and bioluminescent enzyme, e.g., luciferase, simultaneously or contemporaneously and the mixture is allowed to incubate for a second predetermined time period. Cytochrome P450 activity is determined by measuring the amount of luminescence that is generated from the reaction mixture relative to a control (e.g., no P450 enzyme).

[0143] In yet another (and preferred) aspect of the invention, the drug candidate is preferably incubated first with the P450 enzyme for a first predetermined time period to form a first mixture. Thereafter, the first mixture is contacted with the luminogenic molecule, e.g., luciferin derivative, as defined in claim 1 to form a second mixture that is allowed to incubate for a second predetermined time period. The second mixture is then contacted with a bioluminescent enzyme, e.g. luciferase, to form a third mixture which is allowed to incubate for a third predetermined time period. Thereafter, the P450 activity resulting from the interaction of the enzyme with the drug candidate is determined by measuring luminescence after the third predetermined time period relative to a control (e.g., no drug) reaction.

[0144] Any suitable concentration ranges for compound screenings may be used in practicing this invention. For primary library screening, the concentration of test compounds used generally ranges from about 1 to about 100 micromolar, usually about 10 micromolar. Secondary and further screens of primary hit test compounds generally employ wider ranges to establish the dose dependency of the response and depends, in part, on the potency of the test compound.

[0145] In practicing this aspect of the invention, non-ionic detergent is preferably added to the second mixture prior to the addition of luciferase in order to denature or deactivate the P450 enzyme. Suitable detergents and amounts are described above.

[0146] In another embodiment of the invention, a high throughput assay method is provided for screening a plurality of compounds to determine their effect on cytochrome P450 activity. The test compounds are contacted with one or more types of P450 enzymes, the luminogenic molecule, e.g. luciferin derivative, as defined in claim 1 and bioluminescent enzyme, e.g., luciferase, for a predetermined time period. Thereafter, the P450 activity resulting from the interaction of the P450 enzyme with the compounds are determined by measuring luminescence.

[0147] In one aspect of the invention, the compounds are preferably contacted first with the P450 enzymes for a first predetermined time period. Thereafter, the mixture, is contacted with the luminogenic molecule, e.g., luciferin derivative, as defined in claim 1, and bioluminescent enzyme, e.g., luciferase, simultaneously or contemporaneously and the mixture is allowed to incubate for a second predetermined time period. Cytochrome P450 activity is determined by measuring the amount of luminescence that is generated after the second predetermined time period relative to a control (e.g., non-P450 enzyme) reaction. In another (and preferred) aspect of the invention, the compounds are preferably contacted first with the P450 enzymes for a first predetermined time period to form first mixtures. Thereafter, the first mixtures are contacted with the luciferin derivative to form second mixtures that are allowed to incubate a second predetermined time period. The second mixtures are then contacted with luciferase to form a third mixture which is allowed to incubate for a third predetermined time period. Thereafter, the P450 activities resulting from the interaction of the enzyme with the test compounds are determined by measuring luminescence of the reaction mixture relative to a control (e.g., no P450 enzyme) reaction mixture. In practicing this aspect of the invention, non-ionic detergent is preferably added to the second mixture prior to the addition of luciferase. Suitable detergents and amounts are described above.

[0148] For compound screening, P450 is contacted first with the test compound for a predetermined time period prior to addition of the luminogenic molecule, e.g., luciferin derivative, as defined in claim 1. Another approach would involve contacting the P450 with the drug and luciferin simultaneously. Yet another approach would involve contacting the P450 with the luciferin first for a predetermined time period prior to addition of the test compound.

[0149] In another embodiment of the invention, a cell-based method is provided for screening test compound to determine its effect on cytochrome P450 activity of the cell. The test compound is contacted with a cell, the luminogenic molecule, e.g., luciferin derivative, as defined in claim 1, and bioluminescent enzyme, e.g., luciferase, for a predetermined period of time. Thereafter, the P450 activity resulting from the interaction of the cell with the compound is determined by measuring luminescence of the reaction mixture relative to a control (minus test compound) reaction mixture.

[0150] In one aspect of the invention, the compound is preferably contacted first with the cell for a predetermined time period. Thereafter, the cell is contacted with the luciferin derivative as defined in claim 1 and luciferase simultaneously or contemporaneously and the cell is allowed to incubate with the derivative and luciferase for a second predetermined time period. Cytochrome P450 activity of the cell is determined by measuring the amount of luminescence generated from the reaction mixture relative to a control reaction mixture (e.g., minus test compound). In another (and preferred) aspect of the invention, the test compound is preferably contacted first with the cell for a predetermined time period. Thereafter, the exposed cell is then contacted with the luciferin derivative as defined in claim 1 and incubated for a second predetermined time period. The cell is then contacted with luciferase to form a third mixture which is allowed to incubate for a third predetermined time period. Thereafter, the P450 activity of the cell resulting from the interaction of the cell with the test compounds are determined by measuring luminescence of the reaction mixture relative to a control reaction mixture (e.g., minus test compound). In practicing this aspect of the invention, non-ionic detergent is preferably added to the second mixture prior to or at the same time as the addition of luciferase to ensure more complete release of luciferin or luciferin derivative metabolites(s), resulting in a stronger signal and a more sensitive assay. Detergent will rupture the cells and release luciferin. In the absence of detergent, however, luciferin or luciferin derivative metabolite (s) may leak out of the cell due to its cell permeability and this would form the basis for a real-time live cell assay with luciferase and ATP in the medium. Suitable detergents and concentration ranges are described above.

[0151] In practicing this aspect of the invention, suitable cells would include any cell that expresses one or more P450 enzymes and its requisite cofactors such as P450 reductase that utilizes the luciferin derivatives as defined in claim 1. Such cells can be used to examine the effects of test compounds on CYP450 enzyme activities present in the cell at the time the test compound is applied. The cells can also be used to examine the effects of test compounds on the expression of endogenous CYP450 genes or transgenes that encode CYP450s with gene regulatory sequences. In this case test compound-induced changes in gene expression can be detected by measuring changes in the level of p450 enzyme activity. Representative examples include: (a) Primary hepatocytes from human or animal sources (commercially available from several sources: Gentest, Woburn, MA; Clonetics, Inc., San Diego, CA; Xenotech LLC, Lenexa, KS); (b) Hepatocytic cell lines: HepG2, HepG2C3A. Commercially available from Amphioxus Cell Technologies Inc. (Houston, TX) and from American Type Culture Collection (ATCC), THLE-3 Commercially available from ATCC, HepLiu porcine hepatocyte line, commercially available from MultiCell Technologies (Warwick, RI), and BC2 human hepatoma cell line (Gomez-Lechon, M.J. et al (2001) "Expression and induction of a large set of drug-metabolizing enzymes by the highly differentiated human hepatoma cell line BC2", Eur. J. Biochem. 268, 1448-1459); (c) Cells expressing recombinant P450s such as: HepG2 (Yoshitomi, S. et al (2001) "Establishment of the transformants expressing human cytochrome P450 subtypes in HepG2, and their applications on drug metabolism and toxicology", Toxicol. In Vitro 15(3), 245-256), Chinese hamster ovary (CHO) cells (Gabelova, A. et al (2002) "Mutagenicity of 7H-dibenzo(c,g)carbazole and its tissue specific derivatives in genetically engineered Chinese hamster V79 cell lines stably expressing cytochrome p450", Mutation Research, 517(1-2), 135-145), BEAS-2B, SV40 immortalized human bronchial epithelial cells (Coulombe, R.A. et al (2002) "Metabolism and cytotoxicity of aflatoxin B1 in cytochrome P450-expressing human lung cells" J. Toxicol. Env. Health Part A, 65(12), 853-867), MCL-5B lymphoblastoid cell line (commercially available from Gentest, Woburn

MA); and (d) non-mammalian cells such as yeast, bacterial, plant, fungal etc. with native expression of P450(s) or the same having been transformed with an expressible P450 cDNA(s) and P450 reductase.

[0152] For cell-based compound screening, any suitable amount of test compound may be used, depending on the potency of the test compound and toxicity. For primary library screening, the concentration of test compounds used typically ranges from about 1 to about 1000 micromolar, usually about 10-100 micromolar. Any suitable incubation time may be used. Generally, the time period for incubation of a test compound with cells typically ranges from about 1 minute to about 96 hours, usually about 24 hours to about 72 hours.

[0153] Any suitable number of cells may be used for cell-based compound screening, including cell-based high throughput screening. A single cell would be the low end of a general range while for a cell culture receptacle surface with adherent cells, cell confluence or super confluence would be considered to be the upper end of the general range for adherent cells. For suspension cultures, a cell-saturated suspension would be considered the upper end of a general range. In practicing this invention, the preferred number of cells would be the minimum number of cells where signal is detectable above background to a maximum number of cells described for the general range. For the incubation period, the suitable temperature and pH ranges will depend on the requirements of the cell. Generally, most cells are cultured at pH 7.4 and at 37°C, but most cells are viable, at least temporarily, over a range of temperature of 4 to 42°C and at pH ranges of about 6 to 9.

[0154] The cell-based luminescence detection assay can be performed in a number of different ways. For instance, in one embodiment, luciferase and ATP may be added to the cell medium and luminescence could be detected directly as luciferin leaks out of the cells. In this case the temperature, pH, salt concentration, and other growth requirements will depend on the requirements of the cells. Luciferase is generally active at the physiological salt, pH and temperature conditions typically employed in cell culture.

[0155] In another embodiment of the cell-based luminescence detection assay, culture medium may be removed from the culture and added to a luciferase reaction. The conditions for this would be essentially as already described for the second step of a two-step reaction assay described above.

[0156] In yet another embodiment of the cell-based assay of the invention, the cells may be lysed in an appropriate lysis buffer that contains ATP, luciferase along with detergent(s) to ensure lysis and then luminescence is read immediately. For animal cells, a buffer with 0.1-1.0% non-ionic detergents such as Triton X 100 or Tergitol is typically sufficient. Bacteria, plant, fungal or yeast cells are usually more difficult to lyse. Detergents, freeze/thaw cycles, hypotonic buffers, sonication, cavitation or combinations of these methods may be used. The method of lysis must produce a lysate that is compatible with luciferase activity. In the case where detergent is used, the detergent is one that is compatible with active luciferase. Luminescence would be proportional to the luciferin or luciferin derivative metabolite(s) generated by the P450 reaction and then released from the cells. In a representative example of cell-based luminescence detection, a volume of lysis buffer is added to an equivolume of the spent cell culture medium. In a variation of this embodiment of the invention, a cell lysate may be prepared with a lysis buffer that does not contain luciferase and ATP, then an aliquot of the lysate could be added to a one-step or two-step luciferase-based assay as described above.

[0157] In yet another embodiment of the cell-based luminescence detection assay of the invention, a cell that either transiently or stably expresses a recombinant bioluminescent enzyme, e.g., luciferase, may be used. Any conventional method for creating transient or stable transfected cells may be used. An expressible cDNA vector that has been introduced to the cell encodes the luciferase. Luminescence would then evolve as a P450(s) also present in the cell metabolizes the luciferin derivative supplied in the medium. P450 activity may be determined by measuring luminescence after a predetermined time period either *in situ* directly or after lysis. Vectors for making the cells by transient or stable transfection are available from Promega (Madison, WI), Clontech (Pal Alto, CA) and Stratagene (La Jolla, CA).

[0158] In another embodiment of the invention, a tissue-based method is provided for screening a test compound or library of compounds to determine their effect on cytochrome P450 activity of the cell. The test compound is contacted with tissue, the luminogenic molecule, e.g., luciferin derivative, as defined in claim 1 and bioluminescent enzyme, e.g., luciferase for a predetermined period of time. Thereafter, the P450 activity resulting from the interaction of the tissue with the compound is determined by measuring luminescence of the reaction mixture relative to a control (minus test compound) reaction mixture. Representative examples of tissue include, without limitation, liver, intestine, and lung. The tissue may be used in the form of minces or slices such as liver slices. Generally, the same considerations provided above for cell-based assays would be applicable.

[0159] In another embodiment of the invention, a method is provided for screening a test compound to determine its effect on the cytochrome P450 activity of an animal. The test compound and the luminogenic molecule, e.g., luciferin derivative, as defined in claim 1 are administered to the animal. After a predetermined time period, a biological specimen is removed from the animal. Representative biological specimen includes blood and serum, urine, feces, bile, cerebrospinal fluid, lymph, saliva, tears, and mucus (basically any body fluid where CYP450 metabolites might be found). Blood/serum, urine, feces and bile are preferable biological specimens. Blood and feces would likely need to be processed. For instance, blood specimens may be processed to remove cells and produce serum. A fecal specimen may be processed to produce an extract. Most of the other fluids would ideally be added directly to a luciferase assay or may be optionally

diluted prior to addition to the luciferase assay. The specimen is then contacted with luciferase, ATP and Mg^{2+} . After a predetermined time period, P450 activity resulting from the interaction of the animal with the compound is determined by measuring luminescence relative to a control. One type of control would include a specimen obtained from the animal prior to administration of the test compound. This type of control would involve administration of the luciferin derivative without exposure to the drug and the samples and measurements would be taken at a set time. At a later time, after the luciferin is cleared from its system, the test experiment, luciferin derivative plus drug exposure, would be performed on the same animal in the same way. This control has the advantage of using the same animal as test and control. This principle could also be applied to cell-based and tissue slice assays. An alternative approach would be to have separate test and control animals. The test animals would receive luciferin derivative and drug while the control animals would receive only luciferin derivative.

[0160] In one aspect of the invention, the compound and luciferin derivative as defined in claim 1 are preferably administered to the animal either simultaneously or contemporaneously. After a first predetermined time period, a biological specimen is obtained from the animal. The biological specimen is then contacted with luciferase for a second predetermined time period. The effect of the test compound on cytochrome P450 activity of the animal is determined by measuring the luminescence produced from the reaction mixture relative to a control reaction mixture (e.g. specimen obtained from control animals as described above).

[0161] In another aspect of the invention, the compound is preferably administered first to the animal. After a first predetermined time period, the luciferin derivative as defined in claim 1 is then administered to the animal. After a second predetermined time period, a biological specimen is obtained from the animal. The biological specimen is then contacted with luciferase for a predetermined time period. The effect of the test compound on cytochrome P450 activity of the animal is determined by measuring the amount of luminescence that is generated from the luciferase reaction mixture relative to a control (e.g., based on a specimen obtained from control animals as described above).

[0162] The test compounds and luminogenic molecules, e.g., luciferin derivatives, as defined in claim 1 may be formulated by any suitable means and can be used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions, suspensions for injectable administration; and the like. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride, and the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of non-toxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (e.g., liposomes) may be utilized.

[0163] The amount of the test composition required as an *in vivo* dose as well as the amount of luciferin derivative used will depend on the route of administration, the type of animal being treated, and the physical characteristics of the specific animal under consideration. The dose can be tailored to achieve a desired effect, but will depend on such factors as weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognize.

[0164] In non-human animal studies, applications of potential drug candidates are commenced at higher dosage levels, with dosage being decreased until the desired effect is no longer achieved or adverse side effects disappear. The dosage can range broadly depending upon the desired effects and the therapeutic indication. Typically, dosages may be between about 10 microgram/kg and 100 mg/kg body weight, preferably between about 100 microgram/kg and 10 mg/kg body weight. Administration is preferably oral. Oral administration would be preferable, although absorption through skin or mucous membranes, intravenous, subcutaneous or intraperitoneal injection routes may be used. While control of dosage may be problematic in some instances, oral administration would allow the analyst to examine first pass metabolism that is greatly influenced by p450s in the cells lining the gut where the compounds are absorbed. Administration by injection may be better for controlling dose in animals.

[0165] In yet another aspect of the invention, an animal-based high throughput assay for screening compounds is provided. Laboratory animals are useful for defining mechanisms of drug activity and for testing therapeutic regimens. More recently, zebrafish and other teleosts have been found to be particularly useful for *in vivo* high throughput screening of compounds that employ intact animals. As defined herein, the term "teleost" means of or belonging to the Teleostei or Teleostomi, a group consisting of numerous fishes having bony skeletons and rayed fins. Teleosts include, for example, zebrafish, medaka, Giant rerio, and puffer fish. See, for instance, U.S. Patent no. 6,299,858 (Phylonix, Inc., assignee). For instance, test compound and luciferin derivative as defined in claim 1 may be administered to living embryonic teleosts contained in multi-well plates. The teleosts are maintained in a suitable medium such as water. After one or more predetermined time periods, the medium is then contacted with luciferase for a second predetermined time period. The effect of the test compound on cytochrome P450 activity of the animal may be determined by measuring the luminescence produced from the mixture relative to a control reaction mixture absent test compound.

[0166] In another aspect of the invention, the compound is preferably administered first to the teleosts. After a first predetermined time period, the animal is contacted with the luciferin derivative as defined in claim 1. After a second predetermined time period, the medium is then contracted with luciferase. The effect of the test compound on cytochrome P450 activity of the animal may be determined by measuring the luminescence produced from the mixture relative to a

control reaction mixture absent test compound.

[0167] Transgenic animals with a luciferase transgene are also useful in animal-based assays for ascertaining P450 activity and for screening one or more compounds. Luciferase transgenes have been efficiently expressed in livers, mostly in mouse. Xenogen, Inc. and others has developed transgenic animals such as mouse and rat with a luciferase transgene that gets expressed in target tissues such as liver. See, for instance, U.S. Patent Nos. 5,650,135 and 6,217,847. Generally, such transgenic animals are injected with luciferin and imaging technology (in vivo biophotonic imaging) is then used to measure luminescence at the site of luciferase expression in the living, intact animal. Thus, in another aspect of the invention, a transgenic animal having a bioluminescence enzyme, e.g., luciferase, transgene may be administered a luminogenic substrate as defined in claim 1 that will be converted into a substrate for a bioluminescence enzyme in tissues where the appropriate P450 is expressed. If the bioluminescence enzyme, e.g., luciferase, transgene is also expressed in that tissue, light will be produced and such light may be detected by any suitable means. As discussed above, one or more drugs can be tested for P450 effects in such transgenic animals in an animal-based assay. Alternatively, tissue from such transgenic animals can be used in a tissue-based assay. A drug that inhibits P450 enzyme activity will diminish the signal and a drug that induces P450 gene expression will enhance the signal.

[0168] The presence of iPP in a luciferase-based reaction is undesirable because it may affect the reproducibility of the reaction. Pyrophosphate is a product of a luciferase-based reaction with ATP, O₂ and luciferin. Under certain conditions, iPP may accumulate to inhibitory levels. In addition, components of a luciferase-based reaction may contribute inhibitory amounts of iPP. For example, orthophosphate salts used to buffer the reaction may contain iPP as a contaminant. While avoiding the use of phosphate buffered solutions can solve the iPP problem, this is often inconvenient or impractical, particularly in P450 reactions where phosphate buffers are generally used. It has been discovered that the inclusion of a pyrophosphatase such as an inorganic pyrophosphatase enzyme (iPPase) in the luciferase-based reaction reduces or prevents luciferase inhibition by iPP that may already be present in the assay mixture or that may be generated during the course of the luciferase reaction. While this discovery is generally applicable to all luciferase-based reactions where potential inhibition of luciferase by iPP may be a problem, the inclusion of a pyrophosphatase such as iPPase in luminescent cytochrome P450 (P450) assays has been found to be particularly useful. Without being bound by any theory of operation, it is believed that the pyrophosphatase, e.g., iPPase, acts to prevent the build-up of iPP as well as remove it from a solution by hydrolyzing the iPP into orthophosphate.

[0169] In practising this invention, the pyrophosphatase, e.g., iPPase, may be added prior to, simultaneously with, or shortly after luciferase addition. Preferably, the pyrophosphatase, e.g., iPPase, is added to the assay mixture simultaneously with luciferase addition to degrade any iPP already present in the mixture and generated during the luciferase reaction. The amount of pyrophosphatase, e.g., iPPase, used in the reaction is sufficient to remove or eliminate any iPP that may already be present or may be subsequently generated during the course of the luciferase reaction. Generally, in the presence of a pyrophosphatase, e.g., iPPase, the level of iPP in the reaction mixture is eliminated or reduced to an amount which has little or no significant effect on luciferase activity during a luciferase reaction. That is, the level of iPP is low enough to reduce luciferase inhibition to an insignificant level. The iPPase may be derived from a variety of sources including, without limitation, yeast, prokaryotes and mammals. In practising this invention, iPPase derived from *Saccharomyces cerevisiae* is preferred.

[0170] In one embodiment of this invention, the pyrophosphatase, e.g., iPPase, may be included in the cell-free or cell-based luminescent P450 assays described above. In said methods, P450 enzymes convert luciferin derivatives as defined in claim 1 that are pro-substrates for firefly luciferase into luciferin substrates that then react with luciferase to generate light. For instance in the first step of the two-step method described above, a P450 enzyme may be incubated with a luciferin derivative under suitable assay reaction conditions. In a second step, the luciferin substrate generated in the first step is then detected luminogenically when luciferase and ATP are added to the mixture of the first step. Generally, it is convenient to employ KPO₄ buffers in the first step to buffer pH and to provide optimal salt concentration for specific P450 isoforms by varying its concentration. However, some KPO₄ buffers carry sufficient amounts of iPP contaminant which may inhibit luciferase and thus impair luciferin detection. By adding an iPPase to the reaction mixture, inhibition by the presence of any iPP contaminant may be reduced or prevented.

[0171] A pyrophosphatase, e.g., iPPase, may be included in any luciferase reaction, including cell-free or cell-based luciferase assays. The addition of pyrophosphatase, e.g., iPPase, as a reaction component would allow these assays to be performed in conventional phosphate buffers without concern for iPP inhibition of luciferase. Other applications may include the use of iPPase in luciferase assays where the iPP generated by the luciferase reaction accumulates to inhibitory levels.

[0172] In another embodiment of the invention, a kit is provided for determining the activity of a CYP450 or the effect of a substance, e.g., a drug candidate, on cytochrome P450 enzyme activity comprising the luminogenic molecules defined in claims 87 and 96. Such kits may further comprise, in one or more containers, usually conveniently packaged to facilitate use in assays, quantities of various compositions essential for carrying out the assays and methods in accordance with the invention described herein. Thus, the kit may include one or more types of cytochrome P450 enzymes; one or more further luminogenic molecules, e.g., D-luciferin derivatives, that are substrates for one or more

types of cytochrome P450 enzymes and a pro-substrate of a bioluminescent enzyme, e.g., luciferase enzyme; a bioluminescent enzyme, e.g., luciferase enzyme; and directions for using the kit. Optionally, the kit includes ATP, a source of Mg ions, non-ionic detergent, a pyrophosphatase, e.g., iPPase, and/or buffers or any other reaction components to provide a solution at suitable pH and ionic strength. As indicated, the various components can be combined, eg., in solution or a lyophilized mixture, in a single container or in various combinations (including individually) in a plurality of containers. The preferred kit includes vial(s) with P450 substrate as defined in claims 87 and 96 (e.g., D-luciferin derivative), vial(s) containing a mixture of bioluminescent enzyme, e.g., luciferase, and optional iPPase (preferably from *Saccharomyces cerevisiae*) (preferably a lyophilized preparation) and a vial with a dilution buffer that contains ATP for the bioluminescent enzyme, e.g., luciferase (in the case of a lyophilized luciferase/ATP preparation, this buffer is a "reconstitution buffer"). The dilution buffer or reconstitution buffer may also contain the detergent.

[0173] The kits can also include, as well known to the ordinary skilled in the art, various controls and standards, including no P450 (negative control), to ensure the reliability and accuracy of the assays carried out using the kits.

Examples:

[0174] **Materials** Sf9 cell expressed CYP450 preparations (Supersomes™), pooled human liver microsomes and NADPH generating system (NADP⁺, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) were purchased from GenTest (Woburn, MA). The substrates, luciferin 6' methyl (Luc ME), ethyl (Luc EE), chloroethyl (Luc CEE) and benzyl (Luc BE) ethers and dehydroxyluciferin (H-Luc) were manufactured by Promega Biosciences (San Luis Obispo, CA). Luc ME and Luc EE were also purchased from Sigma-Aldrich (St. Louis). The recombinant, mutant of firefly luciferase from *Photuris penusylvanica* was from Promega (17). All chemical reagents and solvents referred to herein are readily available from a number of commercial sources including Aldrich Chemical Co. or Fischer Scientific. NMR spectra were run on a Hitachi 60 MHz R-1200 NMR spectrometer or a Varian VX-300 NMR spectrometer. IR spectra were obtained using a Midac M series FT-IR instrument. Mass spectral data were obtained using a Finnegan MAT 90 mass spectrometer. All melting points are corrected.

Example 1: Synthesis of Luciferin Derivatives

(a) Preparation of 2-cyanobenzothiazole derivatives

[0175] **Luciferol:** A suspension of D-luciferin free acid (0.43 g, 1.53 mmol) in THF (15 mL) was cooled in a -20°C bath (dry ice-isopropanol). To the suspension was added dropwise via syringe a solution of borane-THF (1.8 mL of a 1 M solution in THF, 1.8 mmol). The pale yellow solution was allowed to warm to ambient temperature overnight (about 15 h) under nitrogen. Additional borane-THF (2.5 mL of a 1 M solution in THF, 2.5 mmol) was added and the reaction went an additional 24 h. The excess borane-THF was quenched by the addition of 10% aqueous acetic acid solution and the resulting bilayer was extracted with ethyl acetate (3 x 75 mL). Combined extracts were dried and evaporated to give an orange solid that was purified by chromatography on silica gel (70 g) using 4:1 dichloromethane-methanol. This operation separated the remaining D-luciferin free acid from a less polar product mixture. The less polar product mixture was separated by reverse-phase HPLC on a 1-inch Synergi 4μ MAX-RP 80A column (100 x 21.20 mm) using a methanol-water gradient. Appropriate fractions were pooled and evaporated to provide 10 mg (3% yield) of the desired product as a pale yellow solid. This product was 95.4% pure by HPLC analysis. MS (ESI⁻): m/z 264.4 (M-H)⁻; calc'd: 265.01.

[0176] **2-Cyanobenzothiazole:** A suspension of potassium cyanide (1.50 g, 23.0 mmol) in dimethylsulfoxide (DMSO, 100 mL) was prepared in a 1-L 3-necked flask fitted with a reflux condenser, a heating mantle and an internal temperature probe. The 2-chlorobenzothiazole (2.6 g, 2.0 mL, 15.3 mmol) was added to the reaction flask via pipet and the reaction was heated at 80°C (internal temperature). The reaction was monitored periodically by TLC (9:1 heptane-ethyl acetate). After 5 hours the reaction appeared to be about 50% complete. After 17 hours the reaction was judged complete by TLC analysis. The reaction mixture was allowed to cool to room temperature and was then extracted with ether (5 x 100 mL). The extracts were dried over sodium sulfate and concentrated to give a yellow-orange solid. The crude solid was purified on silica gel (100 g) using 9:1 heptane-ethyl acetate. Fractions containing product were pooled and concentrated to give 1.4 g (57%) of the desired product as a yellow-orange solid. The structure was confirmed by ¹H NMR analysis in DMSO-d₆.

[0177] **6-(2-Chloroethoxy)-2-cyanobenzothiazole:** A suspension of 2-cyano-6-hydroxybenzothiazole (1.0 g, 5.68 mmol) in acetone (5 mL) was prepared in a 50-mL 2-necked round-bottomed flask fitted with a reflux condenser. Anhydrous potassium carbonate (1.57 g, 11.3 mmol) was added to the reaction mixture and the suspension turned to a yellow solution. Then 1-bromo-2-chloroethane (1.06 g, 0.56 mL, 7.38 mmol) was added by syringe and the reaction mixture was heated at reflux using a heated stir plate and oil bath. The reaction was monitored periodically by RP HPLC. After 5 hours the reaction appeared to be about 30% complete. Additional 1-bromo-2-chloroethane was added (2.84 mmol, 0.21 mL) and the reaction was refluxed overnight. HPLC analysis indicated the reaction was about 50% complete.

Additional 1-bromo-2-chloroethane (2.84 mmol, 0.21 mL) and potassium carbonate (0.60 g, 4.37 mmol) were added and the reaction was refluxed for the rest of the day (about 7 h). The reaction was about 64% complete, and it was refluxed overnight. The next morning the reaction was found to be about 84% complete. Additional 1-bromo-2-chloroethane (2.84 mmol, 0.21 mL) and potassium carbonate (0.60 g, 4.37 mmol) were added and the reaction was refluxed for the rest of the day (about 7 h). At this point the reaction was about 92% complete. The reaction mixture was allowed to cool to room temperature and it was then filtered through glass fiber paper and rinsed with acetone. The filtrate was concentrated to give a yellow-brown solid. The crude solid (1.3 g) was purified on silica gel (100 g) using 4:1 heptane-ethyl acetate. Fractions containing product were pooled and concentrated to give 1.0 g (74%) of the desired product as an off-white solid. The structure was confirmed by ¹H NMR analysis in DMSO-d₆.

[0178] *6-(4-Trifluoromethylbenzyloxy)-2-cyanobenzothiazole*: A suspension of 2-cyano-6-hydroxybenzothiazole (1.0 g, 5.68 mmol) in acetone (5 mL) was prepared in a 100-mL 2-necked round-bottomed flask fitted with a reflux condenser. Anhydrous potassium carbonate (0.94 g, 6.82 mmol) was added to the reaction mixture and the suspension turned to a yellow solution. Then 4-(trifluoromethyl)benzyl bromide (1.49 g, 6.25 mmol) was added and the reaction mixture was heated at reflux for 15 h using a heated stir plate and oil bath. The reaction mixture was allowed to cool to ambient temperature and then filtered to remove inorganic salts. The filtrate was concentrated by rotoevaporation to provide 1.7 g (89% yield) of an off-white solid that was used without purification. The structure was confirmed by ¹H NMR analysis in DMSO-d₆.

[0179] *6-(Berulyoxy)-2-cyanobenzothiazole*: A suspension of 2-cyano-6-hydroxybenzothiazole (0.35 g, 2.00 mmol) in acetone (35 mL) was prepared in a 100-mL 2-necked round-bottomed flask fitted with a reflux condenser. Anhydrous potassium carbonate (0.33 g, 2.40 mmol) was added to the reaction mixture and the suspension turned to a yellow solution. Then benzyl bromide (0.41 g, 0.29 mL, 2.40 mmol) was added and the reaction mixture was heated at reflux for 15 h using a heated stir plate and oil bath. The reaction mixture was allowed to cool to ambient temperature and then filtered to remove inorganic salts. The filtrate was concentrated by rotoevaporation to provide 0.51 g of a pale yellow solid. The crude product was purified by flash chromatography on silica gel (24 g) using a mobile phase consisting of a mixture of 4:1 heptane-ethyl acetate to provide 410 mg (77 % yield) of the desired product as a white foam. The structure was confirmed by ¹H NMR analysis in DMSO-d₆.

[0180] *6-(2-Phenylethoxy)-2-cyanobenzothiazole*: A suspension of 2-cyano-6-hydroxybenzothiazole (1.00 g, 5.68 mmol) in acetone (5 mL) was prepared in a 100-mL 2-necked round-bottomed flask fitted with a reflux condenser. Anhydrous potassium carbonate (1.18 g, 8.52 mmol) was added to the reaction mixture and the suspension turned to a yellow solution. Then (2-bromoethyl)benzene (1.37 g, 0.88 mL, 7.38 mmol) was added and the reaction mixture was heated at reflux for using a heated stir plate and oil bath. Progress of the reaction was monitored by HPLC analysis of reaction aliquots. After 15 h of reflux the reaction was about 40% complete. Additional (2-bromoethyl)benzene (0.5 equivalents, 2.84 mmol) and potassium carbonate (0.5 equivalents, 2.84 mmol) were added periodically during the course of two days until the starting material was consumed. The reaction mixture was allowed to cool to ambient temperature and then filtered to remove inorganic salts. The filtrate was concentrated by rotoevaporation to provide 2.2 g of a crude oil. The crude product was purified by flash chromatography on silica gel using an initial mobile phase consisting of a mixture of 9:1 heptane-ethyl acetate. The mobile phase was adjusted to a mixture of 5:1 heptane-ethyl acetate, and then adjusted to a mixture of 7:3 heptane-ethyl acetate to provide 800 mg (50 % yield) of the desired product as an off-white foam. The structure was confirmed by ¹H NMR analysis in DMSO-d₆.

[0181] *6-(Geranyloxy)-2-cyanobenzothiazole*: To a dry 25-ml round-bottomed flask containing acetone (5 mL), anhydrous potassium carbonate (1.2 g, 8.4 mmole), and geranyl bromide (1.5 mL, 7.3 mmole) was added 6-hydroxy-2-cyanobenzothiazole (1 g, 5.6 mmole). The mixture was refluxed under argon with stirring. Reaction progress was monitored by TLC analysis, developing with 2:1 heptane-ethyl acetate. After 20 h, the potassium carbonate was filtered from the cooled reaction mixture. The solution was concentrated *in vacuo* to yield 2.1 g of solid. The solid was further purified by flash chromatography using a mixture of 9:1 heptane-ethyl acetate. Appropriate fractions were pooled and evaporated to yield 0.84 g of solid. The structure was confirmed by ¹H NMR analysis in DMSO-d₆.

[0182] *6-(Prenyloxy)-2-cyanobenzothiazole*: To a dry 25-ml round-bottomed flask containing acetone (5 mL), anhydrous potassium carbonate (1.2 g, 8.4 mmol), and prenyl bromide (839 microliters, 7.3 mmole) was added 6-hydroxy-2-cyanobenzothiazole (1.0 g, 5.6 mmol). The mixture was refluxed under argon with stirring. Reaction progress was monitored by TLC analysis, developing with 2:1 heptane-ethyl acetate. After 28 h, the potassium carbonate was filtered from the cooled reaction mixture. The solution was concentrated *in vacuo* to yield 1.7 g of solid. The solid was further purified by flash chromatography using 9:1 heptane-ethyl acetate and gradually stepping to 4:1 heptane-ethyl acetate. Appropriate fractions were pooled and evaporated to yield 0.8 g of solid. The structure was confirmed by ¹H NMR analysis in DMSO-d₆.

[0183] *6-(2-Picolinyloxy)-2-cyanobenzothiazole*: (Bromomethyl)pyridine hydrobromide (1.87 g, 7.38 mmol) and 2-cyano-6-hydroxybenzothiazole (1.00 g, 5.68 mmol) were suspended in acetone (50 mL). After introduction of potassium carbonate (1.96 g, 14.2 mmol), the suspension was refluxed for 72 h under nitrogen. After cooling down the mixture and filtering the solids, the filtrate was concentrated and the residue was purified by silica gel chromatography using 50%-

75% ethyl acetate in heptane. A yellowish solid was obtained in 72% yield. MS (ESI+): m/z 267.90 (M+H)⁺; calc'd: 268.05.

[0184] *6-(3-Picolinyloxy)-2-cyanobenzothiazole*: To a solution of 2-cyano-6-hydroxybenzothiazole (0.39 g, 2.2 mmol) in acetone (50 mL) was added 3-(bromomethyl)pyridine hydrobromide (0.7 g, 2.76 mmol), cesium carbonate (2.15 g, 6.6 mmol), and a catalytic amount of sodium iodide. After adding 3A molecular sieves, the yellow suspension was refluxed for 40 h under nitrogen. After cooling down the mixture and filtering the solids, the filtrate was concentrated and the residue was purified by silica gel chromatography using 50%-100% ethyl acetate in heptane. A yellowish solid was obtained in 61% yield. MS (ESI+): m/z 267.64 (M+M)⁺; calc'd: 268.05.

[0185] *6-(4-Picolinyloxy)-2-cyanobenzothiazole*. To a solution of 2-cyano-6-hydroxybenzothiazole (1.18 g, 6.71 mmol) in acetone (50 mL) was added 3A molecular sieves and cesium carbonate (3.98 g, 12.2 mmol). The resulting suspension was stirred at room temperature for two hours. Then another equivalent of cesium carbonate (1.99 g, 6.1 mmol) was introduced, followed by addition of 4-(bromomethyl)pyridine hydrobromide (1.0 g, 6.1 mmol) and a catalytic amount of cesium iodide. The resulting yellow suspension was refluxed for 48 h under nitrogen. After cooling down the mixture and filtering the solids, the filtrate was concentrated and the residue was purified by silica gel chromatography, using 30% ethyl acetate in heptane to remove the starting material and then 25% methanol in ethyl acetate. A yellowish solid was obtained in 70% yield. MS (ESI+): m/z 267.74 (M+H)⁺; calc'd: 268.05.

(b) General procedures for the conversion of 2-cyanobenzothiazole derivatives to D-luciferin derivatives.

[0186] A solution of 0.39 M aqueous cysteine hydrochloride monohydrate (1.3 equivalents, based on the quantity of the 2-cyanobenzothiazole derivative) was added dropwise to an equal volume of a 0.39 M solution of potassium carbonate, maintaining the pH at 6-7 by addition of 6 M HCl. In a separate reaction flask the 2-cyanobenzothiazole derivative was dissolved in sufficient methanol to prepare a 0.1 M solution. This solution was purged with nitrogen to remove oxygen. The cysteine/potassium carbonate solution described above was added dropwise to the reaction flask containing the 2-cyanobenzothiazole derivative, maintaining the pH at 6-7 by addition of 6 M HCl. The reaction was monitored by TLC, and when complete the reaction mixture was concentrated by rotoevaporation using a cold water bath (<30 °C).

[0187] *6'-Deoxyluciferin (H-Luc)*. Prepared from 2-cyanobenzothiazole (100 mg, 0.62 mmol) according to the general procedure. The crude solid product was purified by flash chromatography on silica gel (20 g) using 9:1 dichloromethane-methanol to afford 163 mg (99%) of desired product as a pale yellow solid. This material was 96% pure by HPLC analysis. MS (ESI-): m/z 263.40 (M-H)⁻; calc'd: 262.99.

[0188] *Luciferin 6'-(2-chloroethyl) ether (Luc CEE)*. Prepared from 6-(2-chloroethoxy)-2-cyanobenzothiazole (1.0 g, 4.19 mmol) according to the general procedure. The solid product thus obtained was 99.5% pure by HPLC analysis and further purification was not deemed necessary. The yield of this product was 1.36 g (95% yield). MS (ESI+): m/z 342.94 (M+H)⁺; calc'd: 343.00.

[0189] *Luciferin 6'-benzyl ether (Luc BE)*. Prepared from 6-(benzyloxy)-2-cyanobenzothiazole (0.41 g, 1.5 mmol) according to the general procedure. The crude solid product was purified by flash chromatography on silica gel (90 g) using 100 % dichloromethane initially, gradually stepping up to 8:2 dichloromethane-methanol to afford 190 mg (34% yield) of desired product. MS (ESI-): m/z 368.67 (M-H)⁻; calc'd: 369.04.

[0190] *Luciferin 6'-(4-trifluoromethyl)benzyl ether (Luc TFMBE)*. Prepared from 6-(4-trifluoromethylbenzyloxy)-2-cyanobenzothiazole (1.7 g, 5.08 mmol) according to the general procedure. The resulting solid was purified by flash chromatography on silica gel using initially a mixture of 99:1 dichloromethane-methanol, gradually stepping up to 9:1 dichloromethane-methanol. Appropriate fractions were pooled and evaporated to yield 700 mg (31%) of the desired product as a solid.

[0191] *Luciferin 6'-(2-phenylethyl) ether (Luc PEE)*. Prepared from 6-(2-chloroethoxy)-2-cyanobenzothiazole (0.80 g, 2.85 mmol) according to the general procedure. The resulting solid was purified by flash chromatography on silica gel using initially a mixture of 6:3:1 heptane-ethyl acetate-methanol, then a mixture of 5:3:2 methanol-heptane-ethyl acetate. Appropriate fractions were pooled and evaporated to yield 145 mg (14%) of the desired product as a solid. MS (ESI+): m/z 384.52 (M+H)⁺; calc'd 385.07.

[0192] *Luciferin 6'-geranyl ether (Luc GE)*. Prepared from 6-geranyloxy-2-cyanobenzothiazole (0.8 g) according to the general procedure. The resulting solid was purified by flash chromatography on silica gel using 9:1 dichloromethane-methanol. Appropriate fractions were pooled and evaporated to yield 101 mg of solid. The structure was confirmed by ¹H NMR analysis in DMSO-d₆.

[0193] *Luciferin 6'-prenyl ether (Luc PE)*. Prepared from 6-prenyloxy-2-cyanobenzothiazole (0.8 g) according to the general procedure. The resulting solid was first purified by flash chromatography using 9:1 dichloromethane-methanol gradually stepping to 8:2 dichloromethane:methanol. The solid was then repurified by flash chromatography using 2:1 heptane-ethyl acetate. Appropriate fractions were pooled and evaporated to yield 339 mg of yellowish solid. The structure was confirmed by ¹H NMR analysis in DMSO-d₆. Fluorescence HPLC analysis indicated background luciferin, so the solid was further purified by preparative reverse-phase HPLC.

[0194] *Luciferin 6'-(2-picolinyloxy)-2-cyanobenzothiazole (Luc 2PE)*. Prepared from 6-(2-picolinyloxy)-2-cyanobenzothiazole (250 mg,

0.94 mmol) according to the general procedure. The solid product thus obtained was 92.0% pure by HPLC analysis and the yield of this product was 80%. MS (ESI+): m/z 371.55 (M+H)⁺; calc'd 372.04.

[0195] *Luciferin 6'-(3-picolynyl) ether (Luc 3PE)*. Prepared from 6-(3-picolinyloxy)-2-cyanobenzothiazole (250 mg, 0.94 mmol) according to the general procedure. The solid product thus obtained was 99.8% pure by HPLC analysis and further purification was not deemed necessary. The yield of this product was 60%. MS (ESI+): m/z 371.64 (M+M⁺; calc'd 372.04.

[0196] *Luciferin 6'-(4-picolynyl) ether (Luc 4PE)*: Prepared from 6-(4-picolinyloxy)-2-cyanobenzothiazole (267 mg, 1.0 mmol) according to the general procedure except that 5 mL DMF was used to dissolve the starting material. The solid product thus obtained was 96.0% pure by HPLC analysis and further purification was not deemed necessary. The yield of this product was 20%. MS (ESI+): m/z 371.61 (M+H)⁺; calc'd 372.04.

Example 2: Two step CYP450/luciferase reaction and luciferin derivatives evaluation

[0197] In this Example, a procedure for a two-step CYP450/luciferase assay is provided. Luciferin derivatives were evaluated as P450 substrates and luciferase pro-substrates using this procedure. 20 microliter CYP450 reactions were prepared at pH 7.4 in an amount of KPO₄ buffer that is optimal for a given CYP450 isoform (100 mM for CYP1A1, CYP1A2, CYP2B6, CYP2D6 and CYP2E1; 50 mM for CYP2C8 and CYP2C19; 25 mM for 2C9 and for pooled human liver microsomes; 200 mM for CYP3A4). For CYP2A6 100 mM Tris at pH 7.5 was substituted for KPO₄. Reaction mixes also contained 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM MgCl₂ and a luciferin derivative/CYP450 substrate. Reactions were initiated by addition of 0.4 pmoles recombinant human CYP450 co-expressed with CYP450 reductase in Sf9 cell microsomal membranes or 4 microgram pooled human liver microsomes and incubation at 37°C. After an initial incubation period at 37°C 20 microliter CYP450 reactions were mixed with an 80 microliter luciferase reaction mix. The luciferase reaction mix contained 250 micromolar ATP, 5-25 microgram/mL thermostable luciferase from *Photuris pennsylvanica* prepared as described in WO/9914336, published March 25, 1999 which is incorporated by reference in its entirety), 20 mM Tricine pH 7.8, 0.1 mM EDTA, 8 mM MgCl₂, 0.6 mM coenzyme A and 33 mM DTT. Assays were also performed using 50 microliter CYP450 and luciferase reaction volumes (e.g., figure 3, panel B, table 3). Light output was measured immediately in a Turner Reporter, Turner 20/20 or Berthold Orion luminometer. Because the calibration of instruments from different manufacturers varies the quantitation of light output is instrument-specific and direct comparisons between instruments cannot be made.

[0198] O-dealkylation and hydroxylation are common CYP450 catalyzed xenobiotic transformations (9). A panel of recombinant human CYP450 microsome preparations was tested for O-dealkylase activity against luciferin 6' methyl (Luc ME), ethyl (Luc EE), chloroethyl (Luc CEE), benzyl (Luc BE), p-CF₃ benzyl (Luc TFMBE), phenylethyl (Luc PEE), geranyl (Luc GE), 2, 3 and 4 picolynyl (Luc 2PE, Luc 3PE and Luc 4PE) and prenyl (Luc PE) ethers and for hydroxylase activity against dehydroluciferin (H-Luc) (figure 2). These compounds are either inactive in a light generating luciferase reaction or only modestly active as compared to authentic luciferin. The CYP450 activities tested are contained in microsome fractions from insect cells that over-express a single recombinant human CYP450 isoform in combination with NADPH CYP450 reductase. It was reasoned that if CYP450s dealkylated or hydroxylated the luciferin derivatives at the 6' position, authentic luciferin would be generated that could be detected enzymatically in a light generating firefly luciferase reaction as described by the equation in figure 1.

[0199] Luc ME, Luc EE, Luc CEE, Luc BE, H-Luc, Luc TFMBE, Luc PEE, Luc GE, Luc 2PE, Luc 3PE and Luc 4PE and Luc PE were subjected to a two-step assay where they were first incubated with a panel of CYP450 enriched microsomes or control microsomes (with no detectable CYP450 activity) under conditions where the CYP450s are known to be active. The panel included CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. After an initial incubation with CYP450, luciferase and its requisite cofactors were added and light output was monitored (figures 3 and 9). Light output was increased significantly over controls by CYP1A2, CYP2C8 and CYP2C9 with Luc ME, CYP2C8, CYP2C9 and CYP3A4 with Luc BE, CYP1A1, CYP1A2, CYP2C8 and CYP2C9 with Luc EE, by CYP1A1, CYP1A2, CYP2C8 and CYP2C9 with Luc CEE, by CYP1A1, CYP2C8 and CYP2C9 by Luc TFMBE, by CYP3A4 with Luc PE, Luc PEE, by CYP1A1 with Luc GE and by CYP3A4 with Luc PE (figure 3). With Luc 2PE, Luc 3PE and Luc 4PE the most obvious increase in light output was with CYP1A1 and CYP3A4, the effect being most pronounced for both isoforms with Luc 3PE (figure 9) These isoforms apparently dealkylated luciferin 6' alkyl ethers and 6' substituted alkyl ethers to form luciferin while the other isoforms tested did not. When H-Luc was used as substrate CYP1A2 and CYP2C9 increased light output over controls. H-Luc was apparently hydroxylated to form luciferin and within the panel tested the reaction was most significant with the CYP2C9 isoform. Values for isoform/substrate combinations not shown were similar to Sf9 cell membrane controls. For each of the panel screens, background luminescence reflects at least in part the presence of contaminating D-luciferin in the unreacted preparations of d-luciferin derivatives. Luminescent CYP1A1, CYP1A2, CYP2C8, CYP2C9 and CYP3A4 reactions were dose-dependent with respect to substrate (data not shown).

Example 3: Time-dependence of CYP450/substrate incubation in two-step luminescent CYP450 reactions.

[0200] Time courses were performed for incubation of CYP1A2, CYP2C8 and CYP2C9 with Luc ME, CYP2C9 with H-Luc and CYP3A4 with Luc BE (figure 4). Light output measured within 12 minutes of adding a luciferase reaction mix to the CYP450 reaction mix increased in a linear fashion for up to 60 minutes with CYP2C9 and Luc ME or H-Luc and for CYP3A4 with Luc BE. With CYP1A2 and CYP2C8 there was a time dependent increase but the rate of increase declined and was increasing only modestly by 60 minutes for CYP2C8, and not at all for CYP1A2. These time courses mirror the activity of CYP1A2, CYP2C8, CYP2C9 and CYP3A4 with the conventional substrates phenacetin, paclitaxel, diclofenac and testosterone, respectively (10, 11, 12).

Example 4: Time course of light input from two-step luminescent CYP450 reactions.

[0201] In this Example, a luminescent signal was generated after combining luciferase reaction components with a CYP450 reaction and was monitored over time (figure 5). D-luciferin derivatives were incubated in a CYP450 reaction mix for 60 minutes at 37°C before combining with a luciferase reaction mixture. In -CYP450 controls CYP450 Sf9 cell microsomes were replaced with H₂O. Luminescence was read on a Turner Reporter luminometer beginning 3 minutes after combining the reactions and at successive intervals as indicated for 284 minutes. For the CYP450s and substrates tested, the signals were quite stable, decaying with a half-life of greater than 5 hours.

Example 5: One step CYP450/luciferase reaction at room temperature

[0202] In this Example, a procedure for a one-step CYP450/luciferase assay is provided. Luciferin derivatives were evaluated as P450 substrates and luciferase pro-substrates using this procedure. 100 microliter CYP450 reactions were prepared at pH 7.4 in an amount of KPO₄ buffer that is optimal for a given CYP450 isoform (see two step reaction method). Reaction mixes also contained 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.7 mM MgSO₄, 0.6 mM coenzyme A, a luciferin derivative/CYP450 substrate, 250 micromolar ATP and 21 microgram/mL thermostable luciferase from *Photuris pennsylvanica* prepared as described in WO/9914336, published March 25, 1999 which is incorporated by reference in its entirety). Reactions were initiated by addition of 2.0 pmoles recombinant human CYP450 co-expressed with CYP450 reductase in Sf9 cell microsomal membranes (e.g. GenTest Supersomes™) and incubation at room temperature or 37°C. Light output was measured immediately and continuously in a Turner Reporter or Berthold Orion luminometer.

[0203] The one-step assays were performed at room temperature (~22°C) with Luc ME, which was co-incubated with CYP1A2, CYP2C8 and CYP2C9 microsome preparations, and luciferase with its requisite cofactors. Initial baseline luminescence was measured and then light output was monitored over time from the point reactions were initiated by adding CYP450 (figure 6). For each reaction a time dependent increase in light output was observed when CYP450 was included in the reaction mix. The most robust response at room temperature was seen with CYP1A2. Light output increased to a maximum level after about forty minutes, remained steady for about 3 hours and then declined gradually over the remainder of the assay. Light output from CYP2C8 and CYP2C9 room temperature reactions increased gradually for about 3 hours, remained steady for about 1 hour then declined gradually over the remainder of the assay. Similar one-step assays were also performed with H-Luc and CYP2C9 and with Luc EE and CYP2C8 (data not shown).

Example 6: One step CYP450/luciferase reaction at 37°C

[0204] In this Example, the one step assay of Example 5 was performed at 37°C and light was monitored over time from the point where the reactions were initiated by adding CYP450 (figure 7). Cyp1A2, CYP2C8 and CYP2C9 were assayed against Luc ME, CYP2C9 against H-Luc and CYP3A4 against Luc BE. Cyp1A2 and CYP2C9, were similar, increasing to a peak of light output by about 30 minutes and declining thereafter. In a CYP2C8 reaction light output exceeded that from a -CYP450 control but declined from initial values over the course of the reaction in both test and control conditions. CYP2C9 with H-Luc was similar to Luc ME, increasing to a maximum by about 30 minutes and declining thereafter. The difference in light output between CYP3A4 with Luc BE and a -CYP450 control was modest. Light output from -CYP450 controls in each case was likely a consequence of luciferin contamination in the unreacted D-luciferin derivative preparation.

Example 7: Pooled human liver microsomes in two-step luminescent CYP450 reaction

[0205] A pooled human liver microsome preparation containing a mixture of CYP450 activities was used in two-step luminescent CYP450 assays that employed H-Luc, Luc ME, Luc EE and Luc BE (figure 8) as substrates. As compared to Sf9 cell membranes with no CYP450 activity significant amounts of CYP450 activity was detected by the luminescent

method with each substrate. The contributions of individual isoforms to the total light output were implied by inhibition with sulfaphenazole (CYP2C9 inhibitor), alpha-naphthoflavone (CYP1A2 inhibitor) and ketoconazole (CYP2C8 and CYP3A4 inhibitor) (13). Of particular note was the near complete inhibition of light output with H-Luc by the CYP2C9 selective inhibitor sulfaphenazole. Partial inhibition of the H-Luc reaction by 100 micromolar ketoconazole is also consistent with the effect of this inhibitor on CYP2C9 activity. This coupled with the demonstration the H-Luc is selective for CYP2C9 (figure 3) indicates that the microsomal activity against H-Luc is predominantly CYP2C9. The effects of sulfaphenazole and ketoconazole on Luc ME and Luc EE activity are consistent with the presence of CYP2C8 activity because CYP2C8 is active against both of these substrates and inhibited by both inhibitors. Inhibition of Luc BE activity by ketoconazole is consistent with the presence of CYP3A4 and/or CYP2C8 because both isoforms are active against Luc BE and both are inhibited by ketoconazole. The lack of inhibition by alpha-naphthoflavone indicates that there is little or no CYP1A2 activity present in the microsome preparation. The slight stimulation of activity against Luc BE by alpha-naphthoflavone may reflect the presence of CYP3A4 activity because this isoform is stimulated by alpha-naphthoflavone (16).

Example 8: Detection of CYP450 inhibition by known P450 inhibitors

[0206] Luciferin derivatives as substrates for luminescent CYP40 assays should be useful as probes for detecting CYP450 inhibition by drugs or other xenobiotics. To test this hypothesis, known CYP450 inhibitors and certain luciferase derivatives were added to the reactions and IC₅₀s were determined. Inhibitors tested were sulfaphenazole for CYP2C9, alpha-naphthoflavone for CYP1A2, and ketoconazole for CYP2C8 and 3A4. Two-step assays were performed as described in Example 2. These drugs inhibited the reactions in a dose-dependent manner (Table 1). In many cases, the IC₅₀s were comparable to those reported for assays with other substrates (13). The inhibitors were acting on the CYP450s. Inhibition of luciferase was not detected in control assays that used luciferin as substrate (data not shown).

[0207] Table 1 summarizes the inhibition of CYP450 reactions with conventional substrates and D-luciferin derivatives by ketoconazole, alpha-naphthoflavone and sulfaphenazole. CYP450 assays with luciferin derivatives were performed in two steps essentially as described in Example 2. In this case the CYP450s were exposed to inhibitors at concentrations ranging from about 40nM to 10micromolar for 10 minutes prior to exposure to a D-luciferin derivative. IC₅₀s were calculated by non-linear regression analysis with GraphPad Prism software. Entries marked with an asterisk were taken from reference 13.

Table 1. Inhibition of CYP450 reactions by CYP450 inhibitors.

P450/substrate	Alpha-Naphthoflavone	Ketoconazole	Sulfaphenazole
CYP 1A2/Luc ME	0.2	---	---
CYP1A2/ethoxyresorufin*	0.4	---	---
CYP1A2/phenanthren*	3.8	---	---
CYP1A2/imaprine*	0.1	---	---
CYP2C8/Luc EE	---	26.4	---
CYP2C8/phenanthrene*	---	8.9	---
CYP2C9/H-Luc	---	---	0.7
CYP2C9/Luc ME	---	---	0.4
CYP2C9/Luc EE	---	---	0.5
CYP2C9/diazepam*	---	---	0.5
CYP2C9/phenanthrene*	---	---	0.7
CYP3A4/Luc BE	---	0.06	---
CYP3A4/diazepam*	---	0.5	---
CYP3A4/phenanthrene*	---	0.03	---
CYP3A4/testosterone*	---	0.04	---
IC ₅₀ s (micromolar) against CYP450 isoform/substrate reactions are shown. *From reference 13.			

Example 9: CYP450-catalyzed conversion of luciferin derivatives to luciferin

[0208] In this Example, the conversion of Luc ME, H-Luc, Luc CEE and Luc 3PE to luciferin by cytochrome P450 enzymes was confirmed by HPLC analysis (figure 10). 100 micromolar Luc ME, H-Luc, Luc CEE and Luc 3PE was incubated with CYP1A2, CYP2C9, CYP1A1 and CYP3A4, respectively in reaction volumes of 150 microliters at 37°C. At various time intervals, the reactions were stopped by the addition of Tergitol to 0.1% (v/v) and flash freezing in liquid nitrogen. 95 microliter aliquots of each reaction mixture were subjected to fractionation by HPLC. HPLC method: High-performance liquid chromatography was performed on an HP 1050 LC system equipped with a multi-wavelength absorbance (HP 1050 MWD) and fluorescence detector (HP 1046A). Separation was achieved on a 5 micron Adsorbosphere HS C18 column (Alltech Associates) with a solvent gradient of 0.05M KH_2PO_4 / pH 6 (solvent A) and 80:20 acetonitrile/water (solvent B). The gradient conditions used were 15%B to 95%B over 10 min. Substrates were detected by absorbance at either 262 or 330 nm and Luciferin was detected by fluorescence at 520 nm (emission) with an excitation wavelength of 330 nm. The zero time points represent the luciferin content of deoxyluciferin or luciferin 6' methyl ether from no enzyme controls.

Example 10: Detection of CYP450 inhibition by known CYP450 substrates

[0209] Luciferin derivatives as substrates for luminescent CYP450 assays would be useful as probes for detecting other CYP450 substrates. Because two distinct substrates for the same CYP450 isoform will likely compete for the active site, it is possible to characterize known substrates and to identify novel substrates by observing their capacity to inhibit the luminescent reactions with luciferin derivatives. To test this hypothesis, known CYP450 substrates were added to the reactions (Tassaneeyakul, W. et al (1993) "Specificity of substrate and inhibitor probes for human cytochromes P450 1A1 and 1A2", J. Pharmacol. Exp. Ther., 265, 401-407; Mancy, A. et al "Diclofenac and its derivatives as tools for studying human cytochromes p450 active sites: particular efficiency and regioselectivity of p450 2Cs", Biochemistry, 38, 14264-14270). Substrates tested were diclofenac for CYP2C9 and phenacetin for CYP1A1 and CYP1A2. The drugs inhibited the reactions in a dose-dependent manner, thus verifying the expectation that CYP450 substrates can be detected by these luminescent assays (see table below). IC₅₀s were calculated by non-linear regression analysis with the program GraphPad PRISM™ (San Diego, CA). The reactions were performed as described in Example 2 except the first step (CYP450 reaction) was in a 50 microliter reaction volume with 1 picomole of CYP450. In the second step a 50 microliter luciferase reaction was added to give final concentrations of 50 micrograms/mL recombinant, mutant of firefly luciferase from *Photuris pennsylvanica* from Promega (17), 200 micromolar ATP, 0.1% tergitol (v/v), 4.0 mM MgSO_4 and 100 mM Tricine pH 8.4. Figures 11 (a)-(c) illustrate the actual inhibition curves.

Table 2

CYP450 isoform/substrate	Diclofenac	Phenacetin
CYP2C9/H-Luc	13	ND
CYP1A1/Luc CEE	ND	21
CYP1A2/Luc ME	ND	25
Values shown are IC ₅₀ s (micromolar).		

Example 11: Two-step Cyp450/Renilla reaction and coelenterazine derivative evaluation

[0210] In this Example, P450 activity was determined using coelenterazine and coelenterazine derivatives, methoxy-coelenterazine HH and coelenterazine HH, in a two-step reaction system. In these assays, P450 acts on the coelenterazine or coelenterazine derivatives in one of two ways. In the first type of reaction coelenterazine derivatives that are neither substrates for Renilla-type luciferases nor exhibit the characteristic coelenterazine chemiluminescence (luminescence in the absence of luciferase) are altered by P450 to become substrates for Renilla-type luciferase and exhibit chemiluminescence. An example of this type of coelenterazine derivative is methoxy-coelenterazine HH. In the second type of reaction coelenterazine or coelenterazine HH, which exhibit chemiluminescence and are competent substrates for Renilla-type luciferase, are altered by P450 resulting in a loss of chemiluminescence and activity with Renilla-type luciferases. In both types of assay it is possible to detect P450 activity either directly by a change in chemiluminescence or indirectly by a change in bioluminescence from a Renilla-type luciferase.

(I) *Synthesis of coelenterazine derivatives*

[0211] *2-Oxo-3-phenyl-propionaldehyde*. Phenylpyruvic acid (25.0 g, 152.0 mmol) was coevaporated twice with dry pyridine and then redissolved in dry pyridine (250 mL). To this solution was added acetic anhydride (170 mL, 1.8 moles) and the solution was stirred at ambient temperature for 15 h. The reaction progress was monitored by TLC. When the reaction was complete, the solution was evaporated to a viscous syrup. The syrup was dissolved in dichloromethane (700 mL) and then washed three times with 0.1 M aqueous HCl solution (3 x 200 mL). The organic phase was dried over anhydrous sodium sulfate, filtered and evaporated to an amber-colored syrup. This product was purified by flash chromatography on silica gel (250 g) using dichloromethane as mobile phase. Appropriate fractions were pooled and evaporated to afford 24 g of a dry solid. This material was dissolved in THF (150 mL) and the solution was cooled in an ice-water bath. To the solution was added dropwise oxalyl chloride (51 mL, 580 mmol). After 10 min DMF (7.5 mL) was added to the reaction mixture and the reaction was stirred for 4 h at 0 °C. Toluene (100 mL) was added and the reaction mixture was evaporated to give a thick oil. This material was coevaporated twice with toluene and the crude product was dried under vacuum for 5 h. The dried product was dissolved in a 1:1 mixture of THF-dichloromethane (200 mL) and the solution was cooled to -78 °C (dry ice-isopropanol bath) under argon. Then, lithium tri-tert-butoxyaluminumhydride (152 mL of a 1.0 M solution in THF, 152 mmol) was added at a rate such that the internal temperature of the reaction was below -60 °C. After addition was complete, the reaction was stirred below -60 °C for 10 h. The reaction was quenched by the slow addition of 2 M aqueous HCl solution (100 mL) and the mixture was allowed to warm to ambient temperature. The reaction mixture was diluted with dichloromethane (500 mL) and then washed twice with 0.1 M aqueous HCl solution (2 x 100 mL). The organic phase was dried over anhydrous sodium sulfate, filtered and evaporated to give an amber-colored syrup. This material was purified by flash chromatography on silica gel (250 g) starting with 95:5 heptane-ethyl acetate, then with 9:1 heptane-ethyl acetate as mobile phase. Appropriate fractions were pooled and evaporated to afford 13.5 g (80%) of the desired compound.

[0212] *2,8-Dibenzyl-6-phenyl-7H-imidazo[1,2- α]pyrazin-3-one (Coelenterazine HH)*. A solution of 2-amino-3-benzyl-5-phenylpyrazine²⁰ (2.0 g, 8.0 mmol) and 2-oxo-3-phenylpropionaldehyde (3.0 g, 16 mmol) in ethanol (125 mL) was deoxygenated with argon gas for 20 min. To the solution was added concentrated hydrochloric acid (4.0 mL) and the reaction mixture was heated at reflux for 18 h. The reaction was allowed to cool to ambient temperature and then evaporated to a brown solid. The crude product was triturated with ethanol (40 mL) and the resulting solid material was collected by centrifugation and then dried in a vacuum oven to afford 1.28 g (41%) of the desired compound. This product was 80% pure according to HPLC analysis.

[0213] *2,8-Dibenzyl-3-methoxy-6-phenyl-imidazo[1,2- α]pyrazine (Coelenterazine HH methyl ether)*. To a stirred solution of 2,8-dibenzyl-6-phenyl-7H-imidazo[1,2- α]pyrazin-3-one (0.25 g, 0.6 mmol) in dry DMF (10 mL) at ambient temperature under argon was added diisopropylethylamine (1.1 mL, 6.0 mmol) all at once, followed by dropwise addition of methyl iodide (0.4 mL, 6.0 mmol). After stirring for 1 h the reaction was complete by TLC analysis. The reaction mixture was diluted with dichloromethane (75 mL) and washed twice with water. The organic extracts were dried over anhydrous sodium sulfate, filtered and evaporated to provide a brown oil. The crude oil was purified by flash chromatography on silica gel (30 g) using dichloromethane as mobile phase. Appropriate fractions were pooled and evaporated to afford 200 mg (77%) of the desired compound.

(II) *P450 assays*

[0214] P450 reactions (20 microliter) containing 200 mM KPO₄ pH 7.4 (for CYP3A4) or 100 mM KPO₄ pH 7.4 for (CYP1A1, 1A2, 2B6, 2D6, 2E1) or 50 mM KPO₄ pH 7.4 (for CYP2C8, 2C19) or 25 mM KPO₄ pH 7.4 (for CYP2C9) or 100 mM Tris pH 7.5 (for CYP2A6); insect cell microsomes containing baculovirus expressed P450 (1 pmole) and P450 reductase or for no P450 control reactions wild type baculovirus infected insect cell microsomes; 1.3 mM NADP⁺; 3.3 mM Glucose-6-Phosphate; 3.3 mM MgCl₂; 0.4 units/ml Glucose-6-Phosphate Dehydrogenase; and substrate (3 micromolar Coelenterazine, 3 micromolar Coelenterazine HH and 10 micromolar Methoxy-coelenterazine HH) were incubated for 60 min at 37 °C.

[0215] Chemiluminescence of P450 altered coelenterazine or coelenterazine derivatives was determined immediately following addition of 80 microliter of 37.5 mM Hepes pH 7.4; 625 mM KCl; 0.125 mM EDTA pH 8.0; 0.25% Triton X-100; 0.125% Mazu; 1.25% Glycerol; 0.25 mg/mL gelatin to the P450 reactions. Renilla luciferase (1.2 ng/mL) (purchased from Chemicon) was added for detection of bioluminescence of P450 altered coelenterazine or coelenterazine derivatives.

[0216] As shown in Figure 12, there was a large increase in both chemiluminescence (panels C and D) and bioluminescence (panels A and B) following incubation of methyl-coelenterazine HH with CYP1A1. There were modest increases in chemiluminescence and bioluminescence with CYP1A2, 2B6 and 2C19 (2-5X). There was a significant reduction in both chemiluminescence (panels G and H) and bioluminescence (panels E and F) following incubation of coelenterazine HH with CYP1A2 and 2E1. Finally, there was a very large reduction in both chemiluminescence (panels K and L) and bioluminescence (panels I and J) following incubation of coelenterazine with all of the P450 isozymes tested except 2C9

and 2A6.

EXAMPLE 12: Luciferase Protection from inhibitory buffer by the addition of yeast iPPase

[0217] This Example illustrates the reversal of inhibition of a luciferase-based P450 reaction in the presence of inhibitory buffer by iPPase. As defined herein, "inhibitory" refers to a reagent (such as a buffer) that includes iPP in sufficient amounts to inhibit the luciferase reaction. A "non-inhibitory reagent" is a reagent that includes substantially no iPP, as measured by its effect on the luciferase reaction. The iPP content is determined empirically by the finding that firefly luciferase is inhibited by iPP, an inhibition is relieved by addition of iPPase and that the inhibition can be recreated by the addition of PPi.

[0218] P450 reactions (50 microliter) contained: 1 pmole CYP1A2 (control reactions contained SF9 membranes), 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.2 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM MgCl₂, 0.01 mM Luc-ME and either 100 mM KPO₄ pH7.4 (inhibitory buffer) or 100 mM KPO₄ pH 7.4 (non-inhibitory buffer). Reactions were incubated at 37°C for 1 hour. The detection of luciferin generated by the P450 reaction was carried out by addition of equal volume of a reagent containing: thermostable luciferase (100 mg/mL) from *Photuris pennsylvanica* prepared as described in WO/9914336, published March 25, 1999 which is incorporated by reference in its entirety; 400 micromolar ATP, 0.4% Prionex, 40 mM Tricine pH 7.8, 8 mM MgSO₄, 0.2% Tergitol. iPPase (Sigma Company, Catalog No. I1891) was added to some of the reactions. Luminescence was detected using a Berthold Orion Microplate Luminometer.

[0219] As shown in Figure 13 and in Figure 15, yeast inorganic pyrophosphatase was effective in reversing iPP inhibition of luciferase when inhibitory buffer is used.

EXAMPLE 13: Inorganic pyrophosphatases protect luciferase from pyrophosphatase contamination.

[0220] In this experiment, thermostable inorganic pyrophosphatase from three sources, New England Biolabs, Inc. (Beverly, MA, Catalog # M0296), a commercially available yeast inorganic pyrophosphatase from Sigma (Cat No. I1891), and a pyrophosphatase isolated from *Thermus thermophilus* (Tth), isolated by conventional methods, were evaluated for their efficiencies at reversing the effect of iPP contaminated buffer in a luciferase-based P450 reaction.

[0221] In this experiment, P450 reaction mixtures (50 microliter) were prepared. The reactions contained: 1 pmole CYP1A2 (control reactions contained Sf9 membranes), 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.2 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM MgCl₂, 0.01 mM LucME, and either 100 mM KPO₄ pH7.4 (inhibitory buffer) or 100 mM KPO₄ pH 7.4 (non-inhibitory buffer). Reactions were incubated at 37°C for one hour. The detection of luciferin generated by the P450 reaction was carried out by addition of equal volume of a reagent containing 100 mg/mL thermostable Luciferase from *Photuris pennsylvanica* prepared as described in WO/9914336, published March 25, 1999 which is incorporated by reference in its entirety), 400 mM ATP, 0.6% Prionex, 40 mM Tricine pH 7.8, 8 mM MgSO₄, 0.2% Tergitol, 0.02% Mazu. iPPase from New England Biolabs, Inc. (Beverly, MA, Catalog # M0296), Sigma (Cat No. I1891), or *Thermus thermophilus* (Tth), isolated by conventional methods was added to some of the reactions. Following incubation at room temperature for 30 minutes luminescence was detected using a Berthold Orion Microplate Luminometer.

[0222] As shown in Figure 14, inorganic pyrophosphatase from different sources reversed inhibition of luciferase when inhibitory KPO₄ buffer is used in a P450 reactions. Reaction conditions, temperature and enzyme concentrations were found to affect the efficiencies of the various iPPase enzymes in reversing iPP inhibition (data not shown).

EXAMPLE 14: Protection of luciferase from added iPP using yeast iPPase

[0223] This Example illustrates iPPase reversal of inhibition of a luciferase reaction in the presence of added iPP and shows that the performance of 200 mM non-inhibitory KPO₄ Buffer with 3 mM NaPPi is similar to that of 200 mM inhibitory KPO₄ buffer and that iPPase reverses the effect of the added NaPPi.

[0224] The reactions contained: 100 mM Tricine pH 8.4, 10 mM MgSO₄, 0.1% Tergitol, 0.01% Mazu, 50 mg/mL thermostable luciferase (from *Photuris pennsylvanica* prepared as described in WO/9914336, published March 25, 1999 which is incorporated by reference in its entirety), 200 mM ATP, 0.2% Prionex, 0.5 mM luciferin. All reactions contained either 200 mM non-inhibitory KPO₄ pH 7.4 or 200 mM inhibitory KPO₄ pH7.4. iPPase (Sigma I1891) was added to some of the reactions to a final concentration of 2 units/mL. Sodium pyrophosphate (NaPPi) was added to some reactions to a final concentration of 3 mM. The reactions were performed at room temperature. Luminescence was detected using a Berthold Orion Microplate Luminometer.

[0225] As shown in Figure 15 inorganic pyrophosphatase was effective in reversing inhibition of luciferase when inhibitory KPO₄ buffer is used. Without being bound to a mechanism, the inventors observed that the addition of iPP to an otherwise active buffer can recreate an inhibitory buffer, and that the inhibition can be reversed by the addition of iPPase. These findings imply that the inhibitor is iPP.

Example 15: Cell-based luminescent CYP450 assay

[0226] In this Example, a cell-based luminescent CYP450 assay is described. Inducers of CYP450 gene expression were evaluated for their effect on CYP450 activity. Primary hepatocytes from sexually mature male Sprague Dawley rats were obtained cryopreserved from Xenotech, LLC (Kansas City, KS). On the first day cells were thawed as recommended by the supplier and the percentage of live cells estimated by the method of trypan blue exclusion. Approximately 1.5×10^5 cells per cm^2 were seeded on collagen-coated 24-well tissue culture plates. Cells were cultured at 37°C , 95% relative humidity and 5% CO_2 in 0.3 mL/well HepatoZyme SFM medium supplemented with 2 mM L-glutamine and 1X penicillin-streptomycin (Life Technologies, Inc., Rockville, MD). Initially cells were allowed to attach to plates for 6 hours then the medium was replaced with fresh medium supplemented to 0.25 mg/ml with Matrigel™ (BD Biosciences, Bedford, MA). Medium was changed daily.

[0227] On the third day after seeding of cells, culture medium was removed and replaced with 0.3 ml medium containing CYP450 gene inducers or their vehicle controls. On the fourth day medium was removed and replaced with fresh induction or vehicle control medium so that cells were exposed for 2 days.

[0228] On the fifth day induction and vehicle control media were replaced with fresh medium that contained luminogenic CYP450 substrates. At the end of the incubation period with luminogenic substrate, two types of luminescent assays were performed. Both assay formats were possible because the luminogenic substrates enter cells, likely by passive diffusion. The first type of assay is possible because the luciferin product of CYP450 reaction exits cells, again likely by passive diffusion. For the first type of assay a sample of medium was removed and combined with an equal volume of a luciferin detection reagent (200 mM tricine, pH 8.4, 100 micrograms/mL thermostable mutant of firefly luciferase from *Photuris pennsylvanica* (Ultra Glow™ luciferase, available from Promega, Corp.), 400 micromolar ATP, 20 mM MgSO_4 and 2% Tergitol) to initiate a luminescent reaction. For blank determinations for the first assay type luminogenic substrate was withheld from some wells but combined with an aliquot of medium after it was first combined with the luciferin detection reagent. For the second type of assay, an equal volume of luciferin detection reagent was added directly to the cell culture medium to stop CYP450 activity, produce a cell lysate and initiate a luminescent reaction. For blank determinations for the second reaction type luminogenic substrate was withheld from some wells but added to these wells after the luciferin detection reagent was first added. Aliquots of both types of reactions were transferred to white, opaque 96-well plates and luminescence was read on a Fluostar Optima luminometer (BMG, Inc.). Luminescence values from blank wells were subtracted from the values of corresponding wells. The results are shown in Figure 16.

Example 16: Stabilization of luminescent signal using luciferase inhibitors

[0229] In this Example, two luciferase competitive inhibitors, 2-amino-6-methylbenzothiazole (AMBT) or 2-(4-aminophenyl)-6-methylbenzothiazole (APMBT), were evaluated to determine their effect on stabilizing a luminescent signal.

[0230] 50 microliter CYP1A1 reactions (0.5 pmol recombinant CYP1A1 enzyme, $30\mu\text{M}$ Luciferin chloroethyl ether, 100 mM KPO_4 , 1.3 mM NADP^+ , 3.3 mM glucose-6-phosphate, 3.3 mM MgCl_2 , 0.02 unit glucose-6-phosphate dehydrogenase) were incubated at 37°C for 20 min. After the incubation, 50 microliters of a luciferin detection reagent (100 micrograms/mL thermostable luciferase (from *Photuris pennsylvanica*), 400 micromolar ATP, 0.6% Prionex, 2 units/mL iPPase, 200 mM Tricine pH 8.4, 20 mM MgSO_4 , 2% Tergitol) containing either 100 micromolar APMBT, 100 micromolar AMBT, or no inhibitor were added to each CYP1A1 reaction. Luminescence was read immediately and at subsequent 5 minute intervals for 1 hour. The results are shown in Figure 17.

[0231] As shown in Figure 17, inhibition of luciferase by an inhibitor such as 2-(4-aminophenyl)-6-methylbenzothiazole (APMBT) or 2-amino-6-methyl benzothiazole (AMBT) stabilizes the luminescent signal in a luminescent CYP450 assay.

[0232] While the present invention has now been described and exemplified with some specificity, those skilled in the art will appreciate the various modifications, including variations, additions and omissions, that may be made in what has been disclosed herein without departing from the spirit of the invention. Accordingly, it is intended that these modifications also be encompassed by the present invention and that the scope of the present invention be limited solely by the broadest interpretation that lawfully can be accorded the appended claims.

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[0233]

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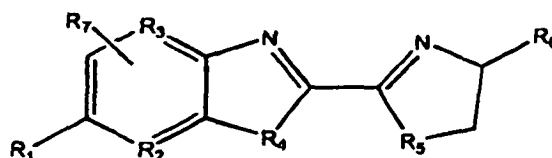
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21. This compound was prepared from acetophenone according to the procedure described by Usami, K. et al. *Tetrahedron*, Vol 52, No. 37, pp 12061-12090, 1996.
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Claims

1. A method for measuring the activity of a cytochrome P450 enzyme comprising:

- (a) contacting a luminogenic molecule that is a luciferin derivative or a coelentraine derivative and a cytochrome P450 substrate and a pro-substrate of a bioluminescent enzyme with at least one cytochrome P450 enzyme and at least one bioluminescent enzyme to produce a reaction mixture; and
- (b) determining cytochrome P450 activity by measuring bioluminescence of the reaction mixture,

wherein the luciferin derivative is selected from derivatives having the formula:



wherein

R₁ represents hydrogen, hydroxyl, amino, C₁₋₂₀ alkoxy, substituted C₁₋₂₀ alkoxy, C₂₋₂₀ alkenyloxy, substituted C₂₋₂₀ alkenyloxy, halogenated C₂₋₂₀ alkoxy, substituted halogenated C₂₋₂₀ alkoxy, C₃₋₂₀ alkynyloxy, substituted C₃₋₂₀ alkynyloxy, C₃₋₂₀ cycloalkoxy, substituted C₃₋₂₀ cycloalkoxy, C₃₋₂₀ cycloalkylamino, substituted C₃₋₂₀ cycloalkylamino, C₁₋₂₀ alkylamino, substituted C₁₋₂₀ alkylamino, di C₁₋₂₀ alkylamino, substituted di C₁₋₂₀ alkylamino, C₂₋₂₀ alkenylamino, substituted C₂₋₂₀ alkenylamino, di C₂₋₂₀ alkenylamino, substituted di C₂₋₂₀ alkenylamino, C₂₋₂₀ alkenyl C₁₋₂₀ alkylamino, substituted C₂₋₂₀ alkenyl C₁₋₂₀ alkylamino, C₃₋₂₀ alkynylamino, substituted C₃₋₂₀ alkynylamino, di C₃₋₂₀ alkynylamino, substituted di C₃₋₂₀ alkynylamino, C₃₋₂₀ alkynyl C₁₋₂₀ alkylamino, substituted C₃₋₂₀ alkynyl C₁₋₂₀ alkylamino, C₃₋₂₀ alkynyl C₂₋₂₀ alkenylamino, or substituted C₃₋₂₀ alkynyl C₂₋₂₀ alkenylamino;

R₂ and R₃ independently represents C or N;

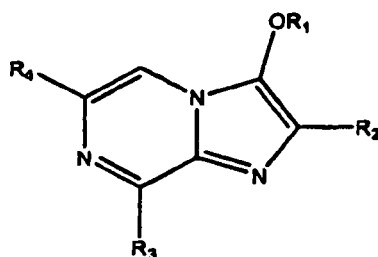
R₄ and R₅ independently represents S, O, NR₈ wherein NR₈ represents hydrogen or C₁₋₂₀ alkyl, CR₉R₁₀ wherein R₉ and R₁₀ independently represent H, C₁₋₂₀ alkyl, or fluorine;

R₆ represents CH₂OH; COR₁₁ wherein R₁₁ represents H, OH, C₁₋₂₀ alkoxide, C₂₋₂₀ alkenyl, or NR₁₂R₁₃ wherein R₁₂ and R₁₃ are independently H or C₁₋₂₀ alkyl; or -OM⁺ wherein M⁺ is an alkali metal or a pharmaceutically acceptable salt; and

R₇ represents H, C₁₋₆ alkyl, C₁₋₂₀ alkenyl, halogen, or C₁₋₆ alkoxide,

with the proviso that R₁ is not OH or NH₂, if R₃ and R₂ are both carbon R₄ and R₅ are both S, R₇ is H, R₆ is COR₁₁ and R₁₁ is OH, such that the luminogenic molecule is not luciferin or aminoluciferin

and wherein the coelentraine derivative is selected from derivatives having a formula:



wherein

R₁ is C₁₋₂₀ alkyl, branched C₃₋₂₀ alkyl, C₃₋₂₀ cycloalkyl, aralkyl, C₁₋₂₀ alkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or diC₁₋₂₀ alkylamino, aralkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or diC₁₋₂₀ alkylamino; and

R₂, R₃, and R₄ are independently hydrogen, C₁₋₂₀ alkyl, C₃₋₂₀ cycloalkyl, branched C₃₋₂₀ alkyl, aralkyl, C₁₋₂₀ alkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or diC₁₋₂₀ alkylamino, aralkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or diC₁₋₂₀ alkylamino, aryl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂ alkylamino, or di C₁₋₂₀ alkylamino.

2. The method according to claim 1 wherein the bioluminescent enzyme is a luciferase and wherein step (a) further includes a pyrophosphatase.
3. The method according to claim 2 wherein the pyrophosphatase is an inorganic pyrophosphatase.
4. The method according to claim 1 wherein the luminogenic molecule, the cytochrome P450 enzyme, and the bioluminescent enzyme are contacted at about the same time.
5. The method according to claim 1 wherein the luminogenic molecule is contacted with at least one cytochrome P450 enzyme to form a first reaction mixture prior to contacting with the bioluminescent enzyme to form a second reaction mixture.
6. The method according to claim 5 wherein the second reaction mixture further comprises a detergent.
7. The method according to claim 6 wherein the detergent is a non-ionic detergent.

8. The method according to claim 1 wherein the cytochrome P450 activity is derived from a cell.
9. The method according to claim 8 wherein the cell expresses the bioluminescent enzyme.
- 5 10. The method according to claim 8 wherein in step(a) the cell is further contacted with a lysis reagent.
11. The method according to claim 8 wherein the cell is lysed prior to step(a).
12. The method according to claim 8 wherein the cell is lysed prior to step(b).
- 10 13. The method according to claim 8 wherein the cell is contacted first with the luminogenic molecule to produce a first reaction mixture prior to contact with the bioluminescent enzyme to produce a second reaction mixture.
14. The method according to claim 1 wherein the cytochrome P450 activity is derived from animal tissue.
- 15 15. The method according to claim 14 wherein the tissue is contacted first with the luminogenic molecule for a first predetermined time period prior to contact with the bioluminescent enzyme to provide a second mixture.
- 20 16. A method for measuring cytochrome P450 enzyme activity in an animal to which the luminogenic molecule as defined in claim 1 has been administered; comprising:
 - (a) contacting a biological sample obtained from the animal with a bioluminescent enzyme to form a reaction mixture; and
 - (b) determining cytochrome P450 activity of the animal by measuring bioluminescence.
- 25 17. The method according to claim 16 wherein the reaction mixture further comprises a detergent.
18. The method according to claim 17 wherein the detergent is non-ionic.
- 30 19. The method according to claim 16 wherein the bioluminescent enzyme is a luciferase and wherein the reaction mixture further comprises a pyrophosphatase.
20. The method according to claim 19 wherein the pyrophosphatase is an inorganic pyrophosphatase.
- 35 21. The method according to claim 16 wherein the animal is a transgenic animal having a bioluminescent enzyme transgene.
22. The method according to claim 21 wherein the bioluminescent enzyme transgene is a luciferase transgene.
- 40 23. The method according to claim 1 further comprising providing a test compound for screening and contacting the compound, the luminogenic molecule, the cytochrome P450 enzyme, and the bioluminescent enzyme to produce the reaction mixture.
24. The method according claim 23 wherein the compound, luminogenic molecule, the cytochrome P450 enzyme, and the bioluminescent enzyme are contacted at about the same time.
- 45 25. The method according claim 23 wherein the compound, luminogenic molecule and at least one cytochrome P450 enzyme are contacted first to form a first reaction mixture prior to contacting with the bioluminescent enzyme to form a second reaction mixture.
- 50 26. The method according to claim 23 wherein the compound is contacted first with the one or more cytochrome P450 enzymes to form a first reaction mixture, the first reaction mixture is then contacted with the luminogenic molecule to form a second reaction mixture, and the second reaction mixture is then contacted with a bioluminescent enzyme to form a third reaction mixture.
- 55 27. The method according to claim 26 wherein the third reaction mixture further includes a detergent.
28. The method according to claim 27 wherein the detergent is a non-ionic detergent.

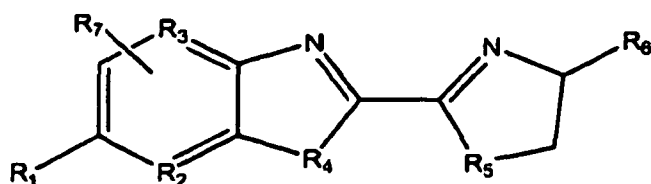
29. The method according to claim 8, further comprising providing a test compound for screening and contacting the test compound, the cell, the luminogenic molecule, and the bioluminescent enzyme to produce a reaction mixture.
30. The method according to claim 29 wherein cell expresses the bioluminescent enzyme.
31. The method according to claim 29 wherein the cell is contacted first with the compound to produce a first reaction mixture prior to contact with the luminogenic molecule to produce a second reaction mixture.
32. The method according to claim 31 wherein the second mixture further comprises a bioluminescent enzyme.
33. The method according to claim 31 wherein the bioluminescent enzyme is added to the second reaction mixture after a predetermined time period.
34. The method according to claim 33 wherein the second reaction mixture further includes a detergent.
35. The method according to claim 34 wherein the detergent is a non-ionic detergent.
36. The method according to claim 14, further comprising providing a test compound for screening and contacting the animal tissue with the test compound, the luminogenic molecule, the bioluminescent enzyme to form a reaction mixture.
37. The method according the claim 36 wherein the animal tissue expresses the bioluminescent enzyme.
38. The method according to claim 36 wherein the tissue is contacted with the test compound to produce a first mixture prior to contact with the luminogenic molecule to produce a second mixture.
39. The method according to claim 38 wherein the second mixture further comprises a bioluminescent enzyme.
40. The method according to claim 38 wherein the bioluminescent enzyme is added to the second reaction mixture after a predetermined time period.
41. The method according to claim 40 wherein the second reaction mixture further includes a detergent.
42. The method according to claim 41 wherein the detergent is a non-ionic detergent.
43. The method according to claim 16 wherein a test compound for screening has been provided and the test compound has been administered to the animal prior to administering the luminogenic compound.
44. The method according to claim 43 wherein the biological sample was taken from the animal just prior to exposure to the test compound.
45. The method according to claim 16 wherein said biological sample comprises blood, serum, bile, urine, feces, or tissue.
46. The method according to claim 43 wherein the animal is a transgenic animal having a bioluminescent enzyme transgene.
47. The method according to claim 46 wherein the bioluminescent enzyme transgene is a luciferase transgene.
48. A high throughput method for rapidly screening a plurality of compounds to determine their effect on cytochrome P450 activity, said method comprising:
 - (a) contacting the compounds to be screened with (i) a luminogenic molecule as defined in claim 1 (ii) one or more cytochrome P450 enzymes; and (iii) one or more bioluminescent enzymes to form reaction mixtures, each reaction mixture having one or more compounds; and
 - (b) determining cytochrome P450 enzyme activity, if any, resulting from the interaction of one or more compounds with one or more cytochrome P450 enzymes by measuring bioluminescence of the reaction mixtures.
49. The method according to claim 48 wherein the compounds are contacted first with the one or more cytochrome

P450 enzymes to form first reaction mixtures, the first reaction mixtures are then contacted with the luminogenic molecule to form second reaction mixtures, and the second reaction mixtures are then contacted with a bioluminescent enzyme to form third reaction mixtures.

- 5 **50.** The method according to claim 49 wherein the third reaction mixture further includes a detergent.
- 51.** The method according to claim 50 wherein the detergent is a non-ionic detergent.
- 10 **52.** The method according to claim 48 wherein the compounds are contacted first with one or more cytochrome P450 enzymes and the luminogenic molecule to form first reaction mixtures prior to contact with one or more bioluminescent enzymes to form a second reaction mixture.
- 53.** The method according to claim 52 wherein the second reaction mixture further comprises a detergent.
- 15 **54.** The method according to claim 52 wherein the detergent is non-ionic.
- 55.** The method according to claim 48 wherein the compounds are contacted simultaneously or contemporaneously with the one or more cytochrome P450 enzymes and the luminogenic molecule to form first reaction mixtures prior to contacting with one or more bioluminescent enzymes to form second reaction mixtures.
- 20 **56.** The method according to claim 48 wherein the bioluminescent enzyme is a luciferase and wherein step (b) further comprises a pyrophosphatase.
- 57.** The method according to claim 56 wherein the pyrophosphatase is an inorganic pyrophosphatase.
- 25 **58.** The method according to claim 48 wherein the cytochrome P450 activity is derived from a cell.
- 59.** The method according to claim 58 wherein the cells express the bioluminescent enzyme.
- 30 **60.** The method according to claim 58 wherein the bioluminescent enzyme from an exogenous source is used.
- 61.** The method according to claim 58 wherein the cells are first contacted with the compounds and luminogenic molecule for a first predetermined time period, then contacted with the bioluminescent enzyme for a second predetermined time period.
- 35 **62.** The method according to claim 61 wherein detergent is present during the second predetermined time period.
- 63.** The method according to claim 62 wherein the detergent is a non-ionic detergent.
- 40 **64.** The method according to claim 58 wherein the cells are first contacted with the compounds for a first predetermined time period, then contacted with the luminogenic molecule and bioluminescent enzyme for a second predetermined time period.
- 45 **65.** The method according to claim 58 wherein the cells, compounds, luminogenic molecule, and bioluminescent enzyme are contacted simultaneously.
- 66.** The method according to claim 48 wherein the cytochrome P450 activity is derived from animal tissue.
- 67.** The method according to claim 66 wherein the tissue expresses at least one bioluminescent enzyme.
- 50 **68.** The method according to claim 66 wherein the tissue is first contacted with the compounds and luminogenic molecule for a first predetermined time period prior to contact with the bioluminescent enzyme.
- 69.** The method according to claim 68, wherein after the first predetermined time period, a detergent is added.
- 55 **70.** The method according to claim 69 wherein detergent and bioluminescent enzyme are added at the same time.
- 71.** The method according to claim 69 wherein detergent is added prior to addition of the bioluminescent enzyme.

72. The method according to claim 66 wherein the tissue is first contacted with the compounds for a first predetermined time period, then contacted with the luminogenic molecule for a second predetermined time period, then contacted with the bioluminescent enzyme for a third predetermined time period.
- 5 73. The method according to claim 72, wherein after the second predetermined time period, a detergent is added.
74. The method according to claim 73 wherein detergent and bioluminescent enzyme are added at the same time.
75. The method according to claim 73 wherein detergent is added prior to bioluminescent enzyme.
- 10 76. The method according to claim 66 wherein the tissue is first contacted with the compounds for a first predetermined time period, then contacted with the luminogenic molecule and bioluminescent enzyme for a second predetermined time period.
- 15 77. The method according to claim 66 wherein the tissue, compounds, luminogenic molecule, and bioluminescent enzyme are contacted simultaneously.
78. The method according to claim 48 wherein the cytochrome P450 enzyme activity is derived from an animal.
- 20 79. The method according to claim 78 wherein the animal is a transgenic teleost that expresses the bioluminescent enzyme.
80. The method according to claim 79 wherein the teleost is first contacted with the compounds and luminogenic molecule for a first predetermined time period prior to contact with the bioluminescent enzyme.
- 25 81. The method according to claim 79 wherein the teleost is first contacted with the compounds for a first predetermined time period, then contacted with the luminogenic molecule for a second predetermined time period, then contacted with the bioluminescent enzyme for a third predetermined time period.
- 30 82. The method according to claim 79 wherein the teleost is first contacted with the compounds for a first predetermined time period, then contacted with the luminogenic molecule and bioluminescent enzyme for a second predetermined time period.
- 35 83. The method according to claim 79 wherein the teleost, compounds, luminogenic molecule, and bioluminescent enzyme are contacted simultaneously.
84. The method according to any one of claims 1, 8, 14, 16, 21, 29, 36, 43, 48, 58, 66, and 78 wherein the the biolumi-
nescent enzyme is a luciferase.
- 40 85. The method of any preceding claim wherein R_4 of the coelentrastazine derivative is aryl or aryl substituted with C_{1-20} alkoxy, hydroxy, halogen, C_{1-20} alkylamino, or C_{1-20} dialkylamino.
86. The method of any preceding claim wherein the luciferin derivative is selected from the group consisting of
45 luciferin 6' 2-chloroethyl ether;
luciferin 6' benzyl ether;
luciferin 6' 4-picolinyl ether;
luciferin 6' 4-trifluoromethylbenzyl ether;
luciferin 6' phenylethyl ether;
luciferin 6' geranyl ether;
50 luciferin 6' prenyl ether,
luciferin 6' 2-picolinyl ether,
luciferin 6' 3-picolinyl ether,
dehydroxyluciferin,
luciferin 6' methyl ether; and
55 luciferin 6' ethyl ether.
87. A kit for determining the effect of a substance on cytochrome P450 enzyme activity comprising:

(a) one or more luminogenic molecules selected from the group consisting of
 luciferin 6' 2-chloroethyl ether;
 luciferin 6' benzyl ether;
 luciferin 6' 4-picolinyl ether;
 luciferin 6' 4-trifluoromethylbenzyl ether;
 luciferin 6' phenylethyl ether,
 luciferin 6' geranyl ether;
 luciferin 6' prenyl ether;
 luciferin 6' 2-picolinyl ether,
 luciferin 6' 3-picolinyl ether; and
 D-luciferin derivatives having a formula:



wherein

R_1 represents amino, halogenated C_{2-20} alkoxy, substituted halogenated C_{2-20} alkoxy, C_{1-20} alkynyloxy, substituted C_{3-20} alkynyloxy, C_{3-20} cycloalkoxy, substituted C_{3-20} cycloalkoxy, C_{3-20} cycloalkylamino, substituted C_{3-20} cycloalkylamino, C_{1-20} alkylamino, substituted C_{1-20} alkylamino, di C_{1-20} alkylamino, substituted di C_{1-20} alkylamino, C_{2-20} alkenylamino, substituted C_{2-20} alkenylamino, di C_{2-20} alkenylamino, substituted di C_{2-20} alkenylamino, C_{2-20} alkenyl C_{1-20} alkylamino, substituted C_{2-20} alkenyl C_{1-20} alkylamino, C_{3-20} alkynylamino, substituted C_{3-20} alkynylamino, di C_{3-20} alkynylamino, substituted di C_{3-20} alkynylamino, C_{3-20} alkynyl C_{1-20} alkylamino, substituted C_{3-20} alkynyl C_{1-20} alkylamino, C_{3-20} alkynyl C_{2-20} alkenylamino, or substituted C_{3-20} alkynyl C_{2-20} alkenylamino;
 R_2 and R_3 independently represents C or N;
 R_4 and R_5 independently represents S, O, or CR_9R_{10} wherein R_9 and R_{10} independently represent H, C_{1-20} alkyl, or fluorine;
 R_6 represents CH_2OH ; COR_{11} wherein R_{11} represents H, OH, C_{2-20} alkenyl, or $NR_{12}R_{13}$ wherein R_{12} and R_{13} are independently H or C_{1-20} alkyl; or $-OM^+$ wherein M^+ is an alkali metal or a pharmaceutically acceptable salt; and
 R_7 represents H, C_{1-20} alkenyl, halogen, or C_{1-6} alkoxide,
 with the proviso that R_1 is not OH or NH_2 , if R_3 and R_2 are both carbon, R_4 and R_5 are both S, R_7 is H, R_6 is COR_{11} and R_{11} is OH such that luminogenic molecule is not luciferin and aminoluciferin; and (b) directions for using the kit.

88. The kit according to claim 87, further comprising one or more bioluminescent enzymes.

89. The kit according to claim 88 wherein the bioluminescent enzyme is a luciferase.

90. The kit according to claim 88 wherein the bioluminescent enzyme is a firefly or Renilla luciferase.

91. The kit according to claim 90 further comprising ATP and magnesium ions.

92. The kit according to claim 91 further comprising a detergent.

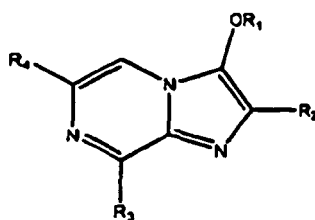
93. The kit according to claim 92 wherein the detergent is non-ionic.

94. The kit according to claim 92 which further comprises a pyrophosphatase.

95. The kit according to claim 94 wherein the pyrophosphatase is an inorganic pyrophosphatase.

96. A kit for determining the effect of a substance on cytochrome P450 enzyme activity comprising:

(a) one or more luminogenic molecules selected from coelenterazine derivatives having the formula:



wherein

R₁ is C₁₋₂₀ alkyl, branched C₃₋₂₀ alkyl, C₃₋₂₀ cycloalkyl, aralkyl, C₁₋₂₀ alkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or diC₁₋₂₀ alkylamino, aralkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or diC₁₋₂₀ alkylamino; and

R₂, R₃, and R₄ are independently hydrogen, C₁₋₂₀ alkyl, C₃₋₂₀ cycloalkyl, branched C₃₋₂₀ alkyl, aryl, aralkyl, C₁₋₂₀ alkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or di C₁₋₂₀ alkylamino, aralkyl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or diC₁₋₂₀ alkylamino, aryl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or di C₁₋₂₀ alkylamino.

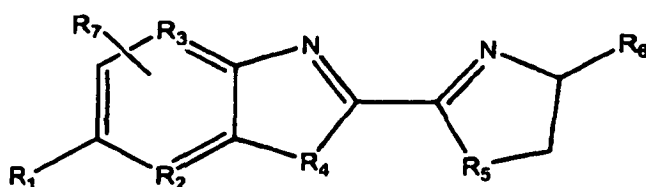
97. The kit according to claim 96 wherein R₄ is aryl or aryl substituted with C₁₋₂₀ alkoxy, hydroxy, halogen, C₁₋₂₀ alkylamino, or C₁₋₂₀ dialkylamino.

98. The kit according to claim 87, further comprising a reversible luciferase inhibitor.

99. The kit according to claim 98, wherein the reversible luciferase inhibitor is 2-(4-aminophenyl)-6-methylbenzothiazole (APMBT) or 2-amino-6-methylbenzothiazole (AMBT).

100. A D-luciferin derivative that is a substrate of a cytochrome P450 enzyme and a pro-substrate of luciferase enzyme, selected from the group consisting of

luciferin 6' 2-chloroethyl ether;
luciferin 6' benzyl ether;
luciferin 6' 4-picolinyl ether;
luciferin 6' 4-trifluoromethylbenzyl ether;
luciferin 6' phenylethyl ether;
luciferin 6' geranyl ether;
luciferin 6' prenyl ether;
luciferin 6' 2-picolinyl ether;
luciferin 6' 3-picolinyl ether; and
D-luciferin derivatives having the formula:



wherein

R₁ represents amino, halogenated C₂₋₂₀ alkoxy, substituted halogenated C₂₋₂₀ alkoxy, C₃₋₂₀ alkynyloxy, sub-

stituted C₃₋₂₀ alkynyloxy, C₃₋₂₀ cycloalkoxy, substituted C₃₋₂₀ cycloalkoxy, C₃₋₂₀ cycloalkylamino, substituted C₃₋₂₀ cycloalkylamino, C₁₋₂₀ alkylamino, substituted C₁₋₂₀ alkylamino, di C₁₋₂₀ alkylamino, substituted di C₁₋₂₀ alkylamino, C₂₋₂₀ alkenylamino, substituted C₂₋₂₀ alkenylamino, di C₂₋₂₀ alkenylamino, substituted di C₂₋₂₀ alkenylamino, C₂₋₂₀ alkenyl C₁₋₂₀ alkylamino, substituted C₂₋₂₀ alkenyl C₁₋₂₀ alkylamino, C₃₋₂₀ alkynylamino, substituted C₃₋₂₀ alkynylamino, di C₃₋₂₀ alkynylamino, substituted di C₃₋₂₀ alkynylamino, C₃₋₂₀ alkynyl C₁₋₂₀ alkylamino, substituted C₃₋₂₀ alkynyl C₁₋₂₀ alkylamino, C₃₋₂₀ alkynyl C₂₋₂₀ alkenylamino, or substituted C₃₋₂₀ alkynyl C₂₋₂₀ alkenylamino;

R₂ and R₃ independently represents C or N;

R₄ and R₅ independently represents S, O, or CR₉R₁₀ wherein R₉ and R₁₀ independently represent H, C₁₋₂₀ alkyl, or fluorine;

R₆ represents CH₂OH; COR₁₁ wherein R₁₁ represents H, OH, C₂₋₂₀ alkenyl, or NR₁₂R₁₃ wherein R₁₂ and R₁₃ are independently H or C₁₋₂₀ alkyl; or -OM⁺ wherein M⁺ is an alkali metal or a pharmaceutically acceptable salt; and

R₇ represents H, C₁₋₂₀ alkenyl, halogen, or C₁₋₆ alkoxide,

with the proviso that R₁ is not OH or NH₂, if R₃ and R₂ are both carbon, R₄ and R₅ are both S, R₇ is H, R₆ is COR₁₁ and R₁₁ is OH, such that the luciferin derivative is not luciferin or aminoluciferin.

101. A composition comprising the D-luciferin derivatives of claim 100.

102. The composition of claim 101, further comprising a pyrophosphatase.

103. The composition according to claim 102, wherein the pyrophosphatase is an inorganic pyrophosphatase.

104. A method for measuring cytochrome P450 activity in an animal which has a bioluminescent enzyme transgene and to which a luminogenic molecule as claimed in Claim 1, or a D-Luciferin derivative as claimed in Claim 100, has been administered, the method comprising determining cytochrome P450 activity by measuring bioluminescence in the living, intact animal.

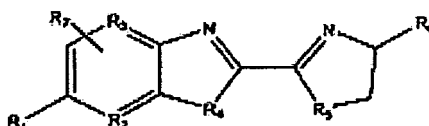
Patentansprüche

1. Verfahren zum Messen der Aktivität eines Cytochrom P450 Enzyms, umfassend:

(a) Kontaktieren eines luminogenen Moleküls, das ein Luciferinderivat oder ein Coelentrazinderivat ist, und eines Cytochrom P450 Substrats und eines Pro-Substrats eines biolumineszenten Enzyms mit mindestens einem Cytochrom P450 Enzym und mindestens einem biolumineszenten Enzym zur Erzeugung eines Reaktionsgemisches; und

(b) Bestimmen der Cytochrom P450 Aktivität durch Messen der Biolumineszenz des Reaktionsgemisches,

wobei das Luciferinderivat ausgewählt ist aus Derivaten mit der Formel:



wobei

R₁ Wasserstoff, Hydroxyl, Amino, C₁₋₂₀-Alkoxy, substituiertes C₁₋₂₀-Alkoxy, C₂₋₂₀-Alkenyloxy, substituiertes C₂₋₂₀-Alkenyloxy, halogeniertes C₂₋₂₀-Alkoxy, substituiertes halogeniertes C₂₋₂₀-Alkoxy, C₃₋₂₀-Alkinyloxy, substituiertes C₃₋₂₀-Alkinyloxy, C₃₋₂₀-Cycloalkoxy, C₃₋₂₀-Cycloalkylamino, substituiertes C₃₋₂₀-Cycloalkylamino, C₁₋₂₀-Alkylamino, substituiertes C₁₋₂₀-Alkylamino, Di-C₁₋₂₀-Alkylamino, substituiertes Di-C₁₋₂₀-Alkylamino, C₂₋₂₀-Alkenylamino, substituiertes C₂₋₂₀-Alkenylamino, Di-C₂₋₂₀-Alkenylamino, substituiertes Di-C₂₋₂₀-Alkenylamino, C₂₋₂₀-Alkenyl-C₁₋₂₀-alkylamino, substituiertes C₂₋₂₀-Alkenyl-C₁₋₂₀-alkylamino, C₃₋₂₀-Alkynylamino, sub-

stituiertes C₃₋₂₀-Alkynylamino, Di-C₃₋₂₀-alkynylamino, substituiertes Di-C₃₋₂₀-alkynylamino, C₃₋₂₀-Alkynyl-C₁₋₂₀-alkylamino, substituiertes C₃₋₂₀-Alkynyl-C₁₋₂₀-alkylamino, C₃₋₂₀-Alkynyl-C₂₋₂₀-alkenylamino oder substituiertes C₃₋₂₀-Alkynyl-C₂₋₂₀-alkenylamino darstellt;

R₂ und R₃ unabhängig C oder N darstellen;

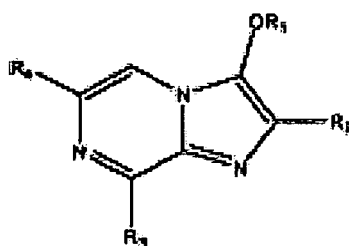
R₄ und R₅ unabhängig S, O, NR₈, wobei NR₈ Wasserstoff oder C₁₋₂₀-Alkyl darstellt, CR₉R₁₀ darstellen, wobei R₉ und R₁₀ unabhängig H, C₁₋₂₀-Alkyl oder Fluor darstellen;

R₆ CH₂OH; COR₁₁, wobei R₁₁ H, OH, C₁₋₂₀-Alkoxid, C₂₋₂₀-Alkenyl oder NR₁₂R₁₃ darstellt, wobei R₁₂ und R₁₃ unabhängig H oder C₁₋₂₀-Alkyl sind; oder -OM⁺ darstellt, wobei M⁺ ein Alkalimetall oder ein pharmazeutisch annehmbares Salz ist; und

R₇ H, C₁₋₆-Alkyl, C₁₋₂₀-Alkenyl, Halogen oder C₁₋₆-Alkoxid darstellt,

unter der Voraussetzung, dass R₁ nicht OH oder NH₂ ist, wenn R₃ und R₂ beide Kohlenstoff sind, R₄ und R₅ beide S sind, R₇ H ist, R₆ COR₁₁ ist und R₁₁ OH ist, so dass das luminogene Molekül nicht Luciferin oder Aminoluciferin ist,

und wobei das Coelentraxinderivat ausgewählt ist aus Derivaten mit einer Formel:



wobei

R₁ C₁₋₂₀-Alkyl, verzweigtes C₃₋₂₀-Alkyl, C₃₋₂₀-Cycloalkyl, Aralkyl, C₁₋₂₀-Alkyl, substituiert mit C₁₋₂₀-Alkoxy, Hydroxy, Halogen, C₁₋₂₀-Alkylamino oder Di-C₁₋₂₀-Alkylamino, Aralkyl, substituiert mit C₁₋₂₀-Alkoxy, Hydroxy, Halogen, C₁₋₂₀-Alkylamino oder Di-C₁₋₂₀-alkylamino ist; und

R₂, R₃ und R₄ unabhängig Wasserstoff, C₁₋₂₀-Alkyl, C₃₋₂₀-Cycloalkyl, verzweigtes C₃₋₂₀-Alkyl, Aryl, Aralkyl, C₁₋₂₀-Alkyl, substituiert mit C₁₋₂₀-Alkoxy, Hydroxy, Halogen, C₁₋₂₀-Alkylamino oder Di-C₁₋₂₀-alkylamino, Aralkyl, substituiert mit C₁₋₂₀-Alkoxy, Hydroxy, Halogen, C₁₋₂₀-Alkylamino oder Di-C₁₋₂₀-alkylamino, Aryl, substituiert mit C₁₋₂₀-Alkoxy, Hydroxy, Halogen, C₁₋₂₀-Alkylamino oder Di-C₁₋₂₀-alkylamino sind.

2. Verfahren nach Anspruch 1, wobei das biolumineszente Enzym eine Luciferase ist und wobei Schritt (a) des Weiteren eine Pyrophosphatase enthält.
3. Verfahren nach Anspruch 2, wobei die Pyrophosphatase eine anorganische Pyrophosphatase ist.
4. Verfahren nach Anspruch 1, wobei das luminogene Molekül, das Cytochrom P450 Enzym und das biolumineszente Enzym ungefähr gleichzeitig in Kontakt gebracht werden.
5. Verfahren nach Anspruch 1, wobei das luminogene Molekül mit mindestens einem Cytochrom P450 Enzym zur Bildung eines ersten Reaktionsgemisches in Kontakt gebracht wird, bevor es mit dem biolumineszenten Enzym zur Bildung eines zweiten Reaktionsgemisches in Kontakt gebracht wird.
6. Verfahren nach Anspruch 5, wobei das zweite Reaktionsgemisch des Weiteren ein Detergens umfasst.
7. Verfahren nach Anspruch 6, wobei das Detergens ein nicht ionisches Detergens ist.
8. Verfahren nach Anspruch 1, wobei die Cytochrom P450 Aktivität von einer Zelle abgeleitet ist.
9. Verfahren nach Anspruch 8, wobei die Zelle das biolumineszente Enzym exprimiert.
10. Verfahren nach Anspruch 8, wobei in Schritt (a) die Zelle ferner mit einem Lyse-Reagens in Kontakt gebracht wird.
11. Verfahren nach Anspruch 8, wobei die Zelle vor Schritt (a) lysiert wird.

12. Verfahren nach Anspruch 8, wobei die Zelle vor Schritt (b) lysiert wird.
13. Verfahren nach Anspruch 8, wobei die Zelle zuerst mit dem luminogenen Molekül in Kontakt gebracht wird, um ein
5 erstes Reaktionsgemisch zu erzeugen, bevor sie mit dem biolumineszenten Enzym in Kontakt gebracht wird, um
ein zweites Reaktionsgemisch zu erzeugen.
14. Verfahren nach Anspruch 1, wobei die Cytochrom P450 Aktivität von einem tierischen Gewebe abgeleitet ist.
15. Verfahren nach Anspruch 14, wobei das Gewebe zuerst über eine erste vorgegebene Zeitperiode mit dem lumino-
10 genen Molekül in Kontakt gebracht wird, bevor es mit dem biolumineszenten Enzym in Kontakt gebracht wird, um
ein zweites Gemisch bereitzustellen.
16. Verfahren zum Messen der Aktivität eines Cytochrom P450 Enzyms in einem Tier, dem das luminogene Molekül,
wie in Anspruch 1 definiert, verabreicht wurde, umfassend:
15 (a) Kontaktieren einer biologischen Probe, die von dem Tier erhalten wurde, mit einem biolumineszenten Enzym
zur Bildung eines Reaktionsgemisches; und
(b) Bestimmen der Cytochrom P450 Aktivität des Tieres durch Messen der Biolumineszenz.
- 20 17. Verfahren nach Anspruch 16, wobei das Reaktionsgemisch des Weiteren ein Detergens umfasst.
18. Verfahren nach Anspruch 17, wobei das Detergens nicht ionisch ist.
19. Verfahren nach Anspruch 16, wobei das biolumineszente Enzym eine Luciferase ist und wobei das Reaktionsge-
25 misch des Weiteren eine Pyrophosphatase umfasst.
20. Verfahren nach Anspruch 19, wobei die Pyrophosphatase eine anorganische Pyrophosphatase ist.
21. Verfahren nach Anspruch 16, wobei das Tier ein transgenes Tier mit einem biolumineszenten Enzym-Transgen ist.
30 22. Verfahren nach Anspruch 21, wobei das biolumineszente Enzym-Transgen ein Luciferase-Transgen ist.
23. Verfahren nach Anspruch 1, des Weiteren umfassend das Bereitstellen einer Testverbindung zum Durchmustern
und das Kontaktieren der Verbindung, des luminogenen Moleküls, des Cytochrom P450 Enzyms und des biolumi-
35 neszenten Enzyms zur Herstellung des Reaktionsgemisches.
24. Verfahren nach Anspruch 23, wobei die Verbindung, das luminogene Molekül, das Cytochrom P450 Enzym und
das biolumineszente Enzym etwa gleichzeitig in Kontakt gebracht werden.
- 40 25. Verfahren nach Anspruch 23, wobei die Verbindung, das luminogene Molekül und mindestens ein Cytochrom P450
Enzym zuerst in Kontakt gebracht werden, um ein erstes Reaktionsgemisch zu bilden, bevor sie mit dem biolumi-
neszenten Enzym in Kontakt gebracht werden, um ein zweites Reaktionsgemisch zu bilden.
- 45 26. Verfahren Nervensprossung 23, wobei die Verbindung zuerst mit einem oder mehreren Cytochrom P450 Enzymen
in Kontakt gebracht wird, um ein erstes Reaktionsgemisch zu bilden, das erste Reaktionsgemisch dann mit dem
luminogenen Molekül in Kontakt gebracht wird, um ein zweites Reaktionsgemisch zu bilden, und das zweite Reak-
tionsgemisch dann mit einem biolumineszenten Enzym in Kontakt gebracht wird, um ein drittes Reaktionsgemisch
zu bilden.
- 50 27. Verfahren nach Anspruch 26, wobei das dritte Reaktionsgemisch des Weiteren ein Detergens enthält.
28. Verfahren nach Anspruch 27, wobei das Detergens ein nicht ionisches Detergens ist.
29. Verfahren nach Anspruch 8, des Weiteren umfassend das Bereitstellen einer Testverbindung zum Durchmustern
und das Kontaktieren der Testverbindung, des luminogenen Moleküls und des biolumineszenten Enzyms zur Her-
55 stellung eines Reaktionsgemisches.
30. Verfahren nach Anspruch 29, wobei die Zelle das biolumineszente Enzym exprimiert.

31. Verfahren nach Anspruch 29, wobei die Zelle zuerst mit der Verbindung in Kontakt gebracht wird, um ein erstes Reaktionsgemisch zu erzeugen, bevor sie mit dem luminogenen Molekül in Kontakt gebracht wird, um ein zweites Reaktionsgemisch zu erzeugen.
- 5 32. Verfahren nach Anspruch 31, wobei das zweite Gemisch des Weiteren ein biolumineszentes Enzym umfasst.
33. Verfahren nach Anspruch 31, wobei das biolumineszente Enzym dem zweiten Reaktionsgemisch nach einer vorgegebenen Zeitperiode zugegeben wird.
- 10 34. Verfahren nach Anspruch 33, wobei das zweite Reaktionsgemisch des Weiteren ein Detergens enthält.
35. Verfahren nach Anspruch 34, wobei das Detergens ein nicht ionisches Detergens ist.
- 15 36. Verfahren nach Anspruch 14, des Weiteren umfassend das Bereitstellen einer Testverbindung zum Durchmustern und das Kontaktieren des tierischen Gewebes mit der Testverbindung, dem luminogenen Molekül, dem biolumineszenten Enzym zur Bildung eines Reaktionsgemisches.
37. Verfahren nach Anspruch 36, wobei das tierische Gewebe das biolumineszente Enzym exprimiert.
- 20 38. Verfahren nach Anspruch 36, wobei das Gewebe mit der Testverbindung in Kontakt gebracht wird, um ein erstes Gemisch zu erzeugen, bevor es mit dem luminogenen Molekül in Kontakt gebracht wird, um ein zweites Gemisch zu erzeugen.
39. Verfahren nach Anspruch 38, wobei das zweite Gemisch des Weiteren ein biolumineszenten Enzym umfasst.
- 25 40. Verfahren nach Anspruch 38, wobei das biolumineszente Enzym dem zweiten Reaktionsgemisch nach einer vorgegebenen Zeitperiode zugegeben wird.
41. Verfahren nach Anspruch 40, wobei das zweite Reaktionsgemisch des Weiteren ein Detergens enthält.
- 30 42. Verfahren nach Anspruch 41, wobei das Detergens ein nicht ionisches Detergens ist.
43. Verfahren nach Anspruch 16, wobei eine Testverbindung zum Durchmustern bereitgestellt wurde und die Testverbindung vor dem Verabreichen der luminogenen Verbindung an das Tier verabreicht wurde.
- 35 44. Verfahren nach Anspruch 43, wobei die biologische Probe dem Tier unmittelbar vor der Belastung mit der Testverbindung entnommen wurde.
- 45 45. Verfahren nach Anspruch 16, wobei die biologische Probe Blut, Serum, Galle, Urin, Fäzes oder Gewebe umfasst.
- 40 46. Verfahren nach Anspruch 43, wobei das Tier ein transgenes Tier mit einem biolumineszenten Enzym-Transgen ist.
47. Verfahren nach Anspruch 46, wobei das biolumineszente Enzym-Transgen ein Luciferase-Transgen ist.
- 45 48. Verfahren mit hohem Durchsatz zum raschen Durchmustern einer Reihe von Verbindungen zur Bestimmung deren Wirkung auf eine Cytochrom P450 Aktivität, wobei das Verfahren umfasst:
 - (a) Kontaktieren der zu durchmusternden Verbindungen mit (i) einem luminogenen Molekül wie in Anspruch 1 definiert, (ii) einem oder mehreren Cytochrom P450 Enzymen; und (iii) einem oder mehreren biolumineszenten Enzymen zur Bildung von Reaktionsgemischen, wobei jedes Reaktionsgemisch eine oder mehrere Verbindungen hat; und
 - (b) Bestimmen der Cytochrom P450 Enzymaktivität, falls vorhanden, die sich aus der Wechselwirkung einer oder mehrerer Verbindungen mit einem oder mehreren Cytochrom P450 Enzymen ergibt, durch Messen der Biolumineszenz der Reaktionsgemische.
- 50 49. Verfahren nach Anspruch 48, wobei die Verbindungen zuerst mit dem einem oder den mehreren Cytochrom P450 Enzymen in Kontakt gebracht werden, um erste Reaktionsgemische zu bilden, die ersten Reaktionsgemische dann mit dem luminogenen Molekül in Kontakt gebracht werden, um zweite Reaktionsgemische zu bilden, und die zweiten
- 55

Reaktionsgemische dann mit einem biolumineszenten Enzym in Kontakt gebracht werden, um dritte Reaktionsgemische zu bilden.

5 **50.** Verfahren nach Anspruch 49, wobei das dritte Reaktionsgemisch des Weiteren ein Detergens enthält.

51. Verfahren nach Anspruch 51, wobei das Detergens ein nicht ionisches Detergens ist.

10 **52.** Verfahren nach Anspruch 48, wobei die Verbindungen zuerst mit einem oder mehreren Cytochrom P450 Enzymen und dem luminogenen Molekül in Kontakt gebracht werden, um erste Reaktionsgemische zu bilden, bevor sie mit einem oder mehreren biolumineszenten Enzymen in Kontakt gebracht werden, um ein zweites Reaktionsgemisch zu bilden.

53. Verfahren nach Anspruch 52, wobei das zweite Reaktionsgemisch des Weiteren ein Detergens umfasst.

15 **54.** Verfahren nach Anspruch 52, wobei das Detergens ein nicht ionisches Detergens ist.

20 **55.** Verfahren nach Anspruch 48, wobei die Verbindungen gleichzeitig oder simultan mit dem einem oder den mehreren Cytochrom P450 Enzymen und dem luminogenen Molekül in Kontakt gebracht werden, um erste Reaktionsgemische zu bilden, bevor sie mit einem oder mehreren biolumineszenten Enzymen in Kontakt gebracht werden, um zweite Reaktionsgemische zu bilden.

56. Verfahren nach Anspruch 48, wobei das biolumineszente Enzym eine Luciferase ist und wobei Schritt (b) des Weiteren eine Pyrophosphatase umfasst.

25 **57.** Verfahren nach Anspruch 56, wobei die Pyrophosphatase eine anorganische Pyrophosphatase ist.

58. Verfahren nach Anspruch 48, wobei die Cytochrom P450 Aktivität von einer Zelle abgeleitet ist.

30 **59.** Verfahren nach Anspruch 58, wobei die Zellen das biolumineszente Enzym exprimieren.

60. Verfahren nach Anspruch 58, wobei das biolumineszente Enzym von einer exogenen Quelle verwendet wird.

35 **61.** Verfahren nach Anspruch 58, wobei die Zellen zuerst mit den Verbindungen und dem luminogenen Molekül über eine erste vorgegebene Zeitperiode in Kontakt gebracht werden, dann mit dem biolumineszenten Enzym über eine zweite vorgegebene Zeitperiode in Kontakt gebracht werden.

62. Verfahren nach Anspruch 61, wobei das Detergens während der zweiten vorgegebenen Zeitperiode vorhanden ist.

40 **63.** Verfahren nach Anspruch 62, wobei das Detergens ein nicht ionisches Detergens ist.

64. Verfahren nach Anspruch 58, wobei die Zellen zuerst mit den Verbindungen über eine erste vorgegebene Zeitperiode in Kontakt gebracht werden, dann mit dem luminogenen Molekül und dem biolumineszenten Enzym über eine zweite vorgegebene Zeitperiode in Kontakt gebracht werden.

45 **65.** Verfahren nach Anspruch 58, wobei die Zellen, die Verbindungen, das luminogene Molekül und die biolumineszenten Enzyme gleichzeitig in Kontakt gebracht werden.

66. Verfahren nach Anspruch 58, wobei die Cytochrom P450 Aktivität von tierischem Gewebe abgeleitet ist.

50 **67.** Verfahren nach Anspruch 66, wobei das Gewebe mindestens ein biolumineszentes Enzym exprimiert.

68. Verfahren nach Anspruch 66, wobei das Gewebe zuerst mit den Verbindungen und dem luminogenen Molekül über eine erste vorgegebene Zeitperiode in Kontakt gebracht wird, bevor es mit dem biolumineszenten Enzym in Kontakt gebracht wird.

55 **69.** Verfahren nach Anspruch 68, wobei nach der ersten vorgegebenen Zeitperiode ein Detergens zugegeben wird.

70. Verfahren nach Anspruch 69, wobei das Detergens und das biolumineszente Enzym gleichzeitig zugegeben werden.

71. Verfahren nach Anspruch 69, wobei das Detergens vor der Zugabe des biolumineszenten Enzyms zugegeben wird.
72. Verfahren nach Anspruch 66, wobei das Gewebe zuerst mit den Verbindungen über eine erste vorgegebene Zeitperiode in Kontakt gebracht wird, dann mit dem luminogenen Molekül über eine zweite vorgegebene Zeitperiode in Kontakt gebracht wird, dann mit dem biolumineszenten Enzym über eine dritte vorgegebene Zeitperiode in Kontakt gebracht wird.
73. Verfahren nach Anspruch 72, wobei nach der zweiten vorgegebenen Zeitperiode ein Detergens zugegeben wird.
74. Verfahren nach Anspruch 73, wobei das Detergens und das biolumineszente Enzym gleichzeitig zugegeben werden.
75. Verfahren nach Anspruch 73, wobei das Detergens vor dem biolumineszenten Enzym zugegeben wird.
76. Verfahren nach Anspruch 66, wobei das Gewebe zuerst mit den Verbindungen über eine erste vorgegebene Zeitperiode in Kontakt gebracht wird, dann mit dem luminogenen Molekül und biolumineszenten Enzym über eine zweite vorgegebene Zeitperiode in Kontakt gebracht wird.
77. Verfahren nach Anspruch 66, wobei das Gewebe, die Verbindungen, das luminogene Molekül und das biolumineszente Enzym gleichzeitig in Kontakt gebracht werden.
78. Verfahren nach Anspruch 48, wobei die Cytochrom P450 Enzymaktivität von einem Tier abgeleitet ist.
79. Verfahren nach Anspruch 78, wobei das Tier ein transgener Knochenfisch (Teleostei) ist, der das biolumineszente Enzym exprimiert.
80. Verfahren nach Anspruch 79, wobei der Knochenfisch zuerst mit den Verbindungen und dem luminogenen Molekül über eine erste vorgegebene Zeitperiode in Kontakt gebracht wird, bevor er mit dem biolumineszenten Enzym in Kontakt gebracht wird.
81. Verfahren nach Anspruch 79, wobei der Knochenfisch zuerst mit den Verbindungen über eine erste vorgegebene Zeitperiode in Kontakt gebracht wird, dann mit dem luminogenen Molekül über eine zweite vorgegebene Zeitperiode in Kontakt gebracht wird, dann mit dem biolumineszenten Enzym über eine dritte Zeitperiode in Kontakt gebracht wird.
82. Verfahren nach Anspruch 79, wobei der Knochenfisch zuerst mit den Verbindungen über eine erste vorgegebene Zeitperiode in Kontakt gebracht wird, dann mit dem luminogenen Molekül und dem biolumineszenten Enzym über eine zweite vorgegebene Zeitperiode in Kontakt gebracht wird.
83. Verfahren nach Anspruch 79, wobei der Knochenfisch, die Verbindungen, das luminogene Molekül und biolumineszente Enzyme gleichzeitig in Kontakt gebracht werden.
84. Verfahren nach einem der Ansprüche 1, 8, 14, 16, 21, 29, 36, 43, 48, 58, 66 und 78, wobei das biolumineszente Enzym Luciferase ist.
85. Verfahren nach einem der vorangehenden Ansprüche, wobei R_4 des Coelentraxinderivats Aryl oder Aryl substituiert mit C_{1-20} -Alkoxy, Hydroxy, Halogen, C_{1-20} -Alkylamino oder C_{1-20} -Dialkylamino ist.
86. Verfahren nach einem der vorangehenden Ansprüche, wobei das Luciferinderivat ausgewählt ist aus der Gruppe bestehend aus:

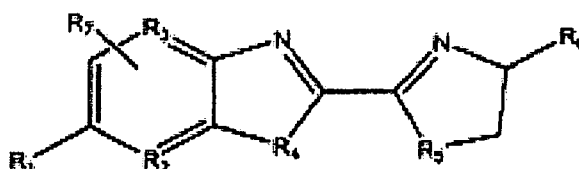
Luciferin 6' 2-Chlorethylether;
Luciferin 6' Benzylether;
Luciferin 6' 4-Picolinylether;
Luciferin 6' 4-Trifluormethylbenzylether;
Luciferin 6' Phenylethylether;
Luciferin 6' Geranylether;
Luciferin 6' Prenylether;
Luciferin 6' 2-Picolinylether;
Luciferin 6' 3-Picolinylether;

Dehydroxyluciferin;
Luciferin 6' Methylether; und
Luciferin 6' Ethylether.

87. Kit zum Bestimmen der Wirkung einer Substanz auf Cytochrom P450 Enzymaktivität, umfassend:

(a) ein oder mehrere luminogene Moleküle, ausgewählt aus der Gruppe bestehend aus

Luciferin 6' 2-Chlorethylether;
Luciferin 6' Benzylether;
Luciferin 6' 4-Picolinylether;
Luciferin 6' 4-Trifluormethylbenzylether;
Luciferin 6' Phenylethylether;
Luciferin 6' Geranylether;
Luciferin 6' Prenylether;
Luciferin 6' 2-Picolinylether;
Luciferin 6' 3-Picolinylether;
D-Luciferasederivaten mit einer Formel:



wobei

R_1 Amino, halogeniertes C_{2-20} -Alkoxy, substituiertes halogeniertes C_{2-20} -Alkoxy, C_{3-20} -Alkinyloxy, substituiertes C_{3-20} -Alkinyloxy, C_{3-20} -Cycloalkoxy, substituiertes C_{3-20} -Cycloalkoxy, C_{3-20} -Cycloalkylamino, substituiertes C_{3-20} -Cycloalkylamino, C_{1-20} -Alkylamino, substituiertes C_{1-20} -Alkylamino, Di- C_{1-20} -alkylamino, substituiertes Di- C_{1-20} -alkylamino, C_{2-20} -Alkenylamino, substituiertes C_{2-20} -Alkenylamino, Di- C_{2-20} -alkenylamino, substituiertes Di- C_{2-20} -Alkenylamino, C_{2-20} -Alkenyl- C_{1-20} -alkylamino, substituiertes C_{2-20} -Alkenyl- C_{1-20} -alkylamino, C_{3-20} -Alkynylamino, substituiertes C_{3-20} -Alkynylamino, Di- C_{3-20} -alkynylamino, substituiertes Di- C_{3-20} -alkynylamino, C_{3-20} -Alkynyl- C_{1-20} -alkylamino, substituiertes C_{3-20} -Alkynyl- C_{1-20} -alkylamino, C_{3-20} -Alkynyl- C_{2-20} -alkenylamino oder substituiertes C_{3-20} -Alkynyl- C_{2-20} -alkenylamino darstellt;

R_2 und R_3 unabhängig C oder N darstellen;

R_4 und R_5 unabhängig S, O oder CR_9R_{10} darstellen, wobei R_9 und R_{10} unabhängig H, C_{1-20} -Alkyl oder Fluor darstellen;

R_6 CH_2OH ; COR_{11} , wobei R_{11} H, OH, C_{2-20} -Alkenyl darstellt, oder $NR_{12}R_{13}$, wobei R_{12} und R_{13} unabhängig H oder C_{1-20} -Alkyl sind; oder - OM^+ darstellt, wobei M^+ ein Alkalimetall oder ein pharmazeutisch annehmbares Salz ist; und

R_7 H, C_{1-20} -Alkenyl, Halogen oder C_{1-6} -Alkoxid darstellt,

unter der Voraussetzung, dass R_1 nicht OH oder NH_2 ist, wenn R_3 und R_2 beide Kohlenstoff sind, R_4 und R_5 beide S sind, R_7 H ist, R_6 COR_{11} ist und R_{11} OH ist, so dass das luminogene Molekül nicht Luciferin und Aminoluciferin ist; und

(b) Anweisungen zur Verwendung des Kits.

88. Kit nach Anspruch 87, des Weiteren umfassend ein oder mehrere biolumineszente Enzyme.

89. Kit nach Anspruch 88, wobei das biolumineszente Enzym eine Luciferase ist.

90. Kit nach Anspruch 88, wobei das biolumineszente Enzym eine Leuchtkäfer-Luciferase oder eine Renilla-Luciferase ist.

91. Kit nach Anspruch 90, des Weiteren umfassend ATP- und Magnesiumionen.

92. Kit nach Anspruch 91, des Weiteren umfassend ein Detergens.

93. Kit nach Anspruch 92, wobei das Detergens nicht ionisch ist.

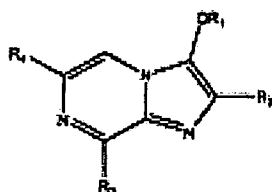
94. Kit nach Anspruch 92, der des Weiteren eine Pyrophosphatase umfasst.

5 95. Kit nach Anspruch 94, wobei die Pyrophosphatase eine anorganische Pyrophosphatase ist.

96. Kit zum Bestimmen der Wirkung einer Substanz auf Cytochrom P450 Enzymaktivität, umfassend:

(a) ein oder mehrere luminogene Moleküle, ausgewählt aus Coelenterazinderivaten mit der Formel:

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wobei

R₁ C₁₋₂₀-Alkyl, verzweigtes C₃₋₂₀-Alkyl, C₃₋₂₀-Cycloalkyl, Aralkyl, C₁₋₂₀-Alkyl, substituiert mit C₁₋₂₀-Alkoxy, Hydroxy, Halogen, C₁₋₂₀-Alkylamino oder Di-C₁₋₂₀-alkylamino, Aralkyl, substituiert mit C₁₋₂₀-Alkoxy, Hydroxy, Halogen, C₁₋₂₀-Alkylamino oder Di-C₁₋₂₀-alkylamino ist; und

25

R₂, R₃ und R₄ unabhängig Wasserstoff, C₁₋₂₀-Alkyl, C₃₋₂₀-Cycloalkyl, verzweigtes C₃₋₂₀-Alkyl, Aryl, Aralkyl, C₁₋₂₀-Alkyl, substituiert mit C₁₋₂₀-Alkoxy, Hydroxy, Halogen, C₁₋₂₀-Alkylamino oder Di-C₁₋₂₀-alkylamino, Aralkyl, substituiert mit C₁₋₂₀-Alkoxy, Hydroxy, Halogen, C₁₋₂₀-Alkylamino oder Di-C₁₋₂₀-alkylamino, Aryl, substituiert mit C₁₋₂₀-Alkoxy, Hydroxy, Halogen, C₁₋₂₀-Alkylamino oder Di-C₁₋₂₀-alkylamino sind.

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97. Kit nach Anspruch 96, wobei R₄ Aryl oder Aryl substituiert mit C₁₋₂₀-Alkoxy, Hydroxy, Halogen, C₁₋₂₀-Alkylamino oder Di-C₁₋₂₀-alkylamino ist.

98. Kit nach Anspruch 87, des Weiteren umfassend einen reversiblen Luciferase-Inhibitor.

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99. Kit nach Anspruch 98, wobei der reversible Luciferase-Inhibitor 2-(4-Aminophenyl)-6-Methylbenzothiazol (APMBT) oder 2-Amino-6-Methylbenzothiazol (AMBT) ist.

100.D-Luciferinderivat, das ein Substrat eines Cytochrom P450 Enzyms und ein Pro-Substrat von Luciferaseenzym ist, ausgewählt aus der Gruppe bestehend aus:

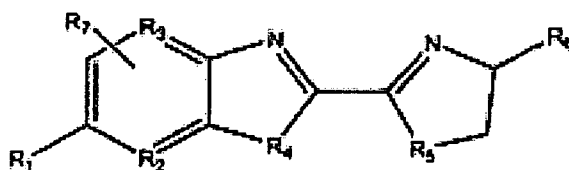
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Luciferin 6' 2-Chlorethylether;
 Luciferin 6' Benzylether;
 Luciferin 6' 4-Picolinylether;
 Luciferin 6' 4-Trifluormethylbenzylether;
 Luciferin 6' Phenylethylether;
 Luciferin 6' Geranylether;
 Luciferin 6' Prenylether;
 Luciferin 6' 2-Picolinylether;
 Luciferin 6' 3-Picolinylether;
 D-Luciferasederivaten mit einer Formel:

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wobei

R_1 Amino, halogeniertes C_{2-20} -Alkoxy, substituiertes halogeniertes C_{2-20} -Alkoxy, C_{3-20} -Alkinyloxy, substituiertes C_{3-20} -Alkinyloxy, C_{3-20} -Cycloalkoxy, substituiertes C_{3-20} -Cycloalkoxy, C_{3-20} -Cycloalkylamino, substituiertes C_{3-20} -Cycloalkylamino, C_{1-20} -Alkylamino, substituiertes C_{1-20} -Alkylamino, Di- C_{1-20} -alkylamino, substituiertes Di- C_{1-20} -alkylamino, C_{2-20} -alkenylamino, substituiertes C_{2-20} -Alkenylamino, Di- C_{2-20} -Alkenylamino, substituiertes Di- C_{2-20} -alkenylamino, C_{2-20} -Alkenyl- C_{1-20} -alkylamino, substituiertes C_{2-20} -Alkenyl- C_{1-20} -alkylamino, C_{3-20} -Alkylamino, substituiertes C_{3-20} -Alkylamino, Di- C_{3-20} -alkylamino, substituiertes Di- C_{3-20} -alkylamino, C_{3-20} -Alkyl- C_{1-20} -alkylamino, substituiertes C_{3-20} -Alkyl- C_{1-20} -alkylamino, C_{3-20} -Alkyl- C_{2-20} -alkenylamino oder substituiertes C_{3-20} -Alkyl- C_{2-20} -alkenylamino darstellt;

R_2 und R_3 unabhängig C oder N darstellen;

R_4 und R_5 unabhängig S, O oder CR_9R_{10} darstellen, wobei R_9 und R_{10} unabhängig H, C_{1-20} -Alkyl oder Fluor darstellen;

R_6 CH_2OH ; COR_{11} , wobei R_{11} H, OH, C_{2-20} -Alkenyl oder $NR_{12}R_{13}$ darstellt, wobei R_{12} und R_{13} unabhängig H oder C_{1-20} -Alkyl sind; oder - OM^+ ist, wobei M^+ ein Alkalimetall oder ein pharmazeutisch annehmbares Salz ist; und

R_7 H, C_{1-20} -Alkenyl, Halogen oder C_{1-6} -Alkoxid darstellt,

unter der Voraussetzung, dass R_1 nicht OH oder NH_2 ist, wenn R_3 und R_2 beide Kohlenstoff sind, R_4 und R_5 beide S sind, R_7 H ist und R_{11} OH ist, so dass das luminogene Molekül nicht Luciferin oder Aminoluciferin ist.

101. Zusammensetzung, umfassend die D-Luciferinderivate von Anspruch 100.

102. Zusammensetzung nach Anspruch 101, des Weiteren umfassend eine Pyrophosphatase.

103. Zusammensetzung nach Anspruch 102, wobei die Pyrophosphatase eine anorganische Pyrophosphatase ist.

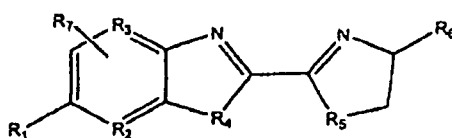
104. Verfahren zum Messen einer Cytochrom P450 Aktivität in einem Tier, das ein biolumineszentes Enzym-Transgen aufweist und dem ein luminogenes Molekül nach Anspruch 1 oder ein D-Luciferinderivat nach Anspruch 100 verabreicht wurde, wobei das Verfahren des Bestimmen der Cytochrom P450 Aktivität durch Messen der Biolumineszenz im lebenden, intakten Tier umfasst.

Revendications

1. Procédé pour mesurer l'activité d'une enzyme cytochrome P450, comprenant :

- (a) la mise en contact d'une molécule luminogène qui est un dérivé de luciférine ou un dérivé de coelentérazine et d'un substrat pour cytochrome P450 et d'un prosubstrat d'une enzyme bioluminescente avec au moins une enzyme cytochrome P450 et au moins une enzyme bioluminescente pour produire un mélange réactionnel ; et
- (b) la détermination de l'activité de cytochrome P450 en mesurant la bioluminescence du mélange réactionnel,

dans lequel le dérivé de luciférine est choisi parmi les dérivés ayant la formule :



dans laquelle

R_1 représente un hydrogène, un hydroxyle, un amino, un alcoxy en C_{1-20} , un alcoxy en C_{1-20} substitué, un alcényloxy en C_{2-20} , un alcényloxy en C_{2-20} substitué, un alcoxy en C_{2-20} halogéné, un alcoxy en C_{2-20} halogéné substitué, un alcynioxy en C_{3-20} , un alcynioxy en C_{3-20} substitué, un cycloalcoxy en C_{3-20} , un cycloalcoxy en C_{3-20} substitué, un cycloalkylamino en C_{3-20} , un cycloalkylamino en C_{3-20} substitué, un alkylamino en C_{1-20} , un alkylamino en C_{1-20} substitué, un di-alkylamino en C_{1-20} , un di-alkylamino en C_{1-20} substitué, un alcénylamino en C_{2-20} , un alcénylamino en C_{2-20} substitué, un di-alcénylamino en C_{2-20} , un di-alcénylamino en C_{2-20} substitué, un alcényl en C_{2-20} alkylamino en C_{1-20} , un alcényl en C_{2-20} alkylamino en C_{1-20} substitué, un alcynylamino en C_{3-20} , un alcynylamino en C_{3-20} substitué, un di-alcynylamino en C_{3-20} , un di-alcynylamino en C_{3-20} substitué, un alcynyl en C_{3-20} alkylamino en C_{1-20} , un alcynyl en C_{3-20} alkylamino en C_{1-20} substitué, un alcynyl en C_{3-20} alcénylamino en C_{2-20} ou un alcynyl en C_{3-20} alcénylamino en C_{2-20} substitué ;

R_2 et R_3 représentent indépendamment C ou N ;

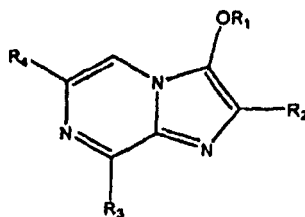
R_4 et R_5 représentent indépendamment S, O, NR_8 où NR_8 représente un hydrogène ou un alkyle en C_{1-20} , CR_9R_{10} où R_9 et R_{10} représentent indépendamment H, un alkyle en C_{1-20} ou du fluor ;

R_6 représente CH_2OH ; COR_{11} où R_{11} représente H, OH, un alcoxyde en C_{1-20} , un alcényle en C_{2-20} ou $NR_{12}R_{13}$ où R_{12} et R_{13} sont indépendamment H ou un alkyle en C_{1-20} ; ou $-OM^+$ où M^+ est un métal alcalin ou un sel pharmaceutiquement acceptable ; et

R_7 représente H, un alkyle en C_{1-6} , un alcényle en C_{1-20} , un halogène ou un alcoxyde en C_{1-6} ,

avec pour condition que R_1 n'est pas OH ou NH_2 , si R_3 et R_2 sont tous les deux du carbone, R_4 et R_5 sont tous les deux S, R_7 est H, R_6 est COR_{11} et R_{11} est OH, de sorte que la molécule luminogène n'est pas une luciférine ou une aminoluciférine

et dans lequel le dérivé de coelentérazine est choisi parmi les dérivés ayant une formule :



dans laquelle

R_1 est un alkyle en C_{1-20} , un alkyle en C_{3-20} ramifié, un cycloalkyle en C_{3-20} , un aralkyle, un alkyle en C_{1-20} substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} , un aralkyle substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} ; et

R_2 , R_3 et R_4 sont indépendamment un hydrogène, un alkyle en C_{1-20} , un cycloalkyle en C_{3-20} , un alkyle en C_{3-20} ramifié, un aryle, un aralkyle, un alkyle en C_{1-20} substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} , un aralkyle substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} , un aryle substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} .

2. Procédé selon la revendication 1, dans lequel l'enzyme bioluminescente est une luciférase et dans lequel l'étape (a) inclut en outre une pyrophosphatase.

3. Procédé selon la revendication 2, dans lequel la pyrophosphatase est une pyrophosphatase inorganique.

4. Procédé selon la revendication 1, dans lequel la molécule luminogène, l'enzyme cytochrome P450 et l'enzyme bioluminescente sont mises en contact environ en même temps.

5. Procédé selon la revendication 1, dans lequel la molécule luminogène est mise en contact avec au moins une enzyme cytochrome P450 pour former un premier mélange réactionnel avant la mise en contact avec l'enzyme bioluminescente pour former un second mélange réactionnel.

6. Procédé selon la revendication 5, dans lequel le second mélange réactionnel comprend en outre un détergent.
7. Procédé selon la revendication 6, dans lequel le détergent est un détergent non ionique.
- 5 8. Procédé selon la revendication 1, dans lequel l'activité de cytochrome P450 est dérivée d'une cellule.
9. Procédé selon la revendication 8, dans lequel la cellule exprime l'enzyme bioluminescente.
- 10 10. Procédé selon la revendication 8, dans lequel, dans l'étape (a), la cellule est en outre mise en contact avec un réactif de lyse.
11. Procédé selon la revendication 8, dans lequel la cellule est lysée avant l'étape (a).
12. Procédé selon la revendication 8, dans lequel la cellule est lysée avant l'étape (b).
- 15 13. Procédé selon la revendication 8, dans lequel la cellule est mise en contact d'abord avec la molécule luminogène pour produire un premier mélange réactionnel avant la mise en contact avec l'enzyme bioluminescente pour produire un second mélange réactionnel.
- 20 14. Procédé selon la revendication 1, dans lequel l'activité de cytochrome P450 est dérivée d'un tissu animal.
15. Procédé selon la revendication 14, dans lequel le tissu est mis en contact d'abord avec la molécule luminogène pendant une première période de temps prédéterminée avant la mise en contact avec l'enzyme bioluminescente pour fournir un second mélange.
- 25 16. Procédé pour mesurer l'activité d'une enzyme cytochrome P450 chez un animal auquel la molécule luminogène selon la revendication 1 a été administrée ; comprenant :
 - 30 (a) la mise en contact d'un échantillon biologique obtenu auprès de l'animal avec une enzyme bioluminescente pour former un mélange réactionnel ; et
 - (b) la détermination de l'activité de cytochrome P450 de l'animal en mesurant la bioluminescence.
17. Procédé selon la revendication 16, dans lequel le mélange réactionnel comprend en outre un détergent.
- 35 18. Procédé selon la revendication 17, dans lequel le détergent est non ionique.
19. Procédé selon la revendication 16, dans lequel l'enzyme bioluminescente est une luciférase et dans lequel le mélange réactionnel comprend en outre une pyrophosphatase.
- 40 20. Procédé selon la revendication 19, dans lequel la pyrophosphatase est une pyrophosphatase inorganique.
21. Procédé selon la revendication 16, dans lequel l'animal est un animal transgénique ayant un transgène d'enzyme bioluminescente.
- 45 22. Procédé selon la revendication 21, dans lequel le transgène d'enzyme bioluminescente est un transgène de luciférase.
23. Procédé selon la revendication 1, comprenant en outre la fourniture d'un composé d'essai à cribler et la mise en contact du composé, de la molécule luminogène, de l'enzyme cytochrome P450 et de l'enzyme bioluminescente pour produire le mélange réactionnel.
- 50 24. Procédé selon la revendication 23, dans lequel le composé, la molécule luminogène, l'enzyme cytochrome P450 et l'enzyme bioluminescente sont mis en contact environ en même temps.
- 55 25. Procédé selon la revendication 23, dans lequel le composé, la molécule luminogène et au moins une enzyme cytochrome P450 sont mis en contact d'abord pour former un premier mélange réactionnel avant la mise en contact avec l'enzyme bioluminescente pour former un second mélange réactionnel.

26. Procédé selon la revendication 23, dans lequel le composé est mis en contact d'abord avec la ou les enzymes cytochromes P450 pour former un premier mélange réactionnel, le premier mélange réactionnel est ensuite mis en contact avec la molécule luminogène pour former un deuxième mélange réactionnel et le deuxième mélange réactionnel est ensuite mis en contact avec une enzyme bioluminescente pour former un troisième mélange réactionnel.
27. Procédé selon la revendication 26, dans lequel le troisième mélange réactionnel inclut en outre un détergent.
28. Procédé selon la revendication 27, dans lequel le détergent est un détergent non ionique.
29. Procédé selon la revendication 8, comprenant en outre un composé d'essai à cribler et la mise en contact du composé d'essai, de la cellule, de la molécule luminogène et de l'enzyme bioluminescente pour produire un mélange réactionnel.
30. Procédé selon la revendication 29, dans lequel la cellule exprime l'enzyme bioluminescente.
31. Procédé selon la revendication 29, dans lequel la cellule est mise en contact d'abord avec le composé pour produire un premier mélange réactionnel avant la mise en contact avec la molécule luminogène pour produire un second mélange réactionnel.
32. Procédé selon la revendication 31, dans lequel le second mélange comprend en outre une enzyme bioluminescente.
33. Procédé selon la revendication 31, dans lequel l'enzyme bioluminescente est ajoutée au second mélange réactionnel après une période de temps prédéterminée.
34. Procédé selon la revendication 33, dans lequel le second mélange réactionnel inclut en outre un détergent.
35. Procédé selon la revendication 34, dans lequel le détergent est un détergent non ionique.
36. Procédé selon la revendication 14, comprenant en outre la fourniture d'un composé d'essai à cribler et la mise en contact du tissu animal avec le composé d'essai, la molécule luminogène, l'enzyme bioluminescente pour former un mélange réactionnel.
37. Procédé selon la revendication 36, dans lequel le tissu animal exprime l'enzyme bioluminescente.
38. Procédé selon la revendication 36, dans lequel le tissu est mis en contact avec le composé d'essai pour produire un premier mélange avant la mise en contact avec la molécule luminogène pour produire un second mélange.
39. Procédé selon la revendication 38, dans lequel le second mélange comprend en outre une enzyme bioluminescente.
40. Procédé selon la revendication 38, dans lequel l'enzyme bioluminescente est ajoutée au second mélange réactionnel après une période de temps prédéterminée.
41. Procédé selon la revendication 40, dans lequel le second mélange réactionnel inclut en outre un détergent.
42. Procédé selon la revendication 41, dans lequel le détergent est un détergent non ionique.
43. Procédé selon la revendication 16, dans lequel un composé d'essai à cribler est fourni et le composé d'essai est administré à l'animal avant d'administrer le composé luminogène.
44. Procédé selon la revendication 43, dans lequel l'échantillon biologique a été prélevé sur l'animal juste avant l'exposition au composé d'essai.
45. Procédé selon la revendication 16, dans lequel ledit échantillon biologique comprend du sang, du sérum, de la bile, de l'urine, des matières fécales ou un tissu.
46. Procédé selon la revendication 43, dans lequel l'animal est un animal transgénique ayant un transgène d'enzyme bioluminescente.

47. Procédé selon la revendication 46, dans lequel le transgène d'enzyme bioluminescente est un transgène de luciférase.
- 5 48. Procédé à haut rendement pour cribler rapidement une pluralité de composés pour déterminer leur effet sur l'activité de cytochrome P450, ledit procédé comprenant :
- 10 (a) la mise en contact des composés à cribler avec (i) une molécule luminogène selon la revendication 1 (ii) une ou plusieurs enzymes cytochromes P450 ; et (iii) une ou plusieurs enzymes bioluminescentes pour former des mélanges réactionnels, chaque mélange réactionnel ayant un ou plusieurs composés ; et
- (b) la détermination de l'activité enzymatique d'un cytochrome P450, s'il y en a, résultant de l'interaction d'un ou plusieurs composés avec une ou plusieurs enzymes cytochromes P450 en mesurant la bioluminescence des mélanges réactionnels.
- 15 49. Procédé selon la revendication 48, dans lequel les composés sont mis en contact d'abord avec la ou les enzymes cytochromes P450 pour former des premiers mélanges réactionnels, les premiers mélanges réactionnels sont ensuite mis en contact avec la molécule luminogène pour former des deuxièmes mélanges réactionnels et les deuxièmes mélanges réactionnels sont ensuite mis en contact avec une enzyme bioluminescente pour former des troisièmes mélanges réactionnels.
- 20 50. Procédé selon la revendication 49, dans lequel le troisième mélange réactionnel inclut en outre un détergent.
51. Procédé selon la revendication 50, dans lequel le détergent est un détergent non ionique.
- 25 52. Procédé selon la revendication 48, dans lequel les composés sont mis en contact d'abord avec une ou plusieurs enzymes cytochromes P450 et la molécule luminogène pour former des premiers mélanges réactionnels avant la mise en contact avec une ou plusieurs enzymes bioluminescentes pour former un second mélange réactionnel.
53. Procédé selon la revendication 52, dans lequel le second mélange réactionnel comprend en outre un détergent.
- 30 54. Procédé selon la revendication 52, dans lequel le détergent est non ionique.
55. Procédé selon la revendication 48, dans lequel les composés sont mis en contact simultanément ou en même temps avec la ou les enzymes cytochromes P450 et la molécule luminogène pour former des premiers mélanges réactionnels avant la mise en contact avec une ou plusieurs enzymes bioluminescentes pour former des seconds mélanges réactionnels.
- 35 56. Procédé selon la revendication 48, dans lequel l'enzyme bioluminescente est une luciférase et dans lequel l'étape (b) comprend en outre une pyrophosphatase.
- 40 57. Procédé selon la revendication 56, dans lequel la pyrophosphatase est une pyrophosphatase inorganique.
58. Procédé selon la revendication 48, dans lequel l'activité de cytochrome P450 est dérivée d'une cellule.
59. Procédé selon la revendication 58, dans lequel les cellules expriment l'enzyme bioluminescente.
- 45 60. Procédé selon la revendication 58, dans lequel l'enzyme bioluminescente d'une source exogène est utilisée.
61. Procédé selon la revendication 58, dans lequel les cellules sont d'abord mises en contact avec les composés et la molécule luminogène pendant une première période de temps prédéterminée, puis mises en contact avec l'enzyme bioluminescente pendant une seconde période de temps prédéterminée.
- 50 62. Procédé selon la revendication 61, dans lequel un détergent est présent pendant la seconde période de temps prédéterminée.
- 55 63. Procédé selon la revendication 62, dans lequel le détergent est un détergent non ionique.
64. Procédé selon la revendication 58, dans lequel les cellules sont d'abord mises en contact avec les composés pendant une première période de temps prédéterminée, puis mises en contact avec la molécule luminogène et

l'enzyme bioluminescente pendant une seconde période de temps prédéterminée.

65. Procédé selon la revendication 58, dans lequel les cellules, les composés, la molécule luminogène et l'enzyme bioluminescente sont mis en contact simultanément.

66. Procédé selon la revendication 48, dans lequel l'activité de cytochrome P450 est dérivée d'un tissu animal.

67. Procédé selon la revendication 66, dans lequel le tissu exprime au moins une enzyme bioluminescente.

68. Procédé selon la revendication 66, dans lequel le tissu est d'abord mis en contact avec les composés et la molécule luminogène pendant une première période de temps prédéterminée avant la mise en contact avec l'enzyme bioluminescente.

69. Procédé selon la revendication 68, dans lequel, après la première période de temps prédéterminée, un détergent est ajouté.

70. Procédé selon la revendication 69, dans lequel le détergent et l'enzyme bioluminescente sont ajoutés en même temps.

71. Procédé selon la revendication 69, dans lequel le détergent est ajouté avant l'addition de l'enzyme bioluminescente.

72. Procédé selon la revendication 66, dans lequel le tissu est d'abord mis en contact avec les composés pendant une première période de temps prédéterminée, puis mis en contact avec la molécule luminogène pendant une deuxième période de temps prédéterminée, puis mis en contact avec l'enzyme bioluminescente pendant une troisième période de temps prédéterminée.

73. Procédé selon la revendication 72, dans lequel, après la deuxième période de temps prédéterminée, un détergent est ajouté.

74. Procédé selon la revendication 73, dans lequel le détergent et l'enzyme bioluminescente sont ajoutés en même temps.

75. Procédé selon la revendication 73, dans lequel le détergent est ajouté avant l'enzyme bioluminescente.

76. Procédé selon la revendication 66, dans lequel le tissu est d'abord mis en contact avec les composés pendant une première période de temps prédéterminée, puis mis en contact avec la molécule luminogène et l'enzyme bioluminescente pendant une seconde période de temps prédéterminée.

77. Procédé selon la revendication 66, dans lequel le tissu, les composés, la molécule luminogène et l'enzyme bioluminescente sont mis en contact simultanément.

78. Procédé selon la revendication 48, dans lequel l'activité d'une enzyme cytochrome P450 est dérivée d'un animal.

79. Procédé selon la revendication 78, dans lequel l'animal est un téléostéen transgénique qui exprime l'enzyme bioluminescente.

80. Procédé selon la revendication 79, dans lequel le téléostéen est d'abord mis en contact avec les composés et la molécule luminogène pendant une première période de temps prédéterminée avant la mise en contact avec l'enzyme bioluminescente.

81. Procédé selon la revendication 79, dans lequel le téléostéen est d'abord mis en contact avec les composés pendant une première période de temps prédéterminée, puis mis en contact avec la molécule luminogène pendant une deuxième période de temps prédéterminée, puis mis en contact avec l'enzyme bioluminescente pendant une troisième période de temps prédéterminée.

82. Procédé selon la revendication 79, dans lequel le téléostéen est d'abord mis en contact avec les composés pendant une première période de temps prédéterminée, puis mis en contact avec la molécule luminogène et l'enzyme bioluminescente pendant une seconde période de temps prédéterminée.

83. Procédé selon la revendication 79, dans lequel le téléostéen, les composés, la molécule luminogène et l'enzyme bioluminescente sont mis en contact simultanément.

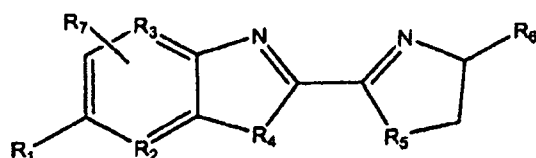
84. Procédé selon l'une quelconque des revendications 1, 8, 14, 16, 21, 29, 36, 43, 48, 58, 66 et 78, dans lequel l'enzyme bioluminescente est une luciférase.

85. Procédé selon l'une quelconque des revendications précédentes, dans lequel le R_4 du dérivé de coelentérazine est un aryle ou un aryle substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} .

86. Procédé selon l'une quelconque des revendications précédentes, dans lequel le dérivé de luciférine est choisi dans le groupe constitué par
l'éther 6'2-chloroéthylrique de luciférine ;
l'éther 6'benzylique de luciférine ;
l'éther 6'4-picolinylique de luciférine ;
l'éther 6'4-trifluorométhylbenzylique de luciférine ;
l'éther 6'phényléthylrique de luciférine ;
l'éther 6'géranylique de luciférine ;
l'éther 6'prénylique de luciférine ;
l'éther 6'2-picolinylique de luciférine,
l'éther 6'3-picolinylique de luciférine,
la déshydroxyluciférine,
l'éther 6'méthylrique de luciférine ; et
l'éther 6'éthylrique de luciférine.

87. Kit pour déterminer l'effet d'une substance sur l'activité enzymatique d'un cytochrome P450, comprenant :

(a) une ou plusieurs molécules luminogènes choisies dans le groupe constitué par
l'éther 6'2-chloroéthylrique de luciférine ;
l'éther 6'benzylique de luciférine ;
l'éther 6'4-picolinylique de luciférine ;
l'éther 6'4-trifluorométhylbenzylique de luciférine ;
l'éther 6'phényléthylrique de luciférine ;
l'éther 6'géranylique de luciférine ;
l'éther 6'prénylique de luciférine ;
l'éther 6'2-picolinylique de luciférine,
l'éther 6'3-picolinylique de luciférine ; et
les dérivés de D-luciférine ayant une formulé



dans laquelle

R_1 représente un amino, un alcoxy en C_{2-20} halogéné, un alcoxy en C_{2-20} halogéné substitué, un alcynyloxy en C_{3-20} , un alcynyloxy en C_{3-20} substitué, un cycloalcoxy en C_{3-20} , un cycloalcoxy en C_{3-20} substitué, un cycloalkylamino en C_{3-20} , un cycloalkylamino en C_{3-20} substitué, un alkylamino en C_{1-20} , un alkylamino en C_{1-20} substitué, un di-alkylamino en C_{1-20} , un di-alkylamino en C_{1-20} substitué, un alcénylamino en C_{2-20} , un alcénylamino en C_{2-20} substitué, un di-alcénylamino en C_{2-20} , un di-alcénylamino en C_{2-20} substitué, un alcényl en C_{2-20} alkylamino en C_{1-20} , un alcényl en C_{2-20} alkylamino en C_{1-20} substitué, un alcénylamino en C_{3-20} , un alcénylamino en C_{3-20} substitué, un di-alcénylamino en C_{3-20} , un di-alcénylamino en C_{3-20} substitué, un alcényl en C_{3-20} alkylamino en C_{1-20} , un alcényl en C_{3-20} alkylamino en C_{1-20} substitué, un alcényl en C_{3-20} alcénylamino en C_{2-20} ou un alcényl en C_{3-20} alcénylamino en C_{2-20} substitué ;

R_2 et R_3 représentent indépendamment C ou N ;

R_4 et R_5 représentent indépendamment S, O ou CR_9R_{10} où R_9 et R_{10} représentent indépendamment H, un alkyle en C_{1-20} ou du fluor ;

R_6 représente CH_2OH ; COR_{11} où R_{11} représente H, OH, un alcényle en C_{2-20} ou $NR_{12}R_{13}$ où R_{12} et R_{13} sont indépendamment H ou un alkyle en C_{1-20} ; ou $-OM^+$ où M^+ est un métal alcalin ou un sel pharmaceutiquement acceptable ; et

R_7 représente H, un alcényle en C_{1-20} , un halogène ou un alcoxyde en C_{1-6} ,

avec pour condition que R_1 n'est pas OH ou NH_2 , si R_3 et R_2 sont tous les deux du carbone, R_4 et R_5 sont tous les deux S, R_7 est H, R_6 est COR_{11} et R_{11} est OH, de sorte que la molécule luminogène n'est pas une luciférine ou une aminoluciférine ; et

(b) des instructions pour utiliser la trousse.

88. Kit selon la revendication 87, comprenant en outre une ou plusieurs enzymes bioluminescentes.

89. Kit selon la revendication 88, dans lequel l'enzyme bioluminescente est une luciférase.

90. Kit selon la revendication 88, dans lequel l'enzyme bioluminescente est une luciférase de luciole ou de Renilla.

91. Kit selon la revendication 90, comprenant en outre de l'ATP et des ions magnésium.

92. Kit selon la revendication 91, comprenant en outre un détergent.

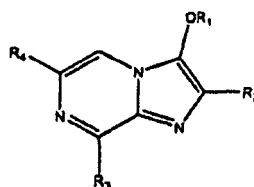
93. Kit selon la revendication 92, dans lequel le détergent est non ionique.

94. Kit selon la revendication 92, qui comprend en outre une pyrophosphatase.

95. Kit selon la revendication 94, dans lequel la pyrophosphatase est une pyrophosphatase inorganique.

96. Kit pour déterminer l'effet d'une substance sur l'activité d'une enzyme cytochrome P450, comprenant :

(a) une ou plusieurs molécules luminogènes choisies parmi les dérivés de coelentérazine ayant la formule :



dans lequel :

R_1 est un alkyle en C_{1-20} , un alkyle en C_{3-20} ramifié, un cycloalkyle en C_{3-20} , un aralkyle, un alkyle en C_{1-20} substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} , un aralkyle substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} ; et

R_2 , R_3 et R_4 sont indépendamment un hydrogène, un alkyle en C_{1-20} , un cycloalkyle en C_{3-20} , un alkyle en C_{3-20} ramifié, un aryle, un aralkyle, un alkyle en C_{1-20} substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} , un aralkyle substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} , un aryle substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} .

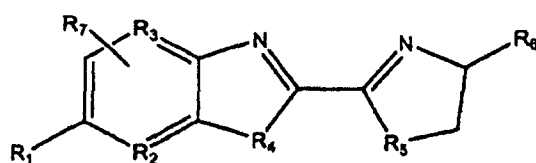
97. Kit selon la revendication 96, dans lequel R_4 est un aryle ou un aryle substitué par un alcoxy en C_{1-20} , un hydroxy, un halogène, un alkylamino en C_{1-20} ou un di-alkylamino en C_{1-20} .

98. Kit selon la revendication 87, comprenant en outre un inhibiteur de luciférase réversible.

99. Kit selon la revendication 98, dans lequel l'inhibiteur de luciférase réversible est le 2-(4-aminophényl)-6-méthylbenzothiazole (APMBT) ou le 2-amino-6-méthylbenzothiazole (AMBT).

100. Dérivé de D-luciférine, qui est un substrat d'une enzyme cytochrome P450 et un prosubstrat de l'enzyme luciférase,

choisi dans le groupe constitué par
 l'éther 6'2-chloroéthylique de luciférine ;
 l'éther 6'benzylique de luciférine ;
 l'éther 6'4-picolinylique de luciférine ;
 l'éther 6'4-trifluorométhylbenzylique de luciférine ;
 l'éther 6'phényléthylique de luciférine ;
 l'éther 6'géranylique de luciférine ;
 l'éther 6'prénylique de luciférine ;
 l'éther 6'2-picolinylique de luciférine,
 l'éther 6'3-picolinylique de luciférine ; et
 les dérivés de D-luciférine ayant la formulé :



dans laquelle

R₁ représente un amino, un alcoxy en C₂₋₂₀ halogéné, un alcoxy en C₂₋₂₀ halogéné substitué, un alcynyloxy en C₃₋₂₀, un alcynyloxy en C₃₋₂₀ substitué, un cycloalcoxy en C₁₋₂₀, un cycloalcoxy en C₃₋₂₀ substitué, un cycloalkylamino en C₃₋₂₀, un cycloalkylamino en C₃₋₂₀ substitué, un alkylamino en C₁₋₂₀, un alkylamino en C₁₋₂₀ substitué, un di-alkylamino en C₁₋₂₀, un di-alkylamino en C₁₋₂₀ substitué, un alcénylamino en C₂₋₂₀, un alcénylamino en C₂₋₂₀ substitué, un di-alcénylamino en C₂₋₂₀, un di-alcénylamino en C₂₋₂₀ substitué, un alcényl en C₂₋₂₀ alkylamino en C₁₋₂₀, un alcényl en C₂₋₂₀ alkylamino en C₁₋₂₀ substitué, un alcynylamino en C₃₋₂₀, un alcynylamino en C₃₋₂₀ substitué, un di-alcynylamino en C₃₋₂₀, un di-alcynylamino en C₃₋₂₀ substitué, un alcynyl en C₃₋₂₀ alkylamino en C₁₋₂₀, un alcynyl en C₃₋₂₀ alkylamino en C₁₋₂₀ substitué, un alcynyl en C₃₋₂₀ alcénylamino en C₂₋₂₀ ou un alcynyl en C₃₋₂₀ alcénylamino en C₂₋₂₀ substitué ;

R₂ et R₃ représentent indépendamment C ou N ;

R₄ et R₅ représentent indépendamment S, O ou CR₉R₁₀ où R₉ et R₁₀ représentent indépendamment H, un alkyle en C₁₋₂₀ ou du fluor ;

R₆ représente CH₂OH ; COR₁₁ où R₁₁ représente H, OH, un alcényle en C₂₋₂₀ ou NR₁₂R₁₃ où R₁₂ et R₁₃ sont indépendamment H ou un alkyle en C₁₋₂₀ ; ou -OM⁺ où M⁺ est un métal alcalin ou un sel pharmaceutiquement acceptable ; et

R₇ représente H, un alcényle en C₁₋₂₀, un halogène ou un alcoxyde en C₁₋₆,

avec pour condition que R₁ n'est pas OH ou NH₂, si R₃ et R₂ sont tous les deux du carbone, R₄ et R₅ sont tous les deux S, R₇ est H, R₆ est COR₁₁ et R₁₁ est OH, de sorte que le dérivé de luciférine n'est pas une luciférine ou une aminoluciférine.

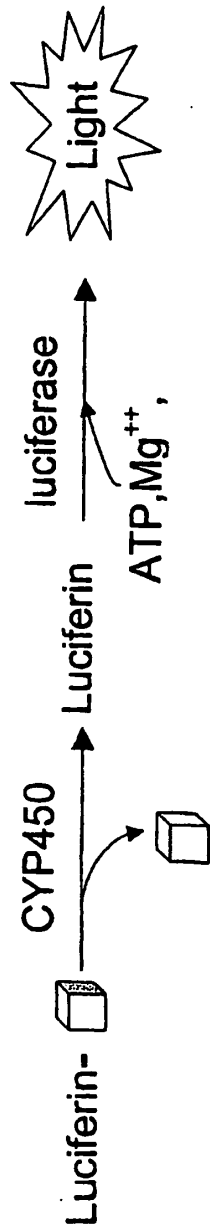
101. Composition comprenant les dérivés de D-luciférine selon la revendication 100.

102. Composition selon la revendication 101, comprenant en outre une pyrophosphatase.

103. Composition selon la revendication 102, dans laquelle la pyrophosphatase est une pyrophosphatase inorganique.

104. Procédé pour mesurer l'activité de cytochrome P450 chez un animal qui a un transgène d'enzyme bioluminescente et auquel une molécule luminogène selon la revendication 1, ou un dérivé de D-luciférine selon la revendication 100, a été administré(e), 1 le procédé comprenant la détermination de l'activité de cytochrome P450 en mesurant la bioluminescence chez l'animal vivant intact.

Figure 1



Examples

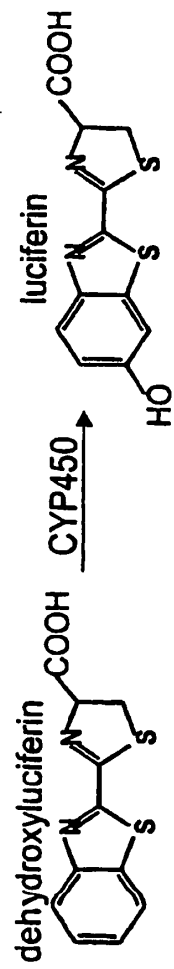
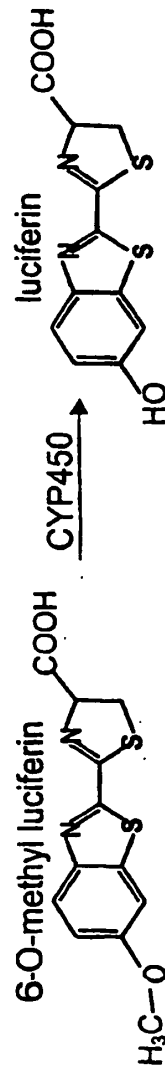
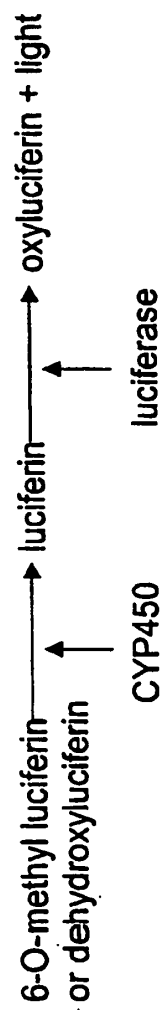


Figure 2

D-luciferin and D-luciferin derivatives.

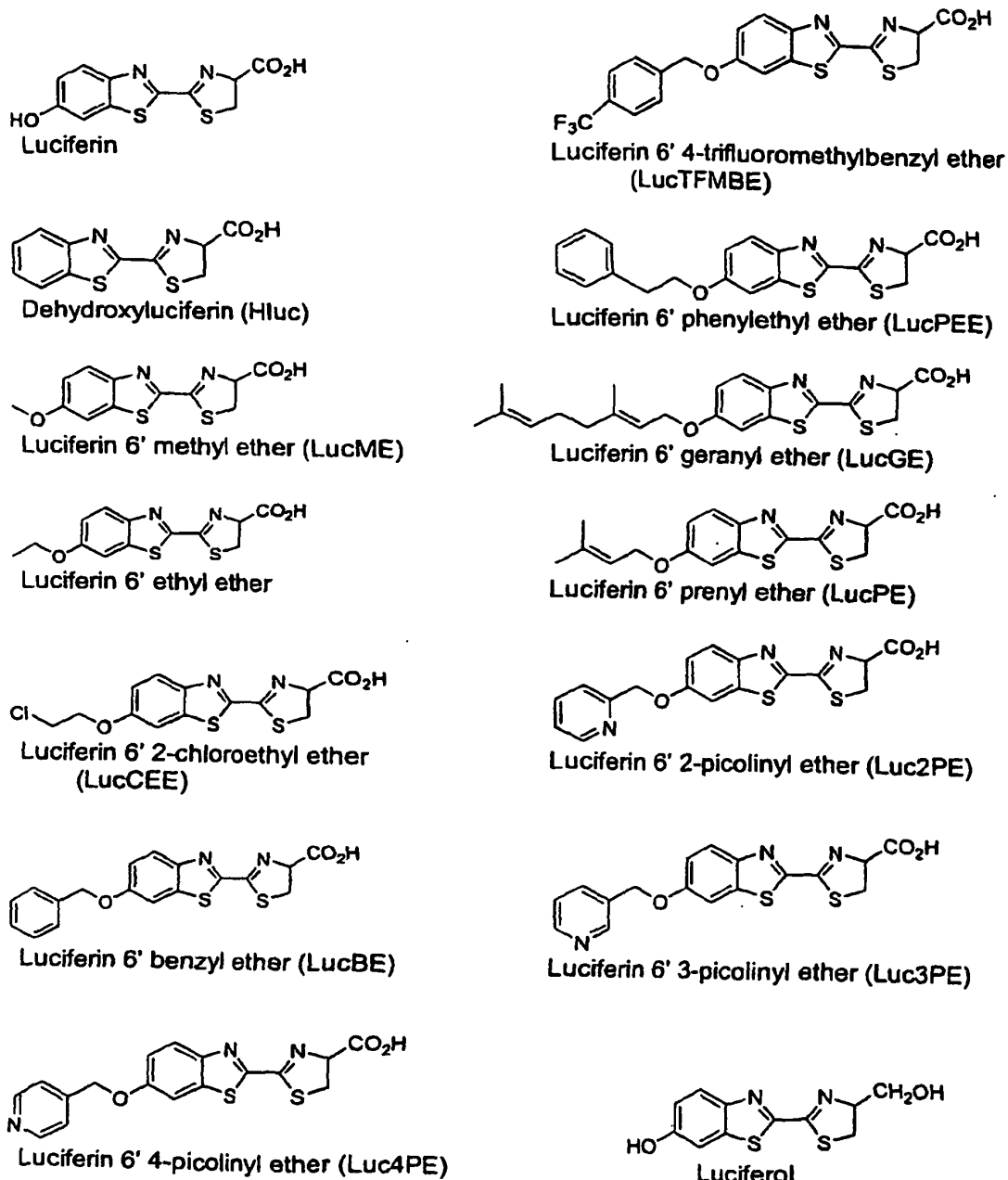
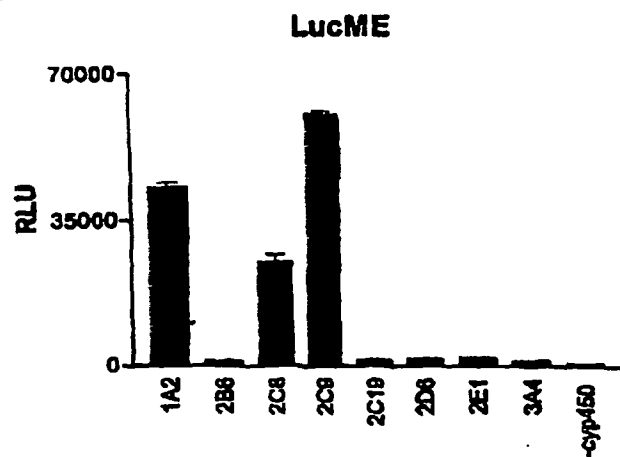
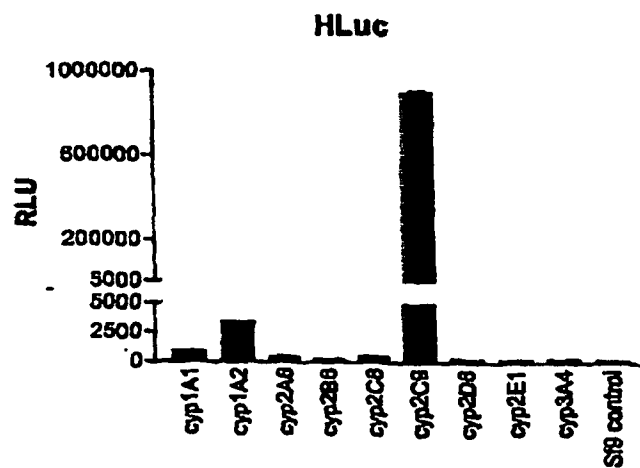
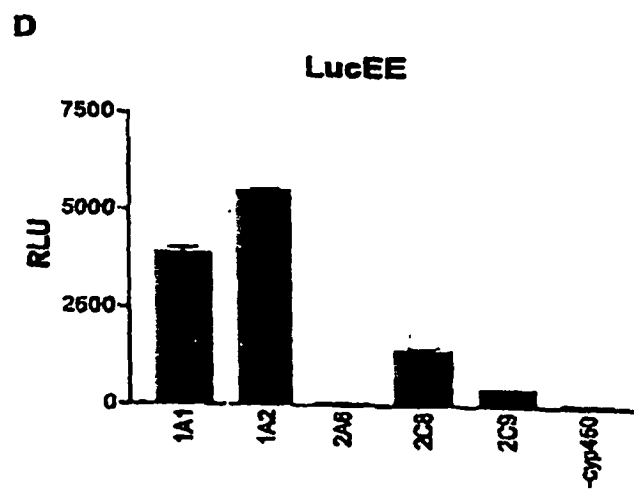
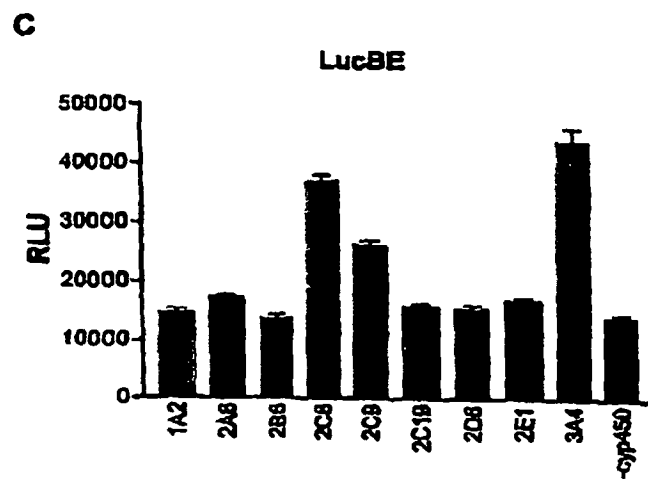
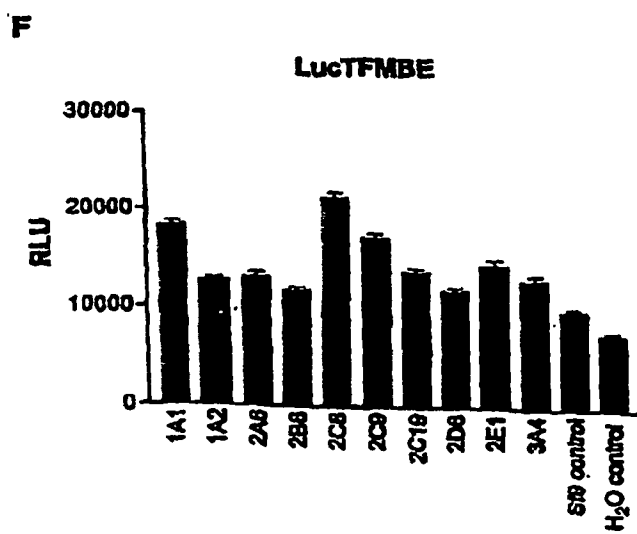
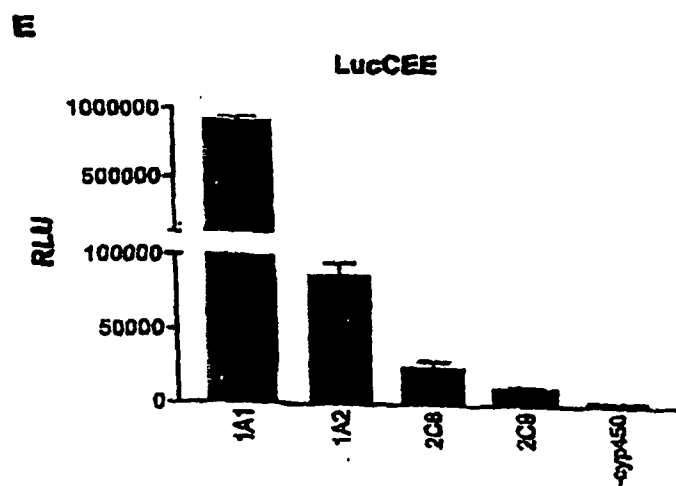


Figure 3

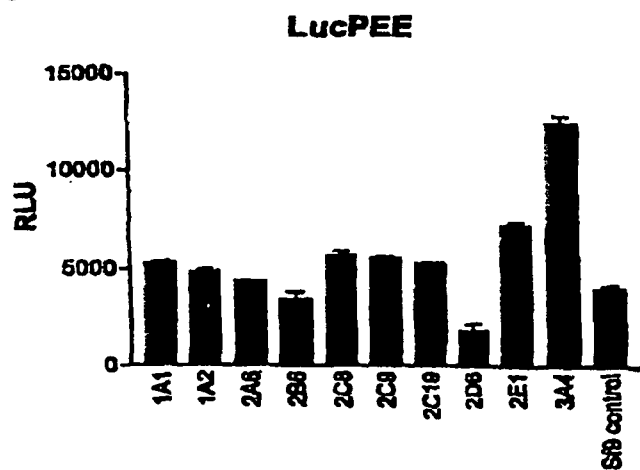
Two-step luminescent cyp450 reactions using D-luciferin derivatives

A**B**

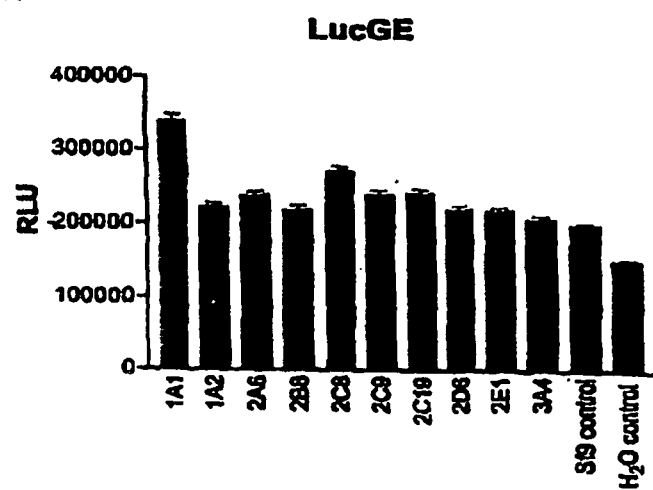




G



H



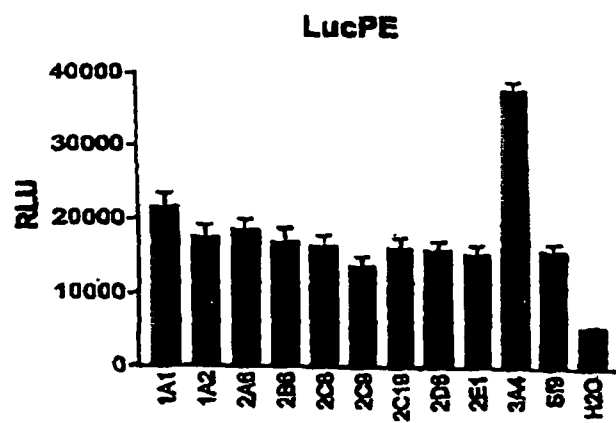


Figure 4

Time-dependence of *cyp450*/substrate incubation in two-step luminescent *cyp450* reactions using D-luciferin derivatives.

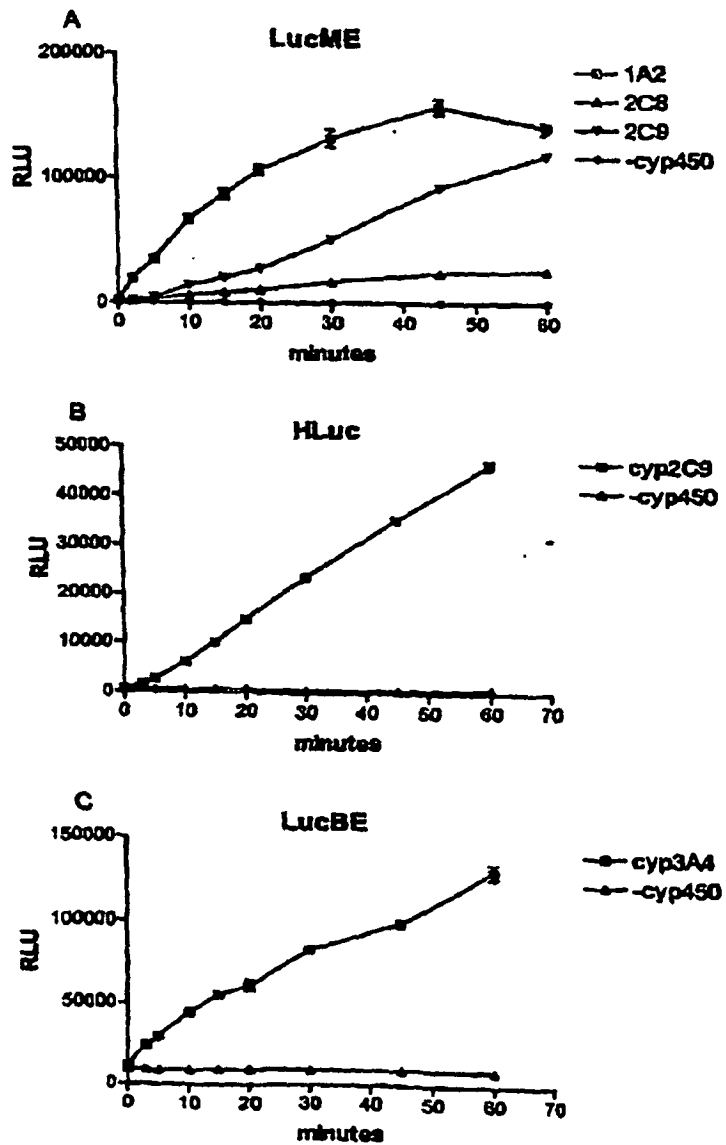


Figure 5

Time course of light output from two-step luminescent cyp450 reactions using LucME.

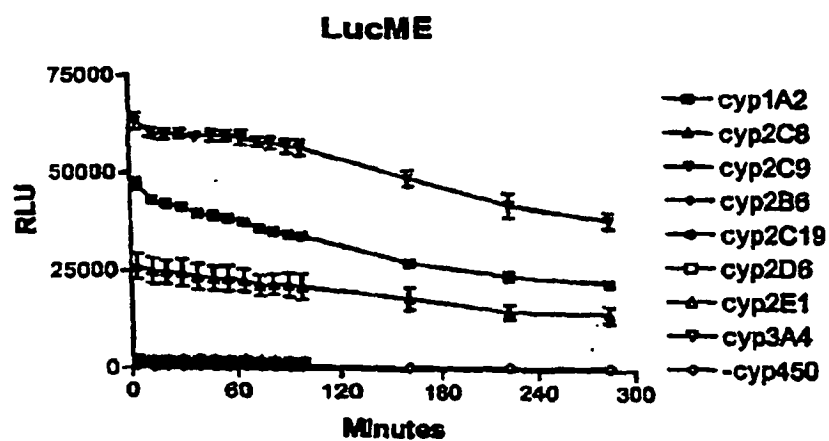


Figure 6

One-step luminescent cyp450 assays at room temperature using LucME.

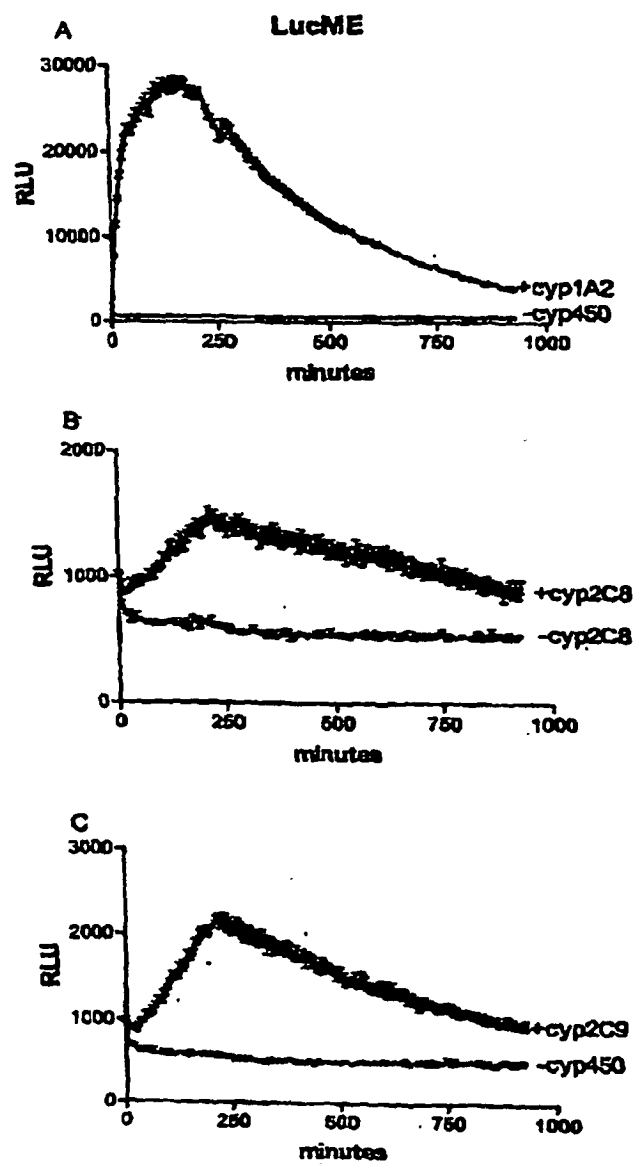


Figure 7

One-step luminescent cyp450 assays at 37°C using D-luciferin derivatives.

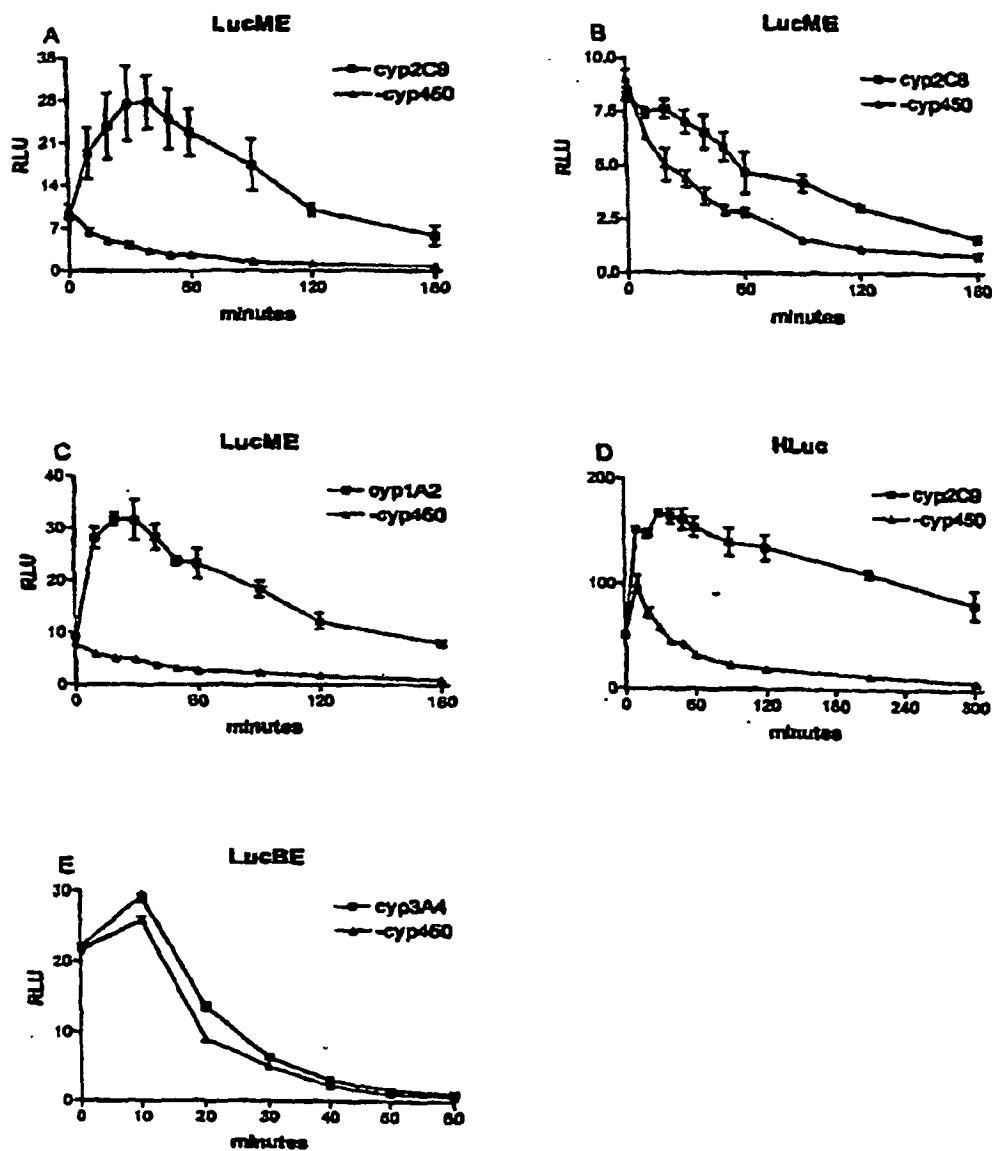


Figure 8

Pooled human liver microsomes in two-step luminescent cyp450 reactions using D-luciferin derivatives.

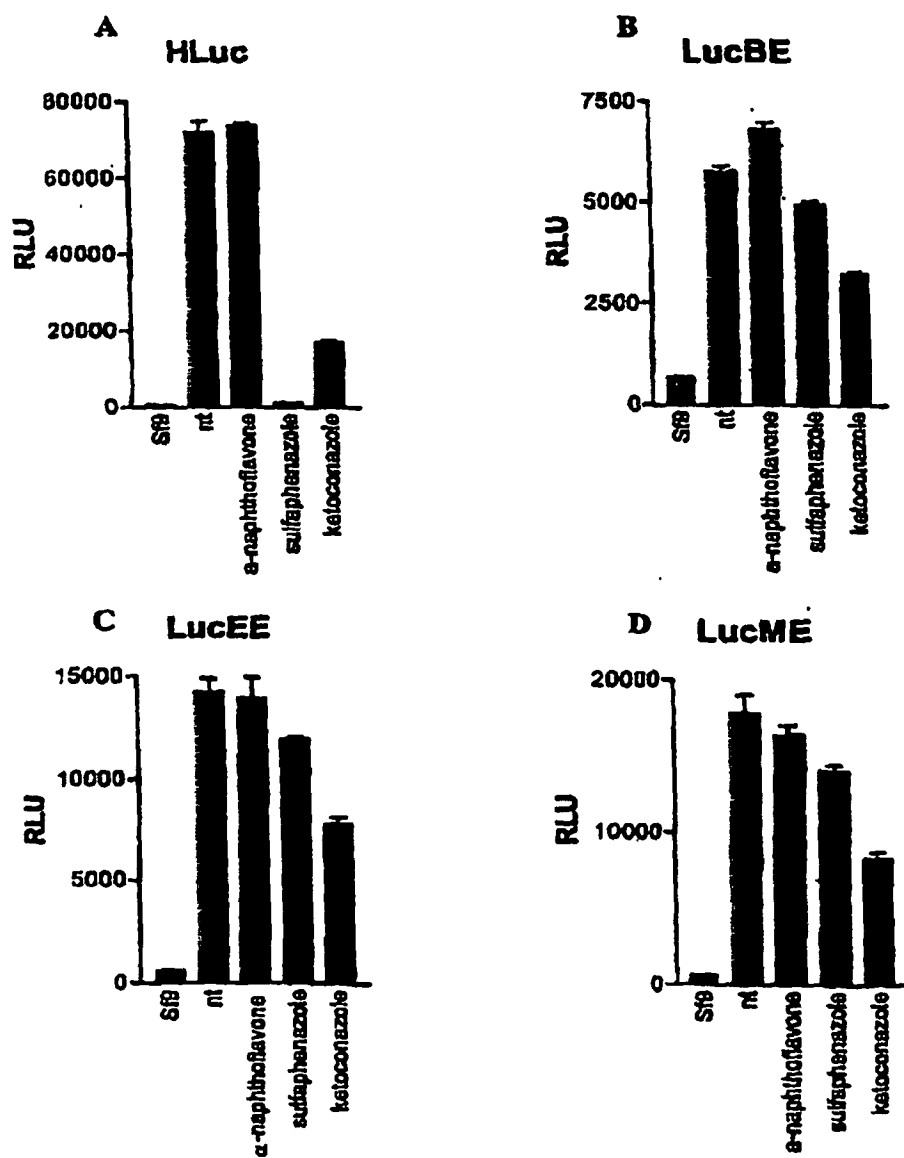


Figure 9

Two-step detection of de-picolinylase activity picolinyl D-luciferin derivatives

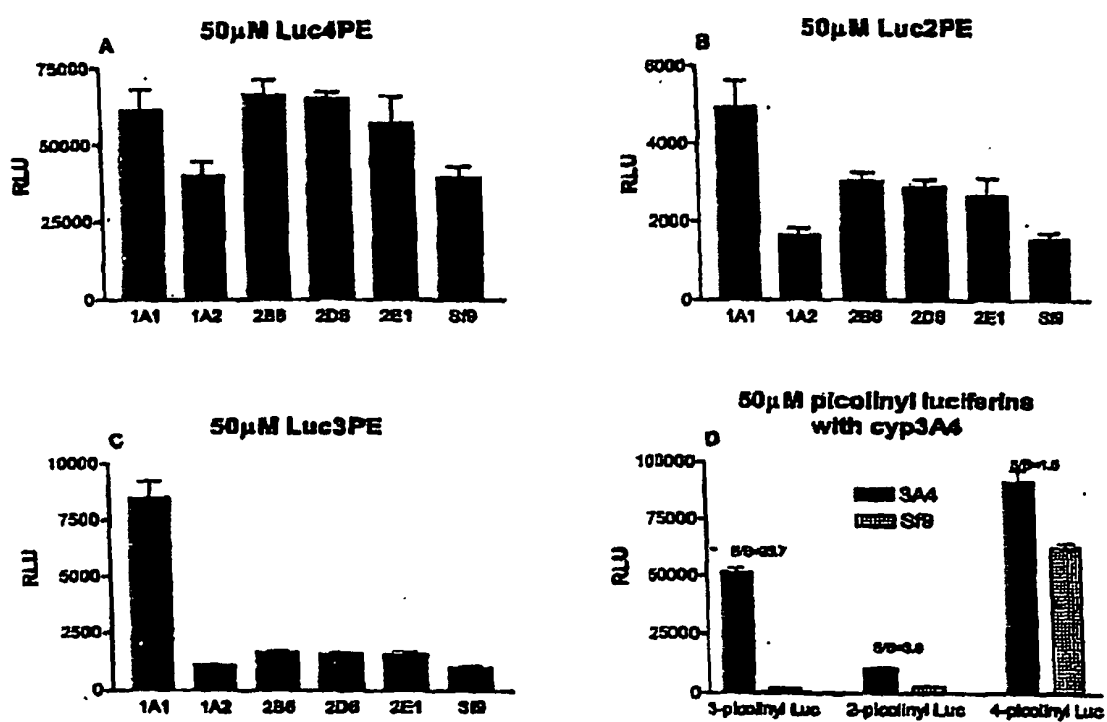
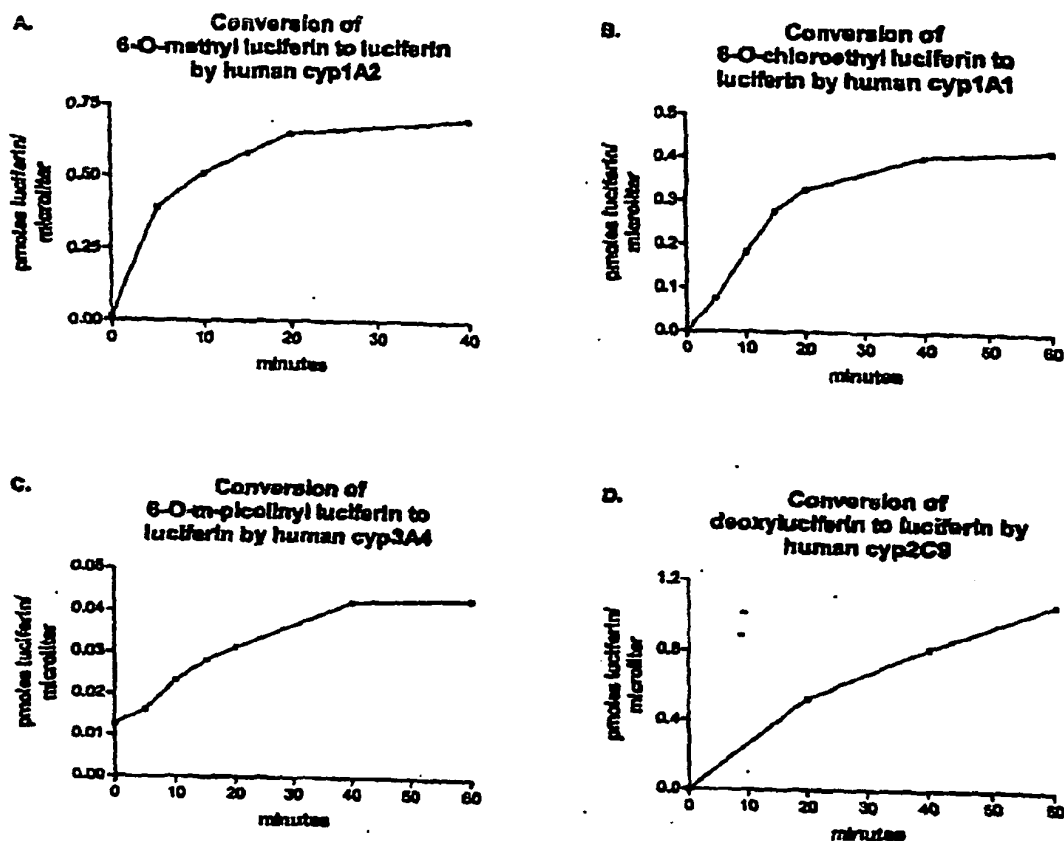


Figure 10

Conversion of D-luciferin derivatives to luciferin by human cyp450s



100 micromolar 6-O-methyl luciferin (panel A.), 100 micromolar 6-O-chloroethyl luciferin (panel B.), 25 micromolar 6-O-m-picolinyl luciferin (panel C.) or 100 micromolar deoxyluciferin (panel D.), was incubated with cyp1A2, cyp1A1, cyp3A4 or cyp2C9, respectively, in reaction volumes of 150 microliters at 37°C. At the indicated times reactions were stopped by addition of tert-butyl alcohol to 0.1% (v/v) and flash freezing in liquid nitrogen. 95 microliters of each reaction was subjected to fractionation by HPLC and luciferin was detected by fluorescence. The zero time points were determined by inactivating the cyp450 with 0.1% tert-butyl alcohol prior to substrate addition (cyp1A1, 1A2 and 3A4) or by determining the luciferin content of a no enzyme control with deoxyluciferin (cyp2C9).

HPLC method: High-pressure liquid chromatography was performed on an HP 1050 LC system equipped with a multi-wavelength absorbance (HP 1050 MWD) and fluorescence detector (HP 1048A). Separation was achieved on a 5 micron Adsorbosphere HS C18 column (Alltech Associates) with a solvent gradient of 0.05M KH_2PO_4 / pH 6 (solvent A) and 80:20 acetonitrile/water (solvent B). The gradient conditions used were 15%B to 95%B over 10 min. Substrates were detected by absorbance at either 282 or 330 nm and Luciferin was detected by fluorescence at 520 nm (emission) with an excitation wavelength of 330 nm.

Figure 11

Inhibition of cyp450 by known cyp450 substrates.

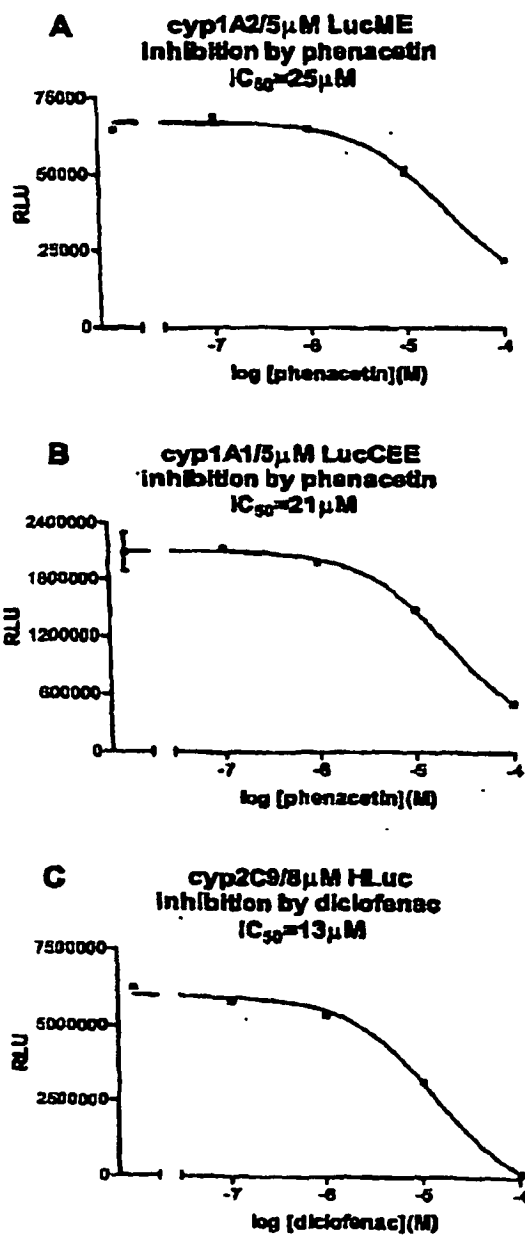


Figure 12

P450 action on methoxy-coelenterazine HH, coelenterazine HH, and coelenterazine by chemiluminescent and bioluminescent detection.

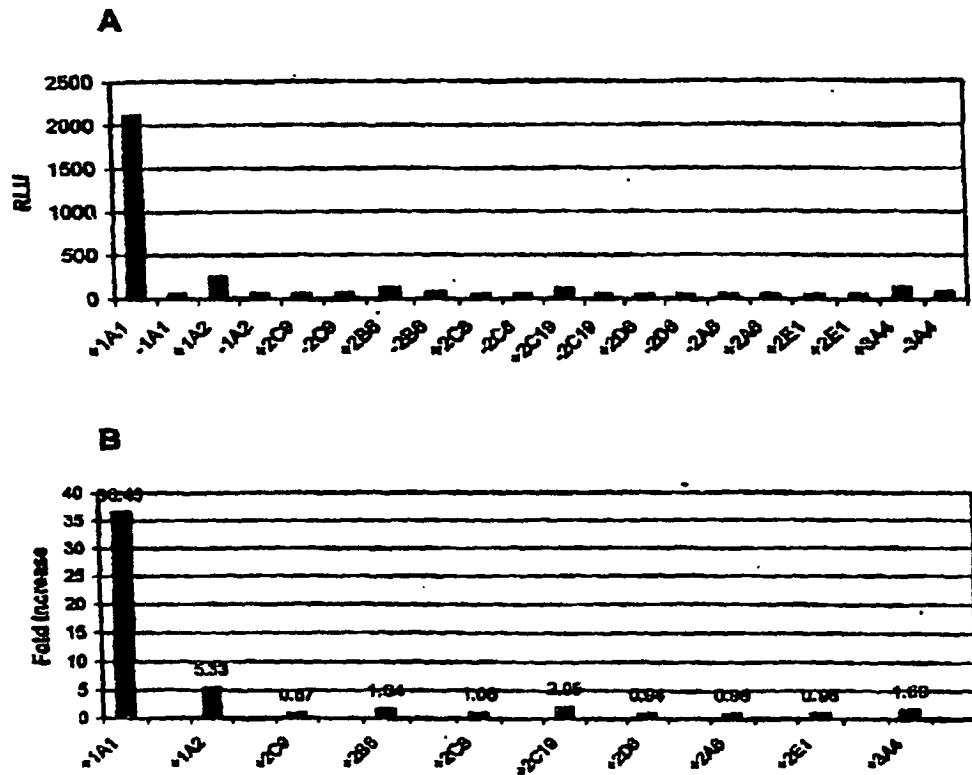


Figure 12 cont.

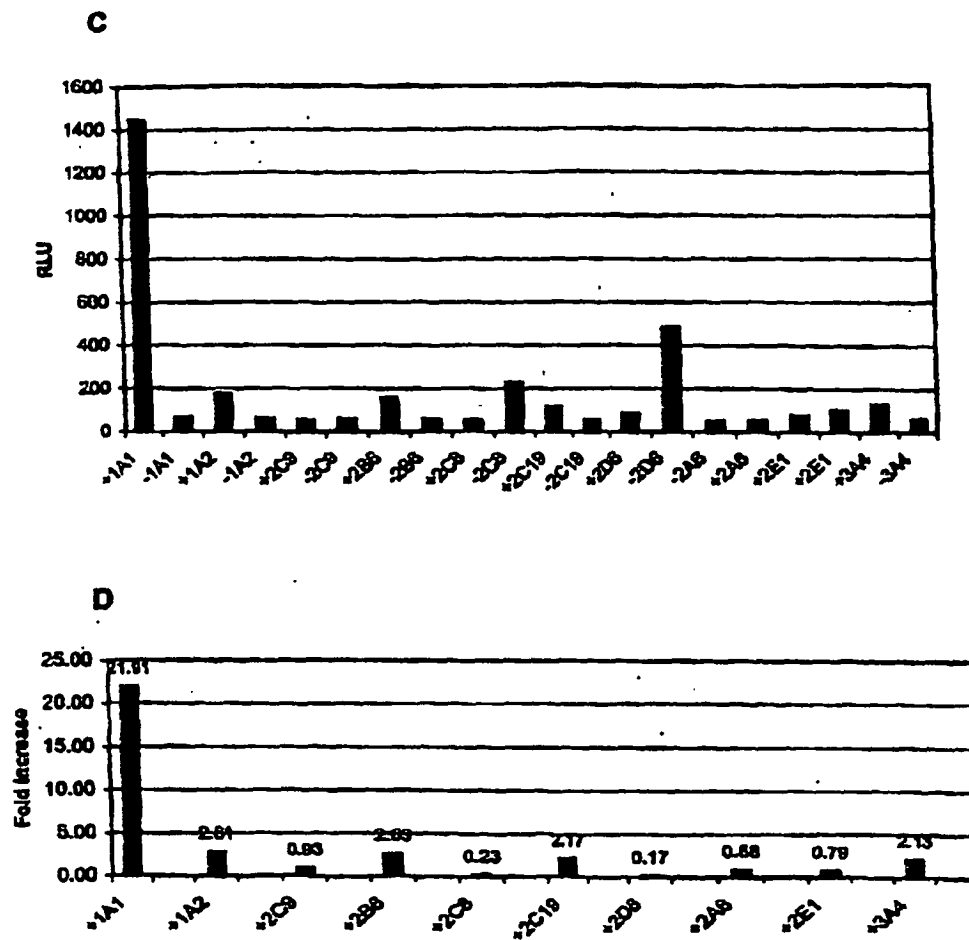


Figure 12 cont.

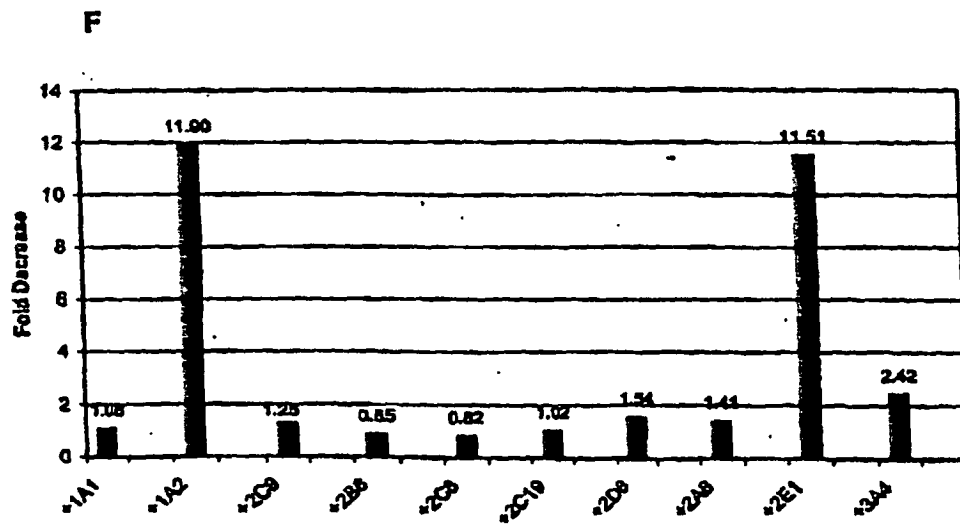
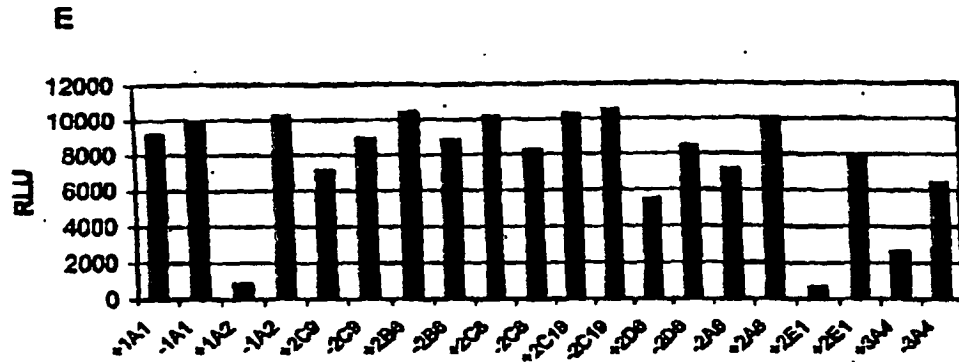


Figure 12 cont.

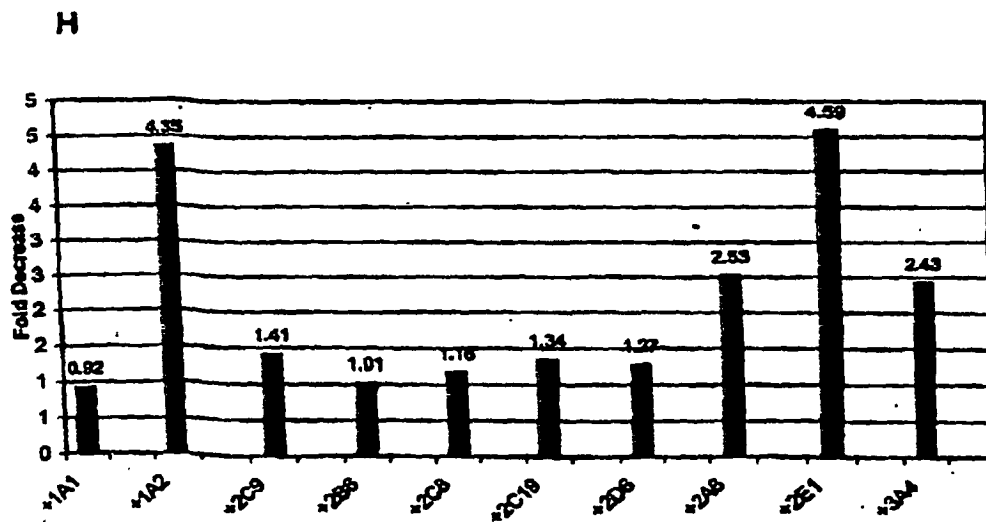
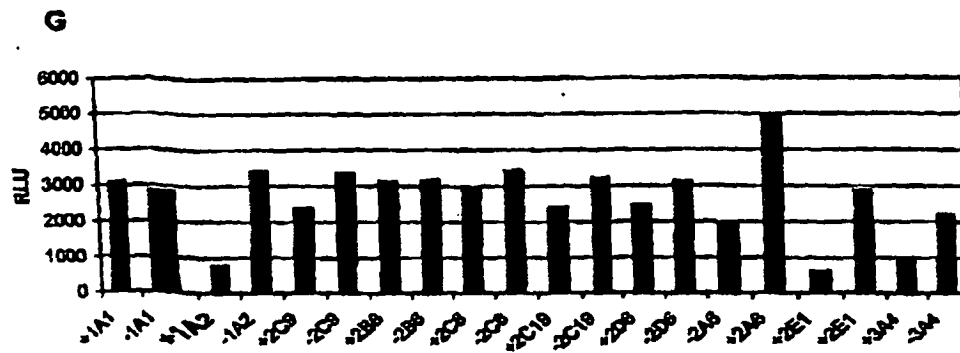


Figure 12 cont.

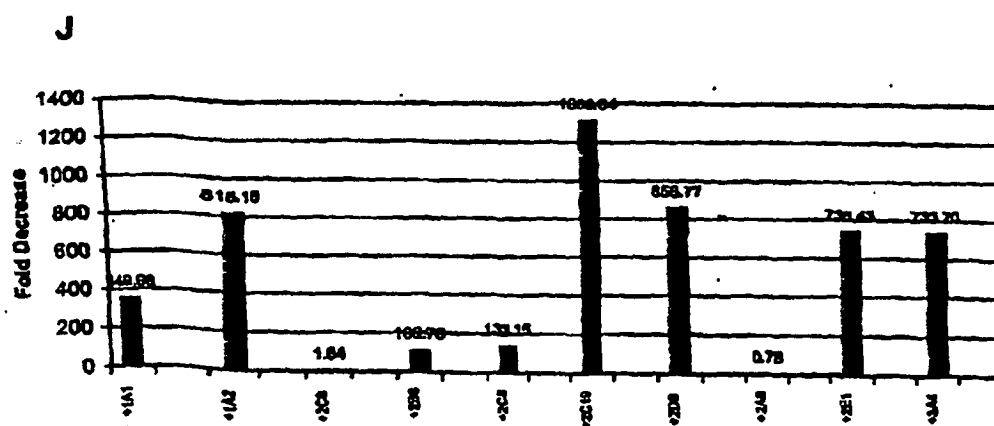
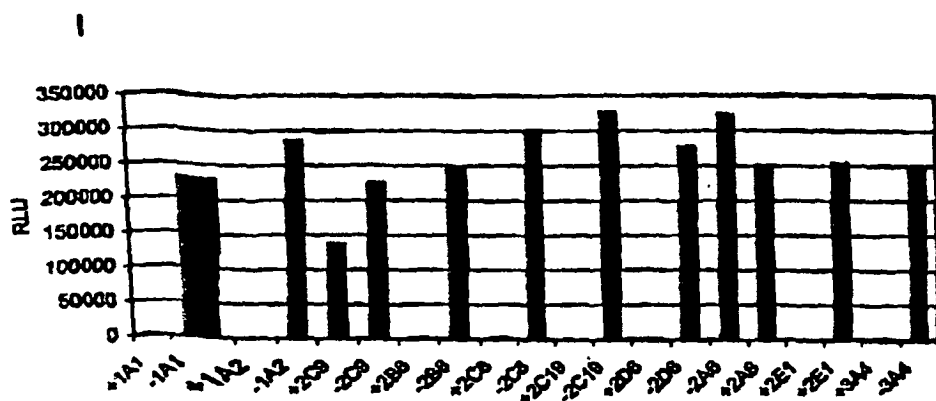


Figure 12 cont.

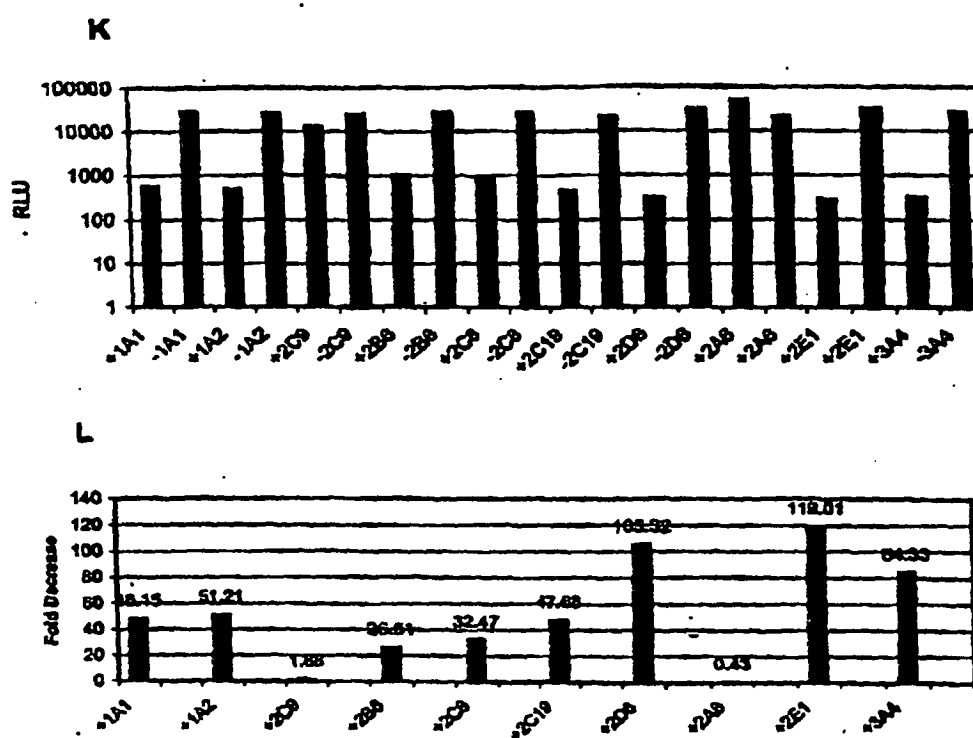


Fig. 13: Pyrophosphatase Activity

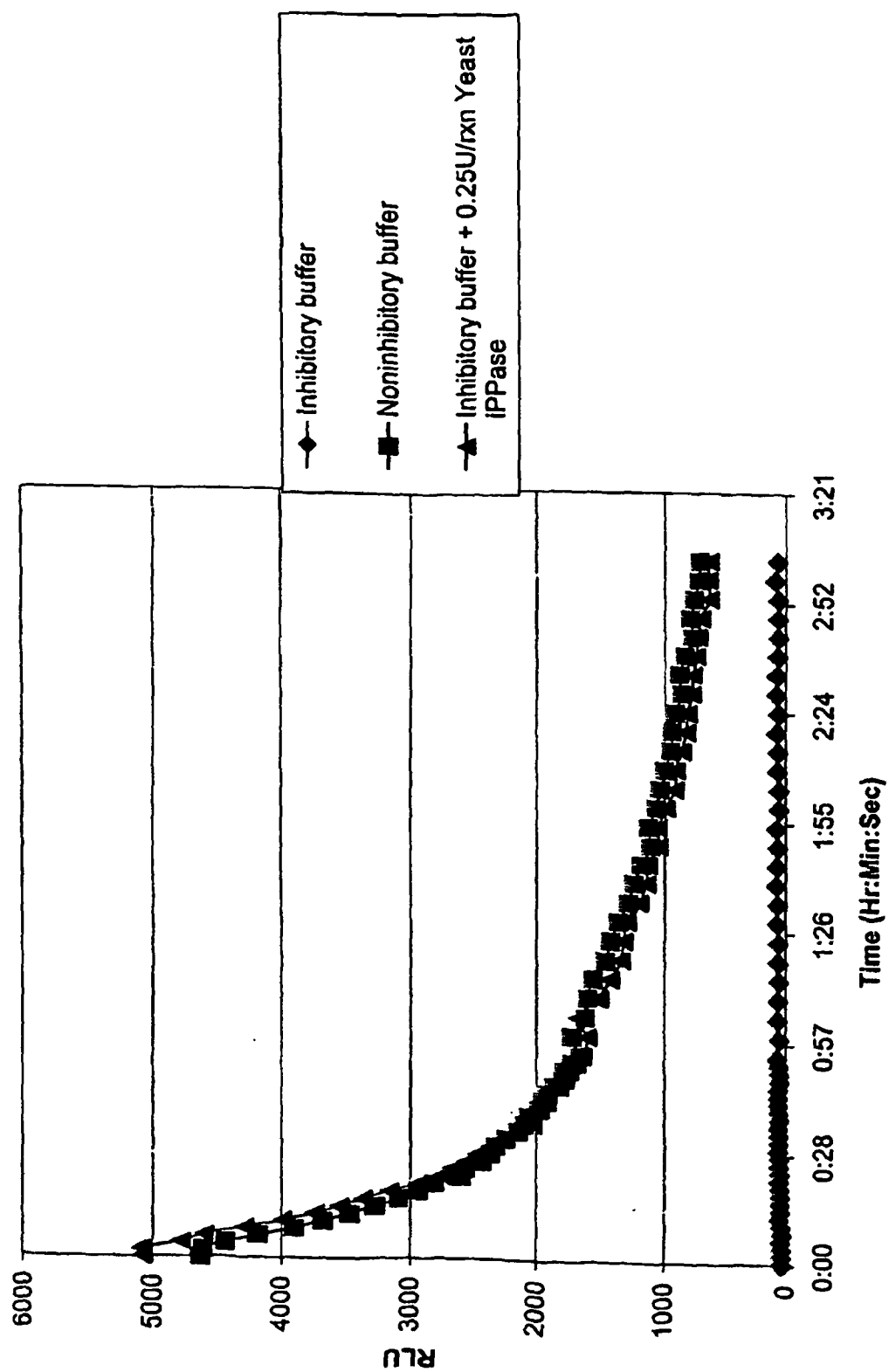


Figure 14

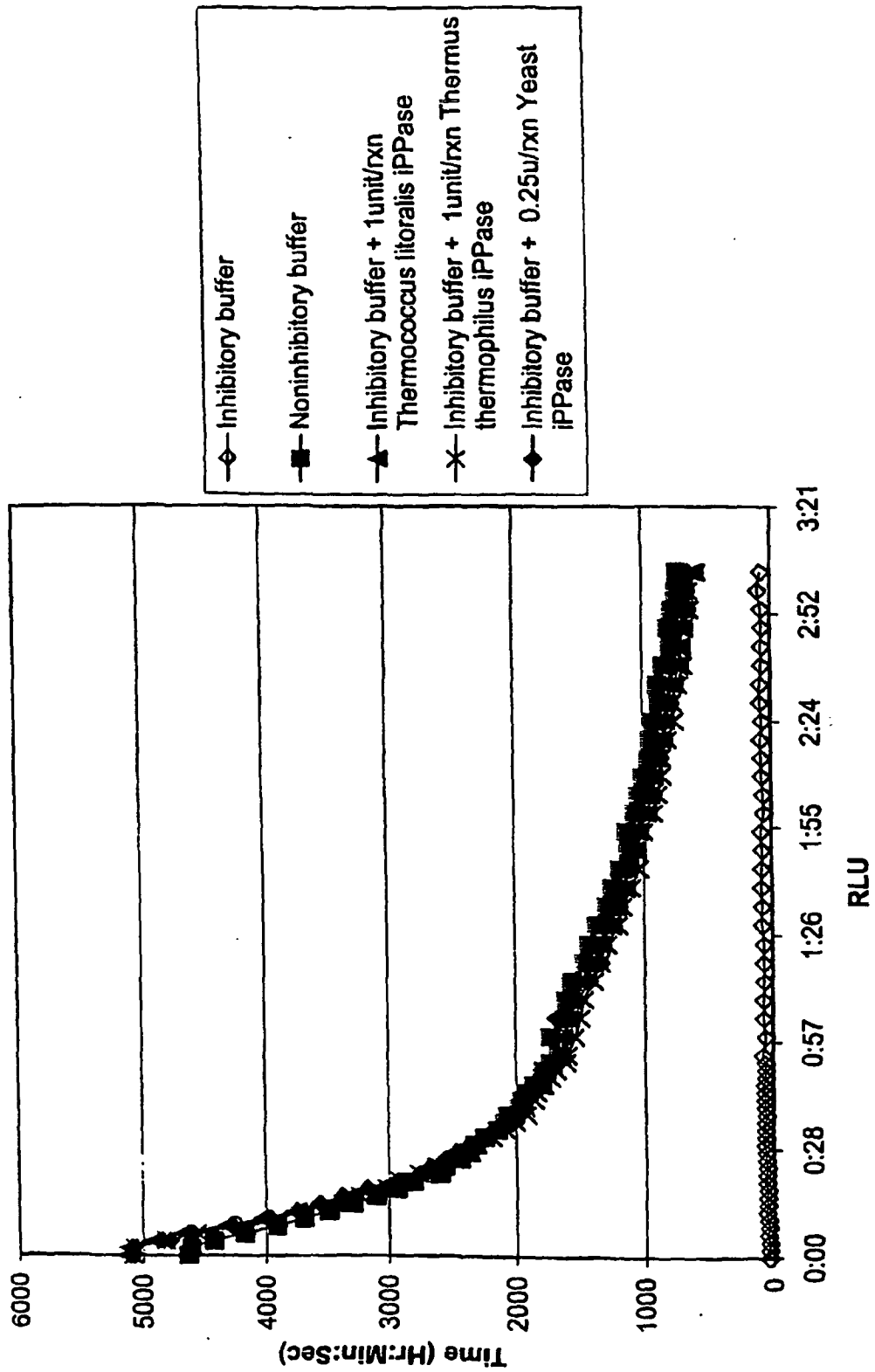


Figure 15

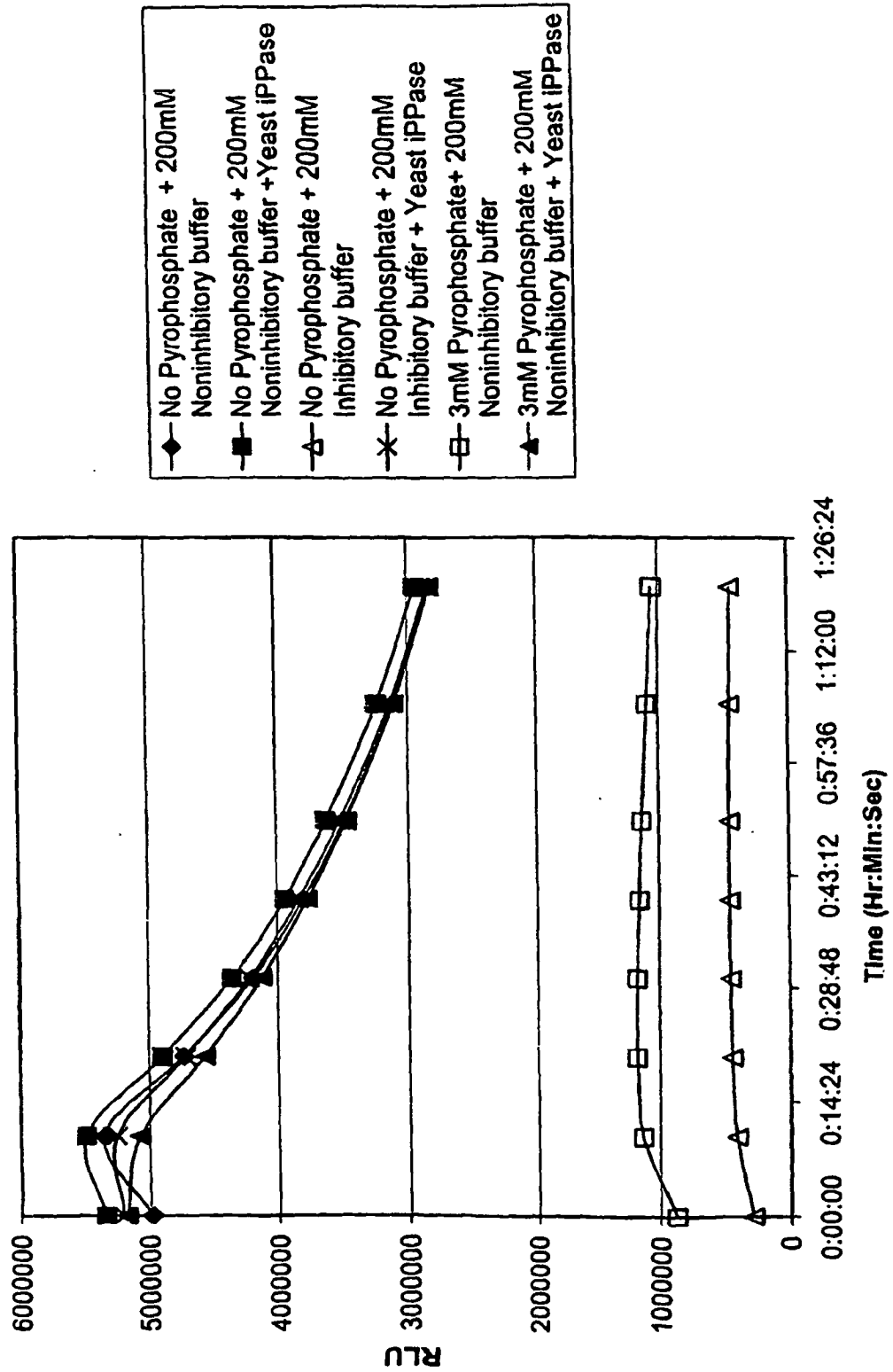


Figure 16

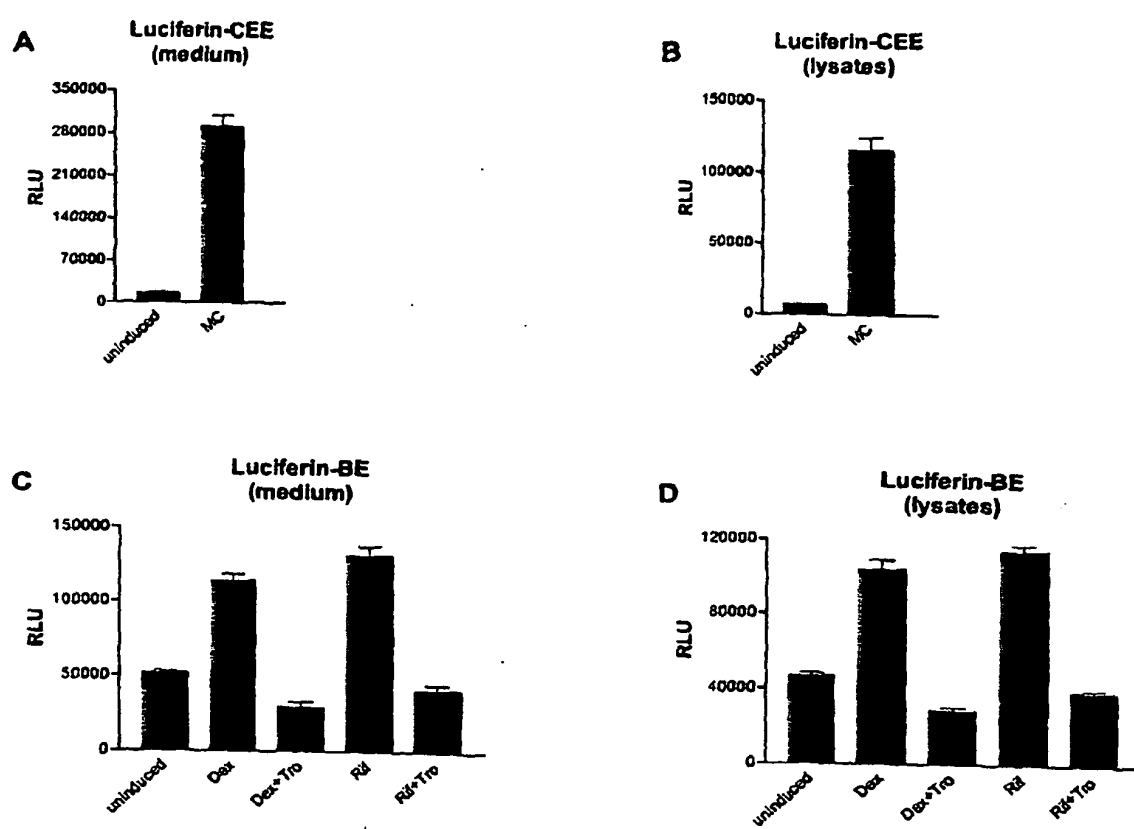
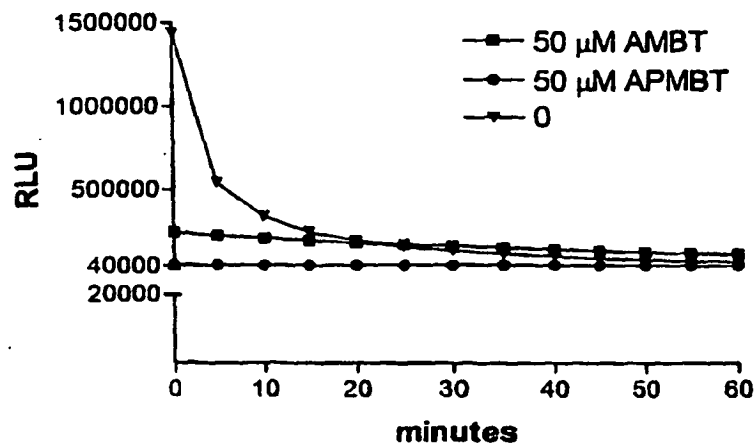


Figure 17

**Stabilization of luminescent
CYP1A1 signal with APMBT
and AMBT**



REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	用于测量细胞色素p450活性的基于发光的方法和探针		
公开(公告)号	EP1546162B1	公开(公告)日	2011-06-22
申请号	EP2003749715	申请日	2003-09-16
[标]申请(专利权)人(译)	蛇药制品有限公司		
申请(专利权)人(译)	PROMEGA公司		
当前申请(专利权)人(译)	PROMEGA公司		
[标]发明人	CALI JAMES J KLAUBERT DIETER DAILY WILLIAM HO SAMUEL KIN SANG FRACKMAN SUSAN HAWKINS ERIKA WOOD KEITH V		
发明人	CALI, JAMES, J. KLAUBERT, DIETER DAILY, WILLIAM HO, SAMUEL, KIN, SANG FRACKMAN, SUSAN HAWKINS, ERIKA WOOD, KEITH, V.		
IPC分类号	C07F9/06 C12N9/02 C12Q1/00 G01N33/53 C12Q1/66 C07D403/04 C07D413/04 C07D417/04 C07D417/14 C12Q1/26 G01N G01N33/58		
优先权	60/412254 2002-09-20 US 60/483309 2003-06-27 US		
其他公开文献	EP1546162A2 EP1546162A4		
外部链接	Espacenet		

摘要(译)

本发明提供了用于分析细胞，组织和动物中的代谢活性以及筛选测试化合物对细胞色素P450活性的影响的化合物，组合物，方法，底物和试剂盒。特别是，使用发光分子的一步法和两步法，例如，使用发光分子。荧光素或腔肠素，即细胞色素P450底物，也是生物发光酶，例如荧光素酶，前底物。本方法还提供了使用荧光素酶稳定剂如可逆荧光素酶抑制剂在基于荧光素酶的测定中稳定和延长发光信号的方法。

